CLINICAL STUDY

Association of *CD40* and *thyroglobulin* genes with later-onset Graves' disease in Taiwanese patients

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Abstract

Objective: Graves' disease (GD) is known to be associated with thyroglobulin (TG) and *CD40* genes. Therefore, we decided to investigate the relationship of age at onset of GD with *CD40* and TG gene susceptibilities in a Taiwanese population.

Design and method: We analyzed the association of TG and *CD40* polymorphisms with age at onset of GD in Taiwanese patients. We stratified patients into those with early onset (<40 years; 30.3 ± 4.8 years; $n = 135$) and later onset (≥ 40 years; 52.3 ± 6.3 years; $n = 80$) and compared the results with those of 141 normal controls.

Results: We found a significant statistical difference in the T/T genotype frequency of E33 single nucleotide polymorphism (SNP) and G/G genotype frequency of E12 SNP when compared with the control group ($P < 0.001$). In addition, the frequencies of the T allele and TT genotype of the *CD40* SNP were found to be significantly increased in GD patients who developed GD aged over 40 years than those below 40 years (allele: $\chi^2 = 5.299$, $P = 0.021$, OR = 1.597; genotype: $\chi^2 = 6.168$, $P = 0.046$). By contrast, the frequencies of genotypes in the TG gene E10, E12, and E33 SNPs were not found to be significantly different in GD patients who developed GD when aged over 40 years when compared with those aged below 40 years.

Conclusions: These data suggest that the T/T genotype and T allele in the *CD40* gene are more likely to be associated with late-onset GD in Taiwanese patients.

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Introduction

Graves' disease (GD) is an autoimmune lymphocyte-mediated disease characterized by clinical hyperthyroidism with diffuse goiter, ophthalmopathy (1, 2), and the presence of anti-thyroid-stimulating hormone (TSH) receptor antibodies (3), although both environmental and genetic factors appear to play a role in disease susceptibility (4). Although the precise mechanism for the pathogenesis of this disorder is not fully understood, the *CD40* and thyroglobulin (TG) genes have been considered to be major genetic factors involved in the development of GD.

An association of the C/T polymorphism in the *CD40* gene with GD in Caucasians (5) and Koreans (6) has recently been established; however, the association could not be confirmed in UK Caucasians (7, 8). Moreover, there is well-established evidence concerning the association of TG gene polymorphisms with GD in Taiwanese (9) and Caucasians (10).

A previous study revealed that different allelic human leukocyte antigen (HLA) associations have

been reported in patients with early onset of GD than those from patients with later onset of GD in a Japanese population (11). In addition, another study suggested that the relative risk for DRB1*0301 was higher for juvenile patients than for adult patients with GD in Caucasian patients (12). Therefore, we decided to investigate the relationship of age at onset of GD with *CD40* and TG gene susceptibilities in a Taiwanese population. This study investigated the association of *CD40* and TG gene polymorphisms with patients who developed GD aged over 40 years and those below 40 years and compared the results with normal controls.

Subject and methods

Subjects

We enrolled 215 Chinese patients with GD (151 females and 64 males; age, 40 ± 13 years) from the Endocrine Clinic and 141 healthy controls (84 females and

57 males; age, 41 ± 12 years) from the Health Care Center of Kaohsiung Medical University Hospital. GD was diagnosed based on clinical and laboratory evidence of hyperthyroidism and diffuse goiter, supported by the presence of TSH-receptor antibodies, or exophthalmos. We excluded patients with a history of radioiodine therapy or previous thyroid surgery. The controls were healthy subjects with no clinical evidence or family history of any autoimmune disease. They were in the euthyroid state according to the laboratory tests and had no obvious goiter, as determined by an experienced research staff member. The patients and control subjects were recruited after giving fully informed written consent. The clinical trial was registered with the Institutional Review Board of Kaohsiung Medical University Hospital, Taiwan, ROC (KMUH-IRB-960130).

Genotypes

DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (RadioMed, Tyngsboro, MA, USA). The TG single nucleotide polymorphisms (SNPs) (E10, E12, and E33) at the TG gene were genotyped by the PCR-restriction fragment length polymorphism method, using the primers listed in Table 1. The CD40 SNP at the CD40 gene was digested using the PCR-restriction fragment length polymorphism method; the primers used were: forward CD40 gene 5'-CCTCTTCCCCGAAGTCTTCC-3' and reverse CD40 gene 5'-GAAACTCCTGCGCGGTGAAT-3'.

PCR was carried out in a 25 µl mixed solution, containing 0.2 µg genomic DNA (from blood leukocytes), 0.4 mmol/l primers, 0.2 mmol/l each of deoxy-ATP, -GTP, -CTP, and -TTP, 2 mmol MgCl₂, 0.5 U Taq DNA polymerase, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3). PCR involved an initial denaturation step for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s at 56 °C, and primer extension for 60 s at 72 °C, followed by a final extension step for 5 min at 72 °C in a thermal-cycler (Gene Amp PCR System 9600; Perkin-Elmer, Foster City, CA, USA). The amplified products of the TG SNPs (E10, E12, and E33) were digested with the restriction enzymes, Bpu10I, BsaAI, and Hpy99I at 37 °C for 4 h (New

England BioLabs, Hitchin, UK; Table 1); the CD40 SNP amplified products were digested with the restriction enzyme StyI at 37 °C for 4 h (New England BioLabs) and analyzed on 3% agarose gel.

Laboratory test

Serum T₄, T₃ and TSH levels were measured by RIA. TSH levels were measured using a one step sandwich assay with a normal range of 0.25–4.0 µU/ml (0.25–4.0 mU/liter; RIA-gnosthTSH; CIS Bio International, Gif-Sur-Yvette, Cedex, France). Serum T₄ and T₃ concentrations were measured using commercial kits (Abbott), with the normal ranges being 12.0–27.5 pmol/l and 1.31–2.74 nmol/l respectively. TSH receptor antibody was measured by radioreceptor assay with a commercial kit (Dia Sorin Inc., Stillwater, MN, USA). The cut-off value for TRAb was 10%.

Statistical analysis

The laboratory data were expressed as means ± s.d. Statistical analysis was performed using the Statistical Package for the Social Science program (SPSS for Windows, Version 10.0; SPSS, Chicago, IL, USA). Allele frequencies were estimated by direct gene counting. Observed numbers of each genotype were compared with those expected for Hardy–Weinberg equilibrium using the χ² test. In this study, a two-tailed P-value less than 0.05 was considered significant.

Results

Association of TG and CD40 gene polymorphisms with susceptibility to GD

The genotype and allele frequencies at E10 SNP158, E12 SNP, E33 SNP, and CD40 SNP in GD patients were compared with those of the controls. The distribution of the genotypes was compatible with that of Hardy–Weinberg equilibrium in both the control and patient groups. There was a significant statistical difference in the T/T genotype of E33 SNP and G/G genotype of E12 SNP

Table 1 SNPs in the TG gene.

SNP	Location in TG gene	Base change	Primers ^a	PCR product size (bp)	RFLP enzyme	RFLP product size (bp)
E10 SNP158	Exon 10	T/C	F: CACCTGCTCATTGTTCTCTCC R: TCTTCACTAGCAGCTTGGCA	264	Bpu10I	TT: 82, 182 CC: 264
E12 SNP	Exon 12	A/G	F: CAGAGCCCACACAGAGCAGG R: AAAAAGGGGTGTCACTTGGC	241	BsaAI	GG: 105, 136 AA: 241
E33 SNP	Exon 33	C/T	F: ATATTGACCAAAGCACCCCC R: ATTAGCCAGTTGCCCTCTCC	375	Hpy99I	CC: 167, 208 TT: 375

^aF, forward primer sequence; R, reverse primer sequence.

when compared with the control group ($P < 0.001$; Table 2). The genotype and allele frequency at the *CD40* SNP in GD patients were not found to be significantly different to those of the controls by the χ^2 test (genotype: $\chi^2 = 0.084$, $P = 0.959$; allele: $\chi^2 = 0.032$, $P = 0.859$; Table 2).

Association of the *CD40* gene polymorphism with the age of the onset of Graves' disease

There were two peaks in the age of onset of GD (Fig. 1). The frequencies of the T allele and TT genotype at the *CD40* SNP were found to be significantly increased in GD patients who developed GD aged over 40 years than those aged below 40 years. (allele: $\chi^2 = 5.299$, $P = 0.021$, OR = 1.597; genotype: $\chi^2 = 6.168$, $P = 0.046$; TT + CT versus CC, $\chi^2 = 5.007$, $P = 0.025$, OR = 2.000; Table 3).

Association of the *TG* gene polymorphism with the age of the onset of Graves' disease

There was no significant difference in the TT genotype frequency of the *TG* gene E10 polymorphism between the patients who developed GD aged above 40 years and those aged below 40 years (genotype: $\chi^2 = 2.972$, $P = 0.226$; Table 4). Similarly, in the *TG* gene E12 and E33 polymorphisms, no significant differences were found between the patients who developed GD aged over 40 years and those below 40 years (data not shown).

Table 2 Allele and genotype frequencies of the *TG* and *CD40* SNPs in patients with Graves' disease (GD) and control subjects.

Alleles/ genotype	Normal control group (N= 141)	Graves' disease group (N=215)	χ^2	P value
TG E10 SNP158				
T	113 (40.1%)	162 (37.7%)	0.413	0.521
C	169 (59.9%)	268 (62.3%)		
TT	16 (11.3%)	18 (8.40%)		
TC	81 (57.4%)	126 (58.6%)	0.896	0.639
CC	44 (31.2%)	71 (33.0%)		
TG E12 SNP				
G	108 (38.3%)	135 (31.4%)	3.609	0.057
A	174 (61.7%)	295 (68.6%)		
GG	15 (10.6%)	6 (2.80%)		
GA	78 (55.3%)	123 (57.2%)	9.747	0.008
AA	48 (34.1%)	86 (40.0%)		
TG E33 SNP				
C	112 (39.7%)	107 (24.9%)	17.593	<0.001
T	170 (60.3%)	323 (75.1%)		
CC	18 (12.8%)	9 (4.20%)		
CT	76 (53.9%)	89 (41.4%)	19.357	<0.001
TT	47 (33.3%)	117 (54.4%)		
CD40 SNP				
T	107 (37.9%)	166 (38.6%)	0.032	0.859
C	175 (62.1%)	264 (61.4%)		
TT	15 (10.6%)	25 (11.6%)		
CT	77 (54.6%)	116 (54.0%)	0.084	0.959
CC	49 (34.8%)	74 (34.4%)

Figure 1 Distribution of age at onset of Graves' disease.

Interaction between the *TG* exon 10 SNP and the *CD40* SNP for the age of the onset of Graves' disease

We analyzed for gene–gene interaction between the associated *TG* and *CD40* SNPs. Case control association analysis of the *TG* SNPs in combination with the *CD40* SNPs showed no evidence of an interaction between *CD40* and *TG* exon 10 SNP.

Discussion

This study had three major findings. Firstly, we found a significant statistical difference in the T/T genotype of E33 SNP and the G/G genotype of E12 SNP when compared with the control group. Secondly, we found that the frequencies of the T allele and TT genotype at the *CD40* SNP were found to be significantly increased in GD patients who developed GD aged above 40 years than those aged below 40 years. Thirdly, no significant difference was found in the frequencies of genotype in the *TG* gene E10, E12 and E33 SNPs between GD patients who developed GD aged above 10 years and those below 40 years.

The prevalence of autoimmune thyroid diseases (AITD) increases with age. Apoptosis plays a pivotal role in the regulation of immune mechanisms in the pathogenesis of AITD. There is considerable evidence that FAS and FAS ligand (FASL) interaction is implicated in the pathogenesis of autoimmune disorders (13, 14). Mysliwiec *et al.* (15) found positive correlations between sFAS and age in GD patients. FAS/FASL and BCL2 signaling pathways seem to be age related and may explain, at least in part, the milder course of Graves' disease in elderly patients. Hiromatsu *et al.* (16) suggest a role of FAS/FASL interaction in the pathogenesis of GD, and their results suggest that the increased expression of FASL in GD thyrocytes, the down-regulation of FAS expression by TSH or possibly by TSH receptor autoantibody, and the overexpression of BCL2, which could render thyrocytes resistant to FASL-mediated elimination, may thus be involved in the pathogenesis of GD. Our studies have confirmed the relationship between the basic pathogenesis of GD and the age at

Table 3 Association between the *CD40* gene polymorphism in patients with Graves' disease (GD) and age at onset.

<i>CD40</i>	Age at onset of GD		Control subjects (N=141)	GD ≥ 40 years versus GD < 40 years	GD ≥ 40 years versus controls
	<40 years (N=135)	≥ 40 years (N=80)			
Genotype					
TT	12 (8.90%)	13 (16.2%)	15 (10.6%)	$\chi^2=6.168$	$\chi^2=2.979$
CT	69 (51.1%)	47 (58.8%)	77 (54.6%)	$P=0.046$	$P=0.225$
CC	54 (40.0%)	20 (25.0%)	49 (34.8%)		
TT+CT	81 (60.0%)	60 (75.0%)	92 (65.2%)	$\chi^2=5.007$	$\chi^2=2.260$
CC	54 (40.0%)	20 (25.0%)	49 (34.8%)	$P=0.025$	$P=0.174$
				OR=2.000 (1.045–3.904)	OR=1.598 (0.834–3.125)
Allele					
T	93 (34.4%)	73 (45.6%)	107 (37.9%)	$\chi^2=5.299$	$\chi^2=2.495$
C	177 (65.6%)	87 (54.4%)	175 (62.1%)	$P=0.021$	$P=0.114$
				OR=1.597 (1.049–2.428)	OR=1.372 (0.908–2.072)

onset of GD, which is known to be associated with an increased risk for Hashimoto thyroiditis (17). This study further demonstrated a relationship between the age at onset of GD and the T/T genotype in the *CD40* gene and the exon 10 T/T genotype in the *TG* gene.

In the previous studies, the associations between age at onset and different HLA genotypes in Caucasians (18), and Japanese (19), and ICAM-1 gene alleles in Caucasians (20) and *CD40* genotypes in Japanese (21), have been reported. Therefore, we evaluated the differences between the frequencies of the *CD40* and *TG* genotypes in Taiwanese patients with GD onset aged below 40 years and those with a later onset of GD. Our study group showed that there are two peaks in the distribution of age at onset of GD. The results suggest that at least two factors may influence GD susceptibility; however, we emphasized that age at onset of GD should be considered in the analysis. No studies to date have examined the association between the age at GD onset and *CD40* and *TG* genotypes in Taiwanese patients. In addition, we wondered whether the age at GD onset may influence the thyroid autoimmunity of GD across populations of different ethnic backgrounds.

The *TG* gene may cause a predisposition to GD by the mechanisms of sequence changes in *TG* may change its antigenicity making it more immunogenic (22). E10 SNP158 is a silent SNP (i.e., it does not cause an amino acid substitution). The E12 SNP has been identified as causing an amino acid substitution (Val to Met). The change from a nonpolar amino acid (Val) to a nonpolar amino acid (Met) would not be expected to change the structure of *TG* in this region. The E33 SNP has been identified as causing a nonconservative amino acid substitution (Trp to Arg), and the change from a hydrophobic amino acid (Trp) to a positively charged hydrophilic amino acid (Arg) would be expected to change the structure of *TG* at this region; however, the precise mechanism for the pathogenesis of this disorder is not fully understood. GD most likely develops as a consequence of a complex interaction between genetic susceptibility and environmental effects.

In conclusion, the frequency of the TT genotype at the *CD40* SNP showed a significant increase in GD patients who developed GD aged above 40 years when compared with those below 40 years in a Taiwanese population. In addition, we performed case control association analysis

Table 4 Association between the *TG* gene E10 polymorphism in patients with Graves' disease and age at onset.

E10	Age at onset of GD		Control subjects (N=141)	GD ≥ 40 years versus GD < 40 years	GD ≥ 40 years versus controls
	<40 years (N=135)	≥ 40 years (N=80)			
Genotype					
TT	8 (5.9%)	10 (12.5%)	15 (10.6%)	$\chi^2=2.972$	$\chi^2=0.582$
TC	80 (59.3%)	46 (57.5%)	77 (54.6%)	$P=0.226$	$P=0.748$
CC	47 (34.8%)	24 (30.0%)	49 (34.8%)		
TT+TC	88 (65.2%)	56 (70.0%)	125 (88.7%)	$\chi^2=0.530$	$\chi^2=11.979$
CC	47 (34.8%)	24 (30.0%)	16 (11.3%)	$P=0.470$	$P=0.001$
				OR=1.246 (0.662–2.376)	OR=0.229 (0.137–0.641)
Allele					
T	96 (35.6%)	66 (41.3%)	107 (37.9%)	$\chi^2=1.387$	$\chi^2=0.469$
C	174 (64.4%)	94 (58.7%)	175 (62.1%)	$P=0.239$	$P=0.494$
				OR=1.273 (0.834–1.938)	OR=1.148 (0.757–1.738)

of the TG SNPs in combination with the CD40 SNPs, which showed no evidence of an interaction between the CD40 and TG E10 SNPs. These findings suggest the obvious differences in the genetic backgrounds of the GD patients in the early-onset and late-onset groups. The CD40 gene may play an important role in the immunogenetic pathogenesis of late-onset GD, and the TT genotype is likely to be associated with late-onset Taiwanese patients.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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