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Association Between the *TAP2* Gene Codon 665 Polymorphism and Graves' Disease

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A total of 95 patients with active Graves' disease (GD) and 105 normal healthy subjects were enrolled in this study, which attempted to determine whether single-site polymorphisms of the transporter associated with antigen processing 2 (*TAP2*) gene contribute to an individual's susceptibility to GD. Such polymorphisms were detected using polymerase chain reaction (PCR)-based restriction analysis. Associations between GD and the three site polymorphisms of the *TAP2* gene at codons 379, 565, and 665 were investigated. The results of the genotype analysis revealed that the frequency of the GG homozygote's presence at codon 665 was lower, and that of the AA homozygote's presence was greater in GD patients (15.8% and 36.8%, respectively) compared to normal controls (34.3% and 16.2%, respectively; $P<0.001$). The OR (OD) for the risk of occurrence for the AA homozygote and AG heterozygote compared to the GG homozygote (as was the case for the GD patients) was respectively 4.941 and 2.117, with respective 95% confidence intervals (CI) of 2.303–10.598 and 1.020–4.369. The allelic analysis also demonstrated reduced G and enhanced A allele frequencies for GD patients compared to controls (respectively 39.5% vs. 59.0% [G allele], and 60.5% vs. 41.0% [A allele]; $P=0.0001$; $OR=2.219$, 95% CI: 1.449–3.395). By contrast, the differences between patient and control groups for the frequency of appearance of genotypes and allelic variants at codon 379 ($P=0.522$ and $P=0.306$, respectively) and codon 565 ($P=0.199$ and $P=0.157$, respectively) did not appear to be significant. These data reveal that the single-site polymorphism of the *TAP2* gene at codon 665 may be an indicator for predicting GD development. J. Clin. Lab. Anal. 20:93–97, 2006. © 2006 Wiley-Liss, Inc.

Key words: codon; Graves' disease; polymorphism; transporter associated with antigen processing

INTRODUCTION

Graves' disease (GD) is a common autoimmune disease of the thyroid and is the major cause of hyperthyroidism in patients younger than age 40. It predominantly affects women, with a worldwide prevalence rate of around 2%, and about one-tenth as many men (1). Clinically, GD is characterized by a diffuse goiter, thyrotoxicosis, infiltrative ophthalmopathy, and occasionally infiltrative dermopathy.

The pathogenesis of GD involves the appearance of intrathyroidal thyrotropin (thyroid stimulating hormone (TSH)) receptor antibodies (TRAb), which are a group of IgG antibodies produced through a concatenation of immunological processes (2). Under the stimulation of interferon-γ, thyroid cells aberrantly express human leukocyte antigen (HLA) class II

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molecules. This allows the thyroid cells to present certain antigens, such as TSH receptors, to the helper T lymphocytes, and thus activate those cells. The activated helper T lymphocytes can then facilitate the proliferation and differentiation of B lymphocytes, with consequent associated antibody formation (3). Thus, it is clear that the expression of HLA molecules upon the presentation of peptide antigens to the body's T lymphocytes plays a critical role in the development of GD.

A specific protein, transporter associated with antigen processing (TAP), is responsible for the translocation of cytosolic antigenic peptides into the endoplasmic reticulum. Within the endoplasmic reticulum, peptides are loaded onto the HLA class I molecules before the thus-produced complexes are delivered to the plasma membrane and recognized by cytotoxic T lymphocytes (CTL). TAP consists of two subunits, TAP1 and TAP2, each of which is composed of two parts: a membrane-spanning domain and a cytoplasmic domain (4). The genes encoding both TAP1 and TAP2 are located within the HLA class II region between the HLA-DP and -DQ loci (5). Hence, it would appear reasonable to suggest that the TAP genes are associated with susceptibility to autoimmune diseases. In fact, some polymorphisms of the TAP genes are specifically involved in various diseases, such as type 1 diabetes mellitus, juvenile rheumatoid arthritis, multiple sclerosis, and Reiter's syndrome (6–9).

Although it would appear that the number of studies investigating the relationship between TAP genes and GD is somewhat limited as presented in the relevant literature, preliminary results have suggested that specific allelic variants of the TAP genes do exist among GD patients of different racial origins (10–12). Further, we recently observed an association between a single-site polymorphism at codon 637 of the *TAP1* gene and an individual's relative susceptibility to GD (13). To further test whether the *TAP1* gene analog, the *TAP2* gene, could be used as a genetic marker to predict the development of GD, we screened three polymorphic sites of the *TAP2* gene (codons 379, 565, and 665), using polymerase chain reaction (PCR)-based restriction analysis to compare GD patients with normal controls from Taiwanese population.

MATERIALS AND METHODS

Patient Selection

A total of 95 unrelated Chinese GD patients (74 females and 21 males, 17–71 years old, mean = 35.3 ± 10.5 years) were enrolled in this study, which extended from July 2003 to November 2003 inclusively. All participating patients were of Han ethnicity and resided in central Taiwan. Hyperthyroidism, diffuse goiter, and positive serum TRAb levels, supported by infiltrative ophthalmopathy and positive serum antimicrosomal and/or antithyroglobulin antibodies, were used as specific indicators to define the presence of GD. The control group consisted of 105 ethnically and geographically matched healthy volunteers over the age of 40 years (83 females and 22 males), all of whom were shown to be euthyroid and had no previous personal or family history of hyperthyroidism or any other autoimmune disease. The study was approved by the institutional ethics committee of our hospital, and informed consent was obtained from each participating subject.

PCR

Genomic DNA was prepared from peripheral blood using a genomic DNA isolation reagent kit (Genomaker Inc., Taipei, Taiwan). PCR was used to identify the three site genotypes at codon 379 [Val (GTA)/Ile (ATA), cDNA 1231], codon 565 [Thr (ACT)/Ala (GCT), cDNA 1693], and codon 665 [Ala (GCA)/Thr (ACA), cDNA 2089] for the *TAP2* gene. PCR for each site polymorphism was performed on a total volume of 50 μL containing genomic DNA (2–6 pmol of each primer), 1 × Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (PerkinElmer; Foster City, CA). The upstream (U) and downstream (D) primers for the three site polymorphisms are listed in Table 1, with U 5′-GAACGTGCCTTGTACCTG CGC-3′ and D 5′-ACCCCCAAGTGCAGCAC-3′ used for codon 379; U 5′-CCGGTTCTGTGAGGAACAAC AGT-3′ and D 5′-GGAGCAAGCTTACAATTTGT-3′ used for codon 565 and U 5′-GGTGATTGCTCACA GGCTGCCG-3′; and D 5′-CACAGCTCTAGGAAA CTC-3′ used for codon 665. These procedures were performed as described by Kuwata et al. (14), Takeuchi et al. (15), and Ismail et al. (16), respectively. PCR amplification was performed using a programmable thermal cycler GeneAmp PCR System 2400 (PerkinElmer). The cycling conditions for the PCR reactions are illustrated in Table 1.

The polymorphisms that arose for the three specific sites were discerned by digestion with *Bst*UI, *Rsa*I, or *Msp*I, respectively. The PCR products thus elicited were mixed separately using the above enzymes and reaction buffer according to the manufacturer's instructions, and both reactions were incubated for 3 hr at 37°C. Following this, 10 μL of each product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The genotypes of the three codons were classified as 1) an excisable allele homozygote (GG for codons 379 and 665, and AA for codon 565, respectively), 2) a non-excisable allele homozygote (AA for

TABLE 1. Primer sequences and conditions for polymerase chain reaction for polymorphisms of the transporter associated with antigen processing (TAP) 2 gene*

Codon	Position (cDNA)	Primer and PCR conditions	PCR product (bp)	Restriction site
379	1231 G/A	U 5′-GAACGTGCCTTGTACCTGCGC-3′	G: 192 + 20	*Bst*UI
	Val (GTA)/	D 5′-ACCCCCAAGTGCAGCAC-3′	A: 212	
	Ile(ATA)	94°C × 5′, 94°C × 20″, 55°C × 20″ and 72°C × 20″, 35 cycles, 72°C × 10′		
565	1693 A/G	U 5′-CCGGTTCTGTGAGGAACAACAGT-3′	A: 132 + 23	*Rsa*I
	Thr (ACA)/	D 5′-GGAGCAAGCTTACAATTTGT-3′	G: 155	
	Ala (GCA)	94°C × 5′, 94°C × 20″, 55°C × 20″ and 72°C × 20″, 35 cycles, 72°C × 10′		
665	2089 G/A	U 5′-GGTGATTGCTCACAGGCTGCCG-3′	G: 207 + 20	*Msp*I
	Ala (GCA)/	D 5′-CACAGCTCTAGGAAACTC-3′	A: 227	
	Thr (ACA)	94°C × 5′, 94°C × 20″, 55°C × 20″ and 72°C × 20″, 35 cycles, 72°C × 10′		

*U and D indicate upstream and downstream primers, respectively.

codons 379 and 665, and GG for codon 565, respectively), or 3) a heterozygote (AG).

Statistical Analysis

The genotype and allelic frequencies of the polymorphisms for the GD and control groups were statistically compared using the chi-square test performed with the Statistical Package for Social Science (SPSS) version 8.01 software (SPSS for Windows; SPSS Inc., Chicago, IL) following examination of allelic frequencies with Hardy-Weinberg equilibrium proportions for genotype frequencies. When the assumption of the chi-square test was violated (i.e., a single cell revealed an expected count of <1, or $>20\%$ of the cells revealed an expected count of <5), Fisher's exact test was used. Results were considered to represent a statistically significant difference when the probability of finding an intergroup difference occurring simply by chance was $<5\%$ ($P<0.05$). Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for disease susceptibility associated with specific genotypes and allelic variants.

RESULTS

In the initial examination for genotype frequencies, the polymorphisms of the three codons were all found to be in Hardy-Weinberg equilibrium for both the patient and control groups.

For the genotype analysis conducted, GD patients, as compared to normal controls, featured a greater frequency of the AA homozygous genotype (respectively 36.8% vs. 16.2%) and a lower frequency of the GG homozygous genotype (respectively 15.8% vs. 34.3%) at codon 665 (chi-square test; $P<0.001$; Table 2). The ORs for the risk of the AA homozygous genotype and AG heterozygous genotype arising, compared to the GG homozygous genotype, for the GD patient group were 4.941 (95% CI: 2.303–10.598) and 2.117 (95% CI: 1.020–4.369), respectively. However, the genotype distributions at codon 379 ($P = 0.522$) and codon 565 ($P = 0.199$) did not differ significantly between the two study groups.

In the allelic analysis we observed in GD patients compared to normal controls a significantly greater A allele frequency (respectively 60.5% vs. 41.0%) and a lower G allele frequency (respectively 39.5% vs. 59.0%) for codon 665 (chi-square test; $P = 0.0001$; Table 3). The OR for the risk of appearance of the A allele in the GD patient group was 2.219, with a 95% CI of 1.449–3.395. As was the case for the genotype analysis, we observed no differences between patient and control groups as regards allelic distributions for codon 379 ($P = 0.306$) and codon 565 polymorphisms ($P = 0.157$).

DISCUSSION

We previously observed that the *TAP1* gene codon 637 A/G polymorphism is associated with the appearance of GD (13). Our current study further demonstrates some form of association between a single-site polymorphism of the *TAP2* gene and GD susceptibility. The data derived from this study reveal an increased frequency for the presence of the AA homozygous genotype and the A allele at codon 665 of the *TAP2* gene for the GD patients compared to normal controls. It would thus appear to be quite reasonable to suggest that the *TAP2* gene could be useful as a genetic marker for predicting GD development.

The relationship between *TAP2* gene polymorphisms and GD for Caucasoid and Chinese populations was previously examined by Rau et al. (10), and Cai et al. (12). In the former study the authors found increased frequencies of homozygous GG genotype and G allelic

TABLE 2. Genotype frequencies for TAP2 gene codons 379, 565, and 665 when comparing healthy control subjects with Graves' disease patients

Genotype	GD patients n = 95 (%)	Controls n = 105 (%)	*P*
Codon 379			0.522[a]
1231*A/1231*A	4 (4.2)	8 (7.6)	
1231*A/1231*G	36 (37.9)	42 (40.0)	
1231*G/1231*G	55 (57.9)	55 (52.4)	
Codon 565			0.199[b]
1693*A/1693*A	3 (3.2)	1 (1.0)	
1693*A/1693*G	22 (23.1)	19 (18.1)	
1693*G/1693*G	70 (73.7)	85 (80.9)	
Codon 665			<0.001[a]
2089*A/2089*A[c]	35 (36.8)	17 (16.2)	
2089*A/2089*G[d]	45 (47.4)	52 (49.5)	
2089*G/2089*G	15 (15.8)	36 (34.3)	

[a]Chi-square test.
[b]Fisher's exact test.
[c]OR for A/A (compared with G/G): 4.941 (95% CI: 2.303–10.598).
[d]OR for A/G (compared with G/G): 2.117 (95% CI: 1.020–4.369).

TABLE 3. Allelic frequencies for TAP2 gene codons 379, 565, and 665 when comparing healthy control subjects with Graves' disease patients

Allelic frequency	GD patients n = 190 (%)	Controls n = 210 (%)	*P*
Codon 379			0.306[a]
Allele A	44 (23.2)	58 (27.6)	
Allele G	146 (76.8)	152 (72.4)	
Codon 565			0.157[a]
Allele A	28 (14.7)	21 (10.0)	
Allele G	162 (85.3)	189 (90.0)	
Codon 665			0.0001[a]
Allele A[b]	115 (60.5)	86 (41.0)	
Allele G	75 (39.5)	124 (59.0)	

[a]Chi-square test.
[b]OR for allele A: 2.219 (95% CI: 1.449–3.395).

variant [GTA (Val)] and decreased frequencies of homozygous AA genotype and A allelic variant [ATA (Ile)] at codon 379 for GD patients compared to normal controls (10). However, Cai et al. (12) did not observe any linkage between *TAP2* gene polymorphisms and GD. In the present study we demonstrated greater frequencies of the AA homozygote and the A allelic variant [ACA (Thr)], and lower frequencies of the GG homozygote and the G allele [GCA (Ala)] at codon 665 for GD patients when compared to controls. The reasons for the discrepancy between the results of our study and those of the previous two investigations is largely unknown; however, it may reflect the effect of racially determined genetic differences on GD pathogenesis for ethnically and geographically distinct sample populations. Also, the possibility of population stratification and statistical artifacts cannot be excluded.

The specific amino acid encoded by the *TAP2* gene codon 665 is located within the cytoplasmic domain of TAP2, close to the region for binding cytosolic peptides (4,17). In theory, regional substitution of the amino acid threonine for alanine may result in alteration of TAP2 conformation, and thus interfere with peptide translocation and HLA molecule expression. This hypothesis appears to be consistent with that proffered by Chen and associates (18), who identified a single-site polymorphism in the cytoplasmic domain of the *TAP1* gene that influenced peptide translocation among human solid tumors and cell lines.

The nature of the association between the *TAP2* gene polymorphism and GD susceptibility remains somewhat unclear. Since most genes within the HLA class II region are in linkage disequilibrium, it is also possible that the *TAP2* gene polymorphism contributes to GD development through linkage disequilibrium with HLA-DQ and

-DP (19,20). The possibility of direct functional activities of the *TAP2* gene polymorphism with respect to GD development should not be excluded, however. There are two possible explanations for such activities. First, the interaction of HLA class I molecules with CTL may be involved in the pathogenesis of GD. Predominantly suppressor/cytotoxic, and not inducer/helper, T lymphocyte infiltration into the thyroids of GD patients has been observed by a number of authors (21–23). CTL can facilitate the deposition of circulating immune complexes and/or their local formation in the thyroid and thereby play a role in GD generation (21). TAP2 plays a critical role in the delivery of peptides and the presentation of HLA class I molecules to CTL. The *TAP2* gene polymorphism referred to herein may thus affect the development of GD via this pathway. Second, the TAP-mediated pathway may present cytosolic antigens to HLA class II-restricted T cells. Although it is generally accepted that TAP is specifically responsible for HLA class I molecule presentation, Malnati et al. (24) observed that TAP is also necessary for HLA class II-restricted presentation of endogenous cytosolic peptides. This finding provides additional evidence of the contribution of the *TAP2* gene polymorphism to the development of GD, a predominantly HLA class II molecule-mediated autoimmune disease.

A reliable genetic marker should lead to earlier diagnosis of disease as well as earlier treatment, and have a positive impact on patient care. Our data indicate that a single-site polymorphism of the *TAP2* gene, codon 665 [GCA (Ala) → ACA (Thr)], may be a candidate genetic marker for screening GD susceptibility. Since direct evidence for the association between the TAP2 and GD susceptibility still appears to be lacking, further study is clearly necessary to elucidate the role of the *TAP2* gene polymorphism with respect to GD pathogenesis.

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