The Association between Polymorphisms of B7 Molecules (CD80 and CD86) and Graves' Ophthalmopathy in a Taiwanese Population

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Objective: This study evaluates whether B7 molecules (CD80 and CD86) could be used as genetic markers for the development of Graves' ophthalmopathy (GO).

Design: Cross-sectional study.

Participants: We included 471 patients with Graves' disease (GD; 200 patients with GO and 271 patients without GO) in a Chinese population in Taiwan.

Methods: An endocrinologist with substantial experience in thyroid diseases identified GO. Blood samples were taken for DNA extraction from GD subjects. The gene polymorphism of *CD80* and *CD86* was genotyped by polymerase chain reaction in each patient.

Main Outcome Measures: Genotypes of CD80 and CD86 polymorphism.

Results: We found that the frequency of C allele at position rs_9831894 of the CD86 gene is different in patients with GD (with and without GO; chi-square test, $P = 0.0017$). In addition, the multifactor dimensionality reduction method was used to identify the best gene–gene interaction to predict the risk of GO. We identified an interaction between CD80_rs9289131 and CD86_rs9872483 (sign test, $P = 0.0010$). Moreover, the G-A haplotype was shown to have a protective effect in the development of ophthalmopathy among patients with GD (odds ratio, 0.63; 95% confidence interval, 0.44–0.90). Moreover, among patients with GO, the patients carrying the G-A haplotype had a lower level of free thyroxine T_4 than those not carrying the G-A haplotype ($P = 0.0001$).

Conclusions: These results suggest that the polymorphisms of the CD86 gene may be used as genetic markers for making the diagnosis and prognosis of GO. Therefore, GO could be a disease with complex genetic factors, resulting from the existing gene–gene interaction found in the present study.

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Graves' disease (GD) is a common autoimmune thyroid disease, in which the body produces antibodies to the receptor of the thyroid-stimulating hormone. Graves' disease is characterized clinically by hyperthyroidism, diffuse goiter, thyroid-specific autoantibodies, Graves' ophthalmopathy (GO), and dermopathy.[1] Recent studies support the involvement of environmental triggers and predisposing genes in the pathogenesis of GD.[2–4]

Affecting 25% to 50% of patients with GD, GO is the most common extrathyroidal manifestation.[4–7] It is considered to be an autoimmune inflammatory disorder affecting the extraocular muscles and the orbital fatty and connective tissues. Recent studies have shown that T-cell–mediated immunity is likely to play an important role in the autoimmune inflammation of ophthalmopathy.[8] Several genes, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4),[9–11] CD28,[12–14] and protein tyrosine phosphatase nonreceptor 22 (*PTPN22*),[15] related to T-cell activation have been investigated, but, to date, only the *CTLA-4* gene has been shown to be associated with GD. From a meta-analysis[16] that included the results of 44 published and unpublished studies, a significant association between the polymorphism of CTLA-4 and GD was found in Asian and Caucasian populations. However, the number of susceptible genes related to T-cell functions remains unknown.

In order to activate native T cells, a specific antigen that can be presented by major histocompatibility complex molecules and antigen–major histocompatibility complex interact with the T-cell receptor are necessary. Moreover, a second signal that includes the interaction between CD28/CTLA-4 and B7 molecules is also required for T-cell activation. B7 molecules are costimulatory molecules expressed on the surface of antigen-presenting cells. The binding of B7 to CD28 on T cells initiates a costimulatory

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signal for T-cell activation, proliferation, differentiation, and the subsequent production of a number of cytokines. In contrast, binding to CTLA-4, which has a higher affinity for B7, downregulates T-cell activation and diminishes the immune response by competing for the binding site of B7 to CD28.[17] Therefore, B7 molecules—B7.1 (CD80) and B7.2 (CD86)—also play important roles in T-cell activation.

From in vitro and in vivo studies, a role of the costimulatory B7 molecules in the regulation of T-cell proliferation and immune response has been suggested.[18–20] Some studies have investigated the association of polymorphisms of B7 molecules with several systemic autoimmune diseases, including multiple sclerosis,[21] rheumatoid arthritis, and systemic lupus erythematosus[22]; however, the role of B7 molecules in organ-specific autoimmune diseases remains unclear. Therefore, in the present study, we investigated the association between single nucleotide polymorphisms (SNPs) in the genes of B7 molecules (CD86 and CD80) and the susceptibility of developing GO in Taiwanese patients with GD.

Methods

Patients and Data Collection

A total of 471 patients with GD visiting at China Medical University Hospital, Taiwan, were enrolled in this study. All participants were interviewed and examined by an endocrinologist with substantial experience in thyroid diseases. The diagnosis of GD was based on clinical symptoms and biochemical confirmation of hyperthyroidism, diffused goiter, and the presence of ≥1 of the following observations: positive results for thyroid-stimulating hormone receptor antibody tests, diffusely increased iodine-131 uptake in the thyroid gland, and exophthalmos. Patients with GD were categorized according to the NOSPECS system recommended by the American Thyroid Association.[23] The GD patients who had proptosis with or without more severe form (classes 3–6) were defined as having GO. The degree of proptosis was measured using an exophthalmometer and was defined as a distance of the apex of the cornea from the lateral orbital rim >18 mm in either eye or a 2-mm difference in the degree of protrusion between the 2 eyes. The data regarding age, gender, history of tobacco use, thyroid gland pathology, and the affected anatomic site were extracted from full medical records. Blood samples were collected by venipuncture for genomic DNA isolation and serologic tests at the time of enrollment in the study. Informed consent was obtained from each participant before his or her inclusion in this study. The study was approved by the ethics committee of China Medical University hospital.

Genomic DNA Extraction and Genotyping

The genomic DNA was extracted from peripheral blood leukocytes using Genomic DNA kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. To select the most representative SNPs by capturing the majority genetic variation, SNP genotype information was downloaded in December 2008 from the HapMap Han Chinese in Beijing + JPT population. HapMap genotypes were analyzed within Haploview and Tag SNPs were selected using the Tagger function. Eight SNPs for each gene (CD80 and CD86) met the criteria were selected. The following are SNPs we selected for analyses. SNPs belong to CD86 gene: rs129055 (A/G), rs11717893 (A/G), rs2715267 (A/C), rs2715272 (A/G), rs4678186 (A/G), rs765945 (A/C), rs9831894 (A/C), and rs9872483 (A/G). SNPs belong to CD80 gene: rs9289131 (A/G), rs7642502 (A/G), rs1523311 (A/G), rs2049502 (A/G), rs2228017 (A/G), rs16829957 (A/G), rs3915165 (A/C), and rs16829988 (A/G). Genotyping was performed using an allele-specific extension and ligation assay according to the manufacturer's instructions (Illumina, San Diego, CA).

Haplotype Analysis

Haplotype frequencies and effects were examined using the statistical package Haplo.stats[24] in software language R (R 2.8.1). The function Haplo.score was used to assess differences in haplotype frequencies between cases and controls and to calculate a global score test that was used to evaluate the overall significance. Effects of individual haplotypes were also examined by comparing the ophthalmopathy risk associated with each inferred haplotype with the risk associated with the highest estimated frequency haplotype. The estimated odds ratio (OR) and 95% confidence intervals (CIs) were obtained using the function Haplo.glm.

Statistical Analyses

The genotype and allele frequency distributions in the polymorphisms in Graves' patients with or without ophthalmopathy were analyzed by the chi-square test or Fisher exact test for differences in proportions. The OR was calculated from genotype frequencies and allelic frequencies with 95% CI by using unconditional logistical regression adjusting for age of diagnosis, gender and smoking history. The multifactor dimensionality reduction (MDR) method with version 1.1.0 of the open source software package (Dartmouth Medical School, Hanover, NH) was used to detect the best locus–locus interaction models. The interaction dendrogram was built according to hierarchical clustering algorithm. The effect of carrying high-risk haplotypes on serology test among the GO group was evaluated by the 2 sample independent *t*-test. All statistical analyses were conducted using SAS statistical software, version 9.1 (SAS Inc., Cary, NC) and $P<0.05$ (2 sided) was used as the level of significance.

Results

We studied 200 GO cases and 271 GO controls, and a total of 16 SNPs were selected from the genes of CD80 (n = 8) and CD86 (n = 8) to perform genotyping and to investigate the effect of B7 (CD80 and CD86) polymorphisms on GO.

Allele and Genotype Frequencies of CD80 and CD86 Polymorphisms

We did not find a significant deviation from the Hardy-Weinberg equilibrium for any SNP ($P>0.05$). This finding indicated that the

Table 3. Summarizing Multifactor Dimensionality Reduction Models for Gene–Gene Interaction in Graves' Ophthalmopathy Risk

Number of Factors	Best Candidate Models	Testing Accuracy (%)	*P*-Value	Cross-Validation Consistency
1	CD86_rs9831894	55.6	0.0107	10/10
2	CD80_rs9289131 CD86_rs9872483	57.2	0.0010	8/10
3	CD80_rs16829957 CD86_rs9831894 CD86_rs6765945	49.0	0.3770	4/10

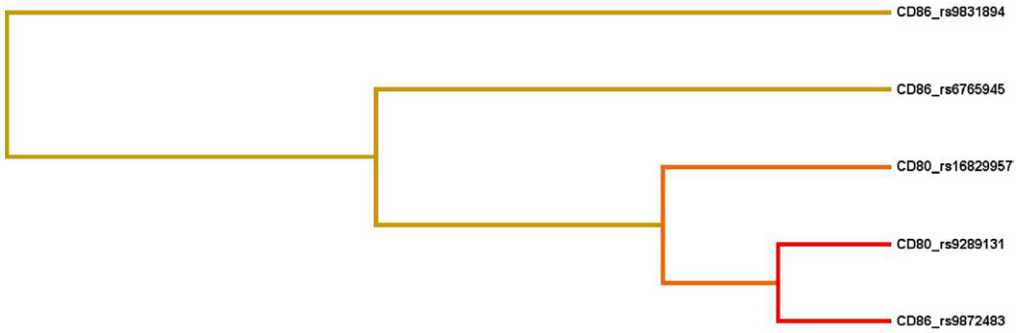

Figure 1. Interaction dendrogram. The interaction dendrogram reveals a strong interaction effect of CD80 and CD86 these 2 genes in modulating the risk of Graves' ophthalmopathy. The location of the longitudinal connecting bars indicates the strength of the dependence: left is weaker and right is stronger. The hierarchical cluster analysis with average linkage place CD80 rs9289131 and CD86 rs9872483 on the same branch.

possibility of a bias resulting from population stratification or a genotyping error was unlikely. In genotype association tests, the polymorphism at position rs9831894 (A→C) in the CD86 gene was statistically associated with GO ($P = 0.0071$). Furthermore, in allele frequency analyses, the frequency of the C allele at position rs9831894 was significantly higher in patients without GO than in those with GO, with an OR of 0.73 (95% CI, 0.55–0.97; Table 1, available online at http://aaojournal.org). However, the distribution of the genotype or the allele frequency of the SNPs within the CD80 gene was not statistically different between the 2 groups (Table 2, available online at http://aaojournal.org).

Multifactor Dimensionality Reduction Analysis

We used MDR analysis to identify the best interaction models among a total of 16 SNPs in the genes of CD80 and CD86. Table 3 summarizes the best interaction models obtained from MDR analysis. Consistent with the individual SNP analysis, in the 1-locus model, the SNP CD86 gene_rs9831894 was the best candidate for predicting a high risk for GO (testing accuracy, 55.6%; cross-validation consistency, 10; sign test, $P = 0.0107$). The best interaction model was a 2-locus model composed of the SNPs CD86 gene _rs9872483 and CD80 gene _rs9289131 (testing accuracy, 57.2%; cross-validation consistency, 8; sign test, $P = 0.0010$). From the interaction dendrogram (Fig 1), a strong synergistic effect of these 2 loci was found in modeling the risk of GO.

Frequencies of the CD80 and CD86 Haplotype

Analysis of the rs9289131 (*CD80* gene) and rs9872483 (*CD86* gene) haplotype was used to investigate a potential gene–gene interaction. The overall global test showed a difference in the frequency of haplotypes between the cases and controls for rs9289131-rs9872483 ($P = 0.0246$). The haplotype G-A was significantly inversely associated with a high risk for GO (OR, 0.63; 95% CI, 0.44–0.90; Table 4). Furthermore, we investigated the effect of G-A haplotypes on clinical serology tests among GO patients. The patients carrying the G-A haplotype had a lower level of free thyroxine (free T_4) compared with those not carrying the G-A haplotype ($P = 0.0001$; Table 5).

Discussion

From previous studies, the importance of immunity-related genes in autoimmune diseases has been discussed, and the crucial role of the costimulatory molecules—CD80 and CD86—for T-cell activation and inhibition has been established. However, the relationship between gene polymorphisms and the susceptibility of developing GO remains unclear. In the present study, we investigated the association between B7 molecules (CD80 and CD86) polymorphisms and GO in a Chinese population in Taiwan.

Both CD80 and CD86 genes are located on chromosome 3q21 and have similar costimulatory ligands to activate T cells. CD86 could be expressed on resting T, B, and natural killer cells, or monocytes.[25] CD86 can be quickly induced on activated macrophages, B cells, and natural killer cells,[26] but not CD80. In addition, the role of costimulation via CD80 or CD86 in the polarization of the T-helper response is different. CD80 preferentially acts as a costimulator for the generation of T-helper 1 cells, whereas CD86 costimulation induces the differentiation toward the T-helper 2 functional phenotype.[27]

Table 4. Haplotype Frequency of CD80_rs9289131 and CD86_rs9872483 among Graves' Disease Patients

Haplotype	Frequency (%)		Haplotype-Specific Test P-Value	OR (95% CI)
	Without GO ($n = 542$)	*With* GO ($n = 400$)		
G-A	39	29	**0.00747**	**0.63 (0.44,0.90)**
A-G	17	16	0.49784	0.80 (0.50, 1.29)
G-G	34	39	0.29026	1
A-A	11	17	0.09272	1.32 (0.85, 2.05)
			$P_{Global\ Score\ Test} = 0.0246$	

CI = confidence interval; GO = Graves' ophthalmopathy; OR = odds ratio.

Table 5. Effect of Carrying the G-A Haplotype on Clinical Serology Test in Graves Disease Ophthalmopathy Patients

	G-A Haplotype		
	With (n = 101), Mean (SD)	*Without (n = 76), Mean (SD)*	**P-Value (*t*-test)**
Free T 4	1.49 (1.09)	2.21 (1.61)	0.0001
TSH	2.46 (8.38)	1.17 (3.12)	>0.05
TRAb*	49.47 (23.47)	54.14 (27.00)	>0.05

*With G-A haplotype (n = 53); without G-A haplotype (n = 4).

To the best of our knowledge, this is the first population-based study to show that the organ-specific autoimmune disease GD was associated with SNPs in the CD86 gene. Moreover, the interaction between CD80 and CD86 genes was found in the present study. However, an association between SNPs in the CD80 gene and GO was not found in the present study, probably because the number of patients diagnosed with GO might have been too small to detect the effect of CD80. A larger sample size should be considered in future studies. From previous studies, CD86 polymorphisms were found to be associated with asthma and related allergic disorders.[28,29] However, Matsushita et al[22] found that polymorphisms in the genes of CD80, CD86, and CTLA-4 were not associated with systemic autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus in a Japanese population.

By using genotype and allele frequencies analyses, we demonstrated that the C genotype at position rs9831894 of the CD86 gene may play a protective role in the development of ophthalmopathy among GD patients. Furthermore, we tested the genotype frequency of rs9831894 between GD patients and a normal population of Han Chinese in Beijing (data from HapMap database) to investigate its protective role in the development of GD. The association with GD was borderline significant ($P = 0.0505$). This could be owing to a lower statistical power in the small Han Chinese in Beijing population (n = 83). These results provide evidence of a protective role of CD86 in the development of ophthalmopathy among GD patients. Furthermore, we investigated the potential interaction between CD80 and CD86 by using MDR analysis. Interestingly, in a 2-locus interaction model, CD80_rs9289131-CD86_rs9872483, was identified as the best model to predict the risk of GO, although none of these SNPs were identified to be associated with GO independently. The protective effects of the G-A haplotype of CD80–CD86 on symptoms of ophthalmopathy was found. Moreover, the higher percentage of GO patients carrying the G-A haplotype had normal levels of free T_4 compared with those not carrying the G-A haplotype (54.46% vs 39.47%; $P = 0.0483$). This finding suggests that the B7 genes play a protective role.

In conclusion, this study provides evidence that the CD86 gene polymorphism is related to GO in GD patients. Furthermore, a protective role of a gene–gene interaction between CD80 and CD86 in the development of GO and in the clinical response was identified.

References

1. Mishra A, Mishra SK. Multicentre study of thyroid nodules in patients with Graves' disease (Br J Surg 2000;87:1111-13) [letter]. Br J Surg 2001;88:313.
2. Gianoukakis AG, Smith TJ. Recent insights into the pathogenesis and management of thyroid-associated ophthalmopathy. Curr Opin Endocrinol Diabetes Obes 2008;15:446–52.
3. Anvari M, Khalilzadeh O, Esteghamati A, et al. Genetic susceptibility to Graves' ophthalmopathy: the role of polymorphisms in proinflammatory cytokine genes. Eye (Lond) 2010; 24:1058–63.
4. Gianoukakis AG, Khadavi N, Smith TJ. Cytokines, Graves' disease, and thyroid-associated ophthalmopathy. Thyroid 2008;18:953–8.
5. Perros P, Neoh C, Dickinson J. Thyroid eye disease. BMJ 2009;338:b560.
6. Kuriyan AE, Phipps RP, Feldon SE. The eye and thyroid disease. Curr Opin Ophthalmol 2008;19:499–506.
7. Khoo TK, Bahn RS. Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies. Thyroid 2007;17:1013–8.
8. Bednarczuk T, Hiromatsu Y, Inoue Y, et al. T-cell-mediated immunity in thyroid-associated ophthalmopathy. Thyroid 2002;12:209–15.
9. Esteghamati A, Khalilzadeh O, Mobarra Z, et al. Association of CTLA-4 gene polymorphism with Graves' disease and ophthalmopathy in Iranian patients. Eur J Intern Med 2009; 20:424–8.
10. Kouki T, Sawai Y, Gardine CA, et al. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. J Immunol 2000;165:6606–11.
11. Yanagawa T, Hidaka Y, Guimaraes V, et al. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. J Clin Endocrinol Metab 1995;80:41–5.
12. Bossowski A, Stasiak-Barmuta A, Urban M. Relationship between CTLA-4 and CD28 molecule expression on T lymphocytes and stimulating and blocking autoantibodies to the TSH-receptor in children with Graves' disease. Horm Res 2005;64:189–97.
13. Bossowski A, Stasiak-Barmuta A, Urban M, Rinderle C. Analysis of costimulatory molecules (CD28-CTLA-4/B7) expression on chosen mononuclear cells in adolescents with Graves' disease during methimazole therapy [in Polish]. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw 2004;10:93–101.
14. Tomer Y, Greenberg DA, Barbesino G, et al. CTLA-4 and not CD28 is a susceptibility gene for thyroid autoantibody production. J Clin Endocrinol Metab 2001;86:1687–93.
15. Ichimura M, Kaku H, Fukutani T, et al. Associations of protein tyrosine phosphatase nonreceptor 22 (PTPN22) gene polymorphisms with susceptibility to Graves' disease in a Japanese population. Thyroid 2008;18:625–30.
16. Kavvoura FK, Akamizu T, Awata T, et al. Cytotoxic T-lymphocyte associated antigen 4 gene polymorphisms and autoimmune thyroid disease: a meta-analysis. J Clin Endocrinol Metab 2007;92:3162–70.
17. Janeway CA, Travers P, Walport M, Schlomchik M. Immunobiology: The Immune System in Health and Disease. 6th ed. New York: Garland Sci.; 2005:328–9.
18. Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. Proc Natl Acad Sci U S A 2004;101:10398–403.
19. Chang TT, Kuchroo VK, Sharpe AH. Role of the B7-CD28/

CTLA-4 pathway in autoimmune disease. Curr Dir Autoimmun 2002;5:113–30.
20. Ziller C, Stoeckel F, Boon L, Haegel-Kronenberger H. Transient blocking of both B7.1 (CD80) and B7.2 (CD86) in addition to CD40-CD40L interaction fully abrogates the immune response following systemic injection of adenovirus vector. Gene Ther 2002;9:537–46.
21. Teutsch SM, Booth DR, Bennetts BH, et al. Association of common T cell activation gene polymorphisms with multiple sclerosis in Australian patients. J Neuroimmunol 2004;148: 218–30.
22. Matsushita M, Tsuchiya N, Oka T, et al. New polymorphisms of human CD80 and CD86: lack of association with rheumatoid arthritis and systemic lupus erythematosus. Genes Immun 2000;1:428–34.
23. Werner SC. Modification of the classification of the eye changes of Graves' disease: recommendations of the Ad Hoc Committee of the American Thyroid Association. J Clin Endocrinol Metab 1977;44:203–4.
24. Schaid DJ, Rowland CM, Tines DE, et al. Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genet 2002;70:425–34.
25. Azuma M, Ito D, Yagita H, et al. B70 antigen is a second ligand for CTLA-4 and CD28. Nature 1993;366:76–9.
26. Hathcock KS, Laszlo G, Pucillo C, et al. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J Exp Med 1994;180:631–40.
27. Battifora M, Pesce G, Paolieri F, et al. B7.1 costimulatory molecule is expressed on thyroid follicular cells in Hashimoto's thyroiditis, but not in Graves' disease. J Clin Endocrinol Metab 1998;83:4130–9.
28. Chen YQ, Shi HZ. CD28/CTLA-4—CD80/CD86 and ICOS—B7RP-1 costimulatory pathway in bronchial asthma. Allergy 2006;61:15–26.
29. Corydon TJ, Haagerup A, Jensen TG, et al. A functional CD86 polymorphism associated with asthma and related allergic disorders. J Med Genet 2007;44:509–15.

Footnotes and Financial Disclosures

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