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A C/T Polymorphism in CD40 Gene is Not Associated with Susceptibility and Phenotype of Graves' Disease in Taiwanese

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Abstract. A single nucleotide polymorphism (SNP) located at position-1 in the Kozak sequence of the CD40 gene has been associated with the development of GD in Caucasian and Koreans. This study investigated possible associated between CD40 SNP and the development of GD in a Taiwanese population. To do this, we enrolled 215 Taiwanese patients with GD and 141 controls from the Endocrine Clinic of Kaohsiung Medical University Hospital. This study investigated the association between gene polymorphism and relapse of hyperthyroidism after the discontinuation of medication in three GD patient groups based on time to relapse and a control group, and compared clinical and laboratory data of patients regrouped in three CD40 SNP genotypes. No significant difference in allele or CD40 SNP genotype frequency was observed between patients with GD and control subjects ($P = 0.859$ and $P = 0.959$, respectively). Furthermore, we analyzed the distribution of CD40 genotypes and three groups based on time to relapse after drug withdrawal. The cutoff points were 9 months, 9 months to 3 years, and more than 3 yr in subgroups of patients with GD divided by clinical and laboratory variables. Although no significant genotype-phenotype associations were found, the T allele and TT genotype frequency was significantly smaller in GD patients who had developed the disease before 35 years old than those who developed it after 35 years old ($x^2 = 6.272$, $P = 0.043$) (TT + CT v.s. CC, $x^2 = 4.951$, $P = 0.030$). These findings suggest that this CD40 gene polymorphism is not associated with GD in Taiwan and is, therefore, not contributing to susceptibility to the disease there.

Key words: Graves' disease, CD40, Gene polymorphisms, SNP

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THE thyroid, a major endocrine gland controlling diverse metabolic pathways is frequently affected by disease. Up to 5% of the overall population suffers from some form of autoimmune thyroid disease (AITD) [1, 2], making it one of the most common autoimmune conditions. Graves' disease (GD) is an autoimmune disorder of the thyroid gland of unknown cause, although both environmental and genetic factors appear to play a role in disease susceptibility [3]. Although autoimmune mechanisms are responsible for the syndrome of Graves' disease, management has been largely directed toward controlling the hyperthyroidism. It is not yet possible to treat the basic pathogenetic factors in GD. There are three therapies used to treat the disease: antithyroid drug therapy, surgery, and radioactive iodine therapy. Antithyroid drug therapy is only palliative, and there is a high relapse rate after withdrawal from antithyroid drugs.

Recently, a functional single nucleotide polymorphism (SNP) in the CD40 gene (20q11.2) at position –1, C to T change (C-1T), affecting the CD40 translation level, has been identified [4, 5]. Jacobson *et al.* produced functional evidence to support the proposed

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Abbreviations: GD, Graves' disease; SNP, single nucleotide polymorphism

role for the CC genotype of the C–T CD40 Kozak singlenucleotide polymorphism (SNP) in susceptibility to Graves' disease (GD) [5]. The authors concluded that the C allele and the CC genotype could be associated with up-regulation of CD40, which may explain the association of the CD40 gene polymorphism with GD. The result was replicated in a small Korean dataset of sixty-six GD patients and fifty-six controls ($P = 0.005$, OR = 1.77) [6]. However, when investigated two independent and much larger UK Caucasian datasets (800 GD patients and 735 control subjects and 451 GD patients and 446 control subjects, respectively), no evidence supporting the association of the CD40 Kozak SNP was reported (genotypic $P = 0.145$ and $P = 0.96$, respectively) [7, 8]. However, Jacobson *et al*. [7] have suggested that the CC genotype of the CD40 Kozak SNP can be associated with GD ($P = 0.05$, OR = 1.22), assuming a recessive mode of inheritance for the CC genotype (where only the CC genotype is associated with disease), when grouped against the TT and CT genotypes.

Given the genetic heterogeneity of GD [9–11], we decided to replicate the association of CD40 polymorphism with GD in a Taiwanese population. In addition, we analyzed the interactions between CD40 geneotypes and controls as well as the association of CD40 polymorphism with the clinical phenotype of GD (initial onset age, smoking, ophthalmopathy, initial serum thyroid hormone levels, antithyroid treatment regimen, initial goiter size, goiter after treatment, initial TSH receptor antibodies, and TSH receptor antibodies after treatment).

This study also investigated the association between gene polymorphism and relapse of hyperthyroidism after medication was discontinued in three GD patient groups and a control group, compare clinical and laboratory data obtained from patients with the three different genotypes of the CD40 gene. We also performed a case-control study of GD patients and control subjects to determine whether CD40 SNP could be related to the development of GD in a Taiwanese population.

Subject and Methods

Patients and controls

We enrolled 215 Chinese patients with GD (151 females and 64 males; age, 40 ± 13 yr) from the Endocrine Clinic and 141 healthy controls (84 females and 57 males; age, 41 ± 12 yr) from the health care center of Kaohsiung Medical University Hospital. GD was diagnosed based on clinical and laboratory evidence of hyperthyroidism and diffuse goitre, supported by the presence of TSH-receptor antibodies and/or antimicrosomal antibodies and/or antithyroglobulin antibodies, or exophthalmos. Only patients who completed a treatment course of at least 1 yr and had adequate follow-up after drug withdrawal were included. We excluded patients with a history of radioiodine therapy or previous thyroid surgery. The controls were healthy subjects without 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 experienced research staff member. The patients and control subjects were recruited after giving fully-informed written consent. The clinical trial was registered with Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-960130).

Treatment and follow-up

Antithyroid treatment was started with methimazole 30 mg or propylthiouracil 300 mg daily. The drug dosage was decreased to two -thirds the initial dose when normal levels of T4 and T3 were achieved, usually 1 to 2 months after beginning treatment. The dose was then titrated gradually to reach a maintenance dose of methimazole 5–10 mg or propylthiouracil 50–100 mg daily by the third to fourth month of treatment. The duration of treatment lasted from one to three years. Antithyroid drug stopped the clinical hyperthyroidism symptoms/signs subsided and thyroid function was found to be euthyroid. The mean times of antithyroid drug treatment were 17.4 ± 7.3, 16.3 ± 6.3, and 12.8 ± 5.9 months for groups 1, 2, and 3, respectively. After drug withdrawal, patients were to asked to come in for follow-ups every 3 months in the first year and then every 6 months thereafter. Relapse was confirmed by clinical presentation and the laboratory data. Common symptoms included palpitation, tremor, body weight loss, and menstrual irregularity. The laboratory data indicating recurrence was having serum T4 and/or T3 levels exceeding the upper limit of the normal range of our laboratory. The 215 patients were divided into three groups according to the time to relapse. Group 1 patients (n = 78) had an early relapse within 9 months

after drug withdrawal. Group 2 (n = 71) had a relapse between 10 and 36 months after stopping treatment. Group 3 (n = 66) had either remained in remission for more than 3 yr until the present time or relapsed after 3 yr of drug withdrawal. The rationale for selection of the cutoff point at 9 months instead of 1 yr for group 1 patients was to ensure an even distribution of number of patients among the three groups. To be sure that the cutoff point of 9 months would not influence our results, we also analyzed the data using 1, 2, and 3 yrs as the cutoff points and found the results to be without difference.

Evaluation of patients

Clinical and laboratory evaluation included CD40 genotype, serum levels of T4, T3, and TSH, antithyroid treatment regimen (methimazole vs. propylthiouracil), and goiter size, and TSH-receptor antibodies at the beginning and end of treatment. Goiter size was determined by palpation and classified into: grade 1, a palpable goiter not reaching the medial edge of the sternocleidomastoid muscle; grade 2, a palpable goiter reaching the sternocleidomastoid muscle but not exceeding the lateral edge; and grade 3, a palpable goiter exceeding the lateral edge of the sternocleidomastoid muscle. The severity of ophthalmopathy was assessed according to the NOSPECS classification [13]. Patients with proptosis, extraocular-muscle dysfunction, exposure keratitis and optic neuropathy (NOSPECS class III and higher) were considered to have clinical evidence. 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, North Chicago, IL), 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). The cut-off value for TRAb was 10%.

Genotype

DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (RadioMed, Tyngsboro, MA.). The CD40 SNP at the CD40 gene were digested using the PCR-restriction fragment length polymorphism method, The primers were:

forward
CD40 gene- 5'-CCTCTTCCCCGAAGTCTTCC-3'
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 initial denaturation step for 5 mins at 95ºC, 35 cycles of denaturation for 30 s at 95ºC, primer annealing for 30 s at 56ºC, and primer extension for 60 s at 72ºC, followed by a final extension step for 5 min at 72ºC in a thermal-cycler(Gene Amp PCR System 9600; Perkin-Elmer, Foster City CA). The amplified products were digested with the restriction enzyme Sty I at 37ºC for 4 hours (New England BioLabs, Hitchin, UK), and analyzed on 3% agarose gel.

Statistics

The laboratory data were expressed as means ± standard deviation (SD).

Statistical analysis were performed using the Statistical Package for the Social Science program (SPSS for Windows, Version 10.0.; SPSS, Chicago). Allele frequencies were estimated by direct gene counting. Observed numbers of each genotype were compared with those expected for Hardy-Weinberg equilibrium using the x^2 test. Comparisons of individual clinical and laboratory variables between groups 1, 2, and 3 were assessed using one-way ANOVA for the continuous data. x^2 test or Fisher exact test was used for categorical data. In this study, a two-tailed *P*-value less than 0.05 was considered significant.

Results

Association of CD40 Gene C/T polymorphism with susceptibility to GD

We measured the difference of genotypic distribution and allele frequencies between the GD patients and controls. The genotype and allele frequency at

Table 1. Allele and genotype frequencies of the CD40 SNP in patients with GD and control subjects

CD40		Graves' disease (n = 215)	Control (n = 141)	χ^2	*P* value
Genotype	TT	25 (11.6%)	15 (10.6%)	0.084	0.959
	CT	116 (54%)	77 (54.6%)		
	CC	74 (34.4%)	49 (34.8%)		
	TT + CT	141 (65.6%)	92 (65.2%)	0.004	1.000
	CC	74 (34.45)	49 (34.8%)		
Allele	T	166 (38.6%)	107 (37.9%)	0.032	0.859
	C	264 (61.4%)	175 (62.1%)		

CD40 SNP in GD patients were compared with those of the controls. The patient groups and controls had similar genotype distributions, as estimated by Hardy-Weinberg equilibrium. Both GD patients and controls had similar allele frequencies and genotype distributions for the CD40 SNP. The GD patients were found not to be significantly different than the controls by the χ^2 test (genotype: $\chi^2 = 0.084$, $P = 0.959$; allele: $\chi^2 = 0.032$, $P = 0.859$) (Table 1). The CC genotype was present in 34.4% of the GD patients and 34.8% of controls and, correspondingly, the TT + CT genotypes were present in 65.6% of patients and 65.2% of controls. No significant difference was found ($\chi^2 = 0.004$, $P = 1.000$).

Comparison of the clinical and laboratory variables among patients with the three different genotypes in CD40 gene

We calculated the differences in clinical and laboratory data among the GD patients with the three genotypic distribution in CD40 SNP. We analyzed differences in age, gender, smoking, ophthalmopathy, initial serum thyroid hormone levels, antithyroid treatment regimen, initial goiter size, goiter after treatment, initial TSH receptor antibodies, TSH receptor antibodies during and after treatment of patients with the three different genotypes in three different CD40 SNP. We compared the clinical and laboratory data of patients belonging to the three different genotypes (C/C, C/T, and T/T) of CD40SNP. We found no significant difference with regard to any of the clinical and laboratory data (Table 2).

Association of CD40 Gene C/T polymorphism with relapse of hyperthyroidism after discontinuation of medication among the three groups of GD patients

The 215 patients were divided into three groups based on time to relapse after drug withdrawal, with cutoff points being 9 months, 9 months to 3 years, and more than 3 years. We then measured the difference of genotypic distribution and allele frequencies among the three groups of GD patients. All three patient groups had similar genotype distributions, this site as estimated by Hardy-Weinberg equilibrium. The three GD patients groups were found to have no significant differences by the χ^2 test (genotype: $\chi^2 = 1.776$, $P = 0.777$; allele: $\chi^2 = 1.211$, $P = 0.546$) (Table 3).

Comparison of the clinical and laboratory variables among patients with the three groups of GD patients

The 215 patients were divided into three groups based on time to relapse after drug withdrawal, cutoff points being 9 months, 9 months to 3 years, and more than 3 years. We calculated the differences in clinical and laboratory data among the GD patients by the three groups. We analyzed differences in age, gender, initial onset age, smoking, ophthalmopathy, initial serum thyroid hormone levels, antithyroid treatment regimen, initial goiter size, goiter after treatment, initial TSH receptor antibodies, and TSH receptor antibodies during and after treatment of patients by patient group. We compared the clinical and laboratory data of patients belonging to the three different groups based on time to relapse after drug withdrawal by genotypes. We found no significant difference with regard to any of these measures (Table 4).

Table 2. Comparison of the clinical and laboratory variables among patients with the three different genotypes in CD40 SNP

CD40	C/C	C/T	T/T	*P* value
Age (yr) ± SD	38.4 ± 12.5	40.2 ± 13.9	44.9 ± 14.6	n.s
Sex (F/M)	53/21	80/36	18/7	n.s
Initial onset Age (yr) ± SD	32.6 ± 12.9	34.9 ± 14.2	39.2 ± 12.3	n.s
Initial T_4 (μg/dl) ± SD	15.5 ± 26.1	8.6 ± 20.1	6 ± 16.6	n.s
Goiter size before treatment	n.s			
Grade 1	2	4	0	
Grade 2	21	34	9	
Grade 3	51	78	16	
TBII before treatment	n.s			
Positive	74	116	25	
Negative	0	0	0	
Goiter size after treatment	n.s			
Grade 1	16	25	5	
Grade 2	42	74	16	
Grade 3	15	17	4	
TBII before treatment	n.s			
Positive	29	44	6	
Negative	45	72	19	
Duration of therapy (month) ± SD	14.9 ± 10.8	16.6 ± 14.2	17.8 ± 17.8	n.s
Antithyroid treatment (MTZ/PTU)	56/18	87/29	17/8	n.s
Opththalmopathy	29/45	47/69	8/27	n.s
Smoking	16/58	16/100	5/20	n.s
Family history	28/46	42/74	9/16	n.s

Table 3. Gene polymorphism at the CD40 SNPs and relapse of hyperthyroidism after discontinuation of medication in patients with GD

CD40		Group 1 (n = 78)	Group 2 (n = 71)	Group 3 (n = 66)	χ^2	*P* value
Genotype	TT	8 (10.3%)	11 (15.5%)	6 (9.1%)	1.776	0.777
	CT	42 (53.8%)	38 (35.5%)	36 (54.5%)		
	CC	28 (35.9%)	22 (31%)	24 (36.4%)		
	TT + CT	50 (64.1%)	49 (69%)	42 (63.6%)	0.557	0.757
	CC	28 (35.9%)	22 (31%)	24 (36.4%)		
Allele	T	58 (37.2%)	60 (42.3%)	48 (36.4%)	1.211	0.546
	C	98 (62.8%)	82 (57.7%)	84 (63.6%)		

Group1–3 according to the cutoff points of 9 m, 9 m–3 years, and more than 3 yr.

Association of the CD40 Gene polymorphism with the age of the onset of Graves' disease

The T allele and TT genotype frequency was significantly smaller in GD patients who developed GD before 35 years old than those who developed the disease after 35 years old ($\chi^2 = 6.272$, $P = 0.043$) (TT + CT v.s. CC, $\chi^2 = 4.951$, $P = 0.030$) (Table 5).

Discussion

This study had three major findings. First, the CD40 gene polymorphism may be not associated with GD in the Taiwanese. Second, the CD40 gene polymorphism may not influence the progress of disease or the treatment outcome of Taiwanese GD patients. Third, the T allele and TT genotype frequency was significantly smaller in GD patients who developed GD before 35 years old than those who developed the disease after 35

Table 4. Comparison of the clinical and laboratory variables among patients with the three groups of GD patients in CD40 SNP

CD40	Group 1	Group 2	Group3	*P* value
Age (yr) ± SD	37.3 ± 13.7	41.3 ± 12.4	42.2 ± 14.2	n.s
Sex (F/M)	53/25	54/17	44/22	n.s
Initial onset Age(yr) ± SD	34.3 ± 13.1	35.7 ± 13.1	33.8 ± 14.9	n.s
Initial T_4 (μg/dl) ± SD	10.3 ± 21.9	11.3 ± 21	10.6 ± 24	n.s
Goiter size before treatment	n.s			
Grade 1	5	0	1	
Grade 2	20	33	12	
Grade 3	53	38	53	
TBII before treatment	n.s			
Positive	78	71	66	
Negative	0	0	0	
Goiter size after treatment	n.s			
Grade 1	17	18	11	
Grade 2	48	44	40	
Grade 3	13	9	15	
TBII before treatment	n.s			
Positive	34	26	19	
Negative	44	45	47	
Duration of therapy (month) ± SD	16.6 ± 16.5	13.1 ± 5.1	18.8 ± 15.5	n.s
Antithyroid treatment (MTZ/PTU)	58/20	54/17	48/18	n.s
Opththalmopathy	27/51	31/40	26/40	n.s
Smoking	11/67	13/68	13/53	n.s
Family history	29/49	27/44	23/43	n.s

Group1–3 according to the cutoff points of 9 m, 9 m–3 years, and more than 3 yr.

Table 5. Association between the CD40 gene polymorphism in patients with Graves' disease and age at onset

CD40	Age at onset of GD		Control subjects (N=141)	GD≥35 yr vs. GD<35 yr	GD≥35 yr vs. Control
	<35 yr (N = 120)	≥35 yr (N = 95)			
Genotype					
TT	10 (8.3%)	15 (15.8%)	15 (10.6%)	$\chi^2 = 6.272$	$\chi^2 = 2.582$
CT	61 (50.8%)	55 (57.9%)	77 (54.6%)	$P = 0.043$	$P = 0.275$
CC	49 (40.9%)	25 (26.3%)	49 (34.8%)		
TT + CT	71 (59.2%)	70 (73.7%)	92 (65.2%)	$\chi^2 = 4.951$	$\chi^2 = 1.877$
CC	49 (40.8%)	25 (26.3%)	49 (34.8%)	$P = 0.030$	$P = 0.199$
Allele					
T	81 (33.8%)	85 (44.7%)	107 (37.9%)	$\chi^2 = 5.401$	$\chi^2 = 2.171$
C	159 (66.2%)	105 (55.3%)	175 (62.1%)	$P = 0.022$	$P = 0.152$

years old.

CD40, an important immunomodulatory gene, is reported to be a likely susceptibility gene for GD in Caucasians [4], Koreans [6], and Japanese [12, 13], though the association could not be confirmed in the United Kingdom (UK) Caucasians [7, 8] and Taiwanese. Similarly, the D727E SNP was associated with GD in a Russian [14] but not a Caucasian [15] or Singapore population [16]. Ho *et al*. [16] reported that a SNP in intron 1 of the TSHR gene was associated with GD in the same study. More specifically, Hitomi *et al*. [17] reported the SNP JST022302 and several adjacent SNPs in intron 7 of the TSHR gene to be significantly associated with GD in Japanese. These results were compatible with the observation that the overall prevalence rate of GD is different among different ethnic groups [18]. From the results of these above studies, we can understand the susceptive genes for Graves'

disease are highly likely polygenic. It has been demonstrated that the CD40 is expressed and functional on thyrocytes [19] and that the thyroidal expression of CD40 is upregulated in the context of GD [20]. It is also known that under certain circumstances, the thyrocyte can express MHC class II molecules and can act as a facultative APC [21–22]. Therefore we investigated whether the SNP in the 5' UTR of the CD40 gene could be associated with GD in Taiwanese.

GD is characterized by remissions and relapses. Many factors affect the duration of remission once anti-thyroid therapy is stopped [23]. The relapse rate of Graves' disease after antithyroid drugs treatment in Taiwanese patients is high [24], especially in those with a history of recurrence. Wang et al. [25, 26] have pointed out that patients with a second occurrence had a higher relapse rate than those with a first occurrence (84% v.s. 43%). The relapse rate was 100% among patients with two or more previous relapses. Although the Graves' disease is a curable disease, its relapse rate is very high. This study investigated the association between gene polymorphism and relapse of hyperthyroidism after medication was discontinued in three GD patient groups and a control group, and compared clinical and laboratory data obtained with patients with the three different genotypes with the CD40 SNPs. Our study has sufficient power to exclude association with the size of effect seen in the study by Tomer and Greenberg [27]. Our study sample size of 356 (215 GD patients and 141 controls) will have a greater than 90% (95.8%) power of detecting a difference in means of 1.000 assuming that the common standard deviation is 2.000 using a two group t-test with a 0.050 two-sided significance level [28]. No significant difference in allele or genotype frequency of the CD40 SNP was observed between patients with GD and control subjects ($P = 0.859$ and $P = 0.959$, respectively). In additional, no significant genotype-phenotype associations were found in Taiwanese.

The reasons why we have failed to replicate the initial findings of the association are numerous. It seems clear from our data that the Kozak SNP is not associated with GD in Taiwanese. First, the positive result could be due to a random chance event because of the small sample size used in the original study. Second, the original positive finding could be the result of the 'first time effect' phenomenon whereby association of a gene is overestimated when first detected. Third, the differences could have arisen as a result of different ethnic, racial and geographical background of the populations used in each study. Although it is impossible to know exactly why we have failed to replicate the initial findings of association, the CD40 gene itself remains a good candidate gene for susceptibility to GD. Further studies performed in adequately sized datasets are required in other populations.

In conclusion, GD is an autoimmune disease thought to be caused by several genetic factors, one being the CD40 gene polymorphism. However, our results show this polymorphism of the CD40 gene is not associated with GD in the Taiwanese. In additional, the CD40 gene polymorphism may not influence the progress or the outcome of the treatment of GD in the Taiwanese. However, In our study, the T allele and TT genotype frequency was significantly smaller in GD patients who developed GD before 35 years old than those who developed the disease after that age ($x^2 = 6.272$, $P = 0.043$) (TT + CT v.s. CC, $x^2 = 4.951$, $P = 0.030$). Our findings differed from those of Mukai *et al.* who showed that the TT genotype frequency was significantly smaller in GD patients who developed GD after 40 years old than those who developed before that age [13]. The differences may have be occurred because of differences in - ethnic, racial or geographical background. Despite the attractiveness of the CD40 gene as a susceptibility locus for GD, further studies using large datasets in other populations functional studies are indicated.

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