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Influence of the TSH Receptor Gene on Susceptibility to Graves' Disease and Graves' Ophthalmopathy

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Background: A large gene region, called GD-1, was first described by this laboratory as linked to Graves' disease (GD) and included the gene for the thyroid-stimulating hormone receptor (*TSHR*). Recent studies have now suggested an association of *TSHR* intronic polymorphisms with GD. We have taken the opportunity to examine a population of well-characterized patients with autoimmune thyroid disease (AITD) typed for an additional thyroid susceptibility gene, the immunoregulatory gene for cytotoxic T-lymphocyte antigen 4 (*CTLA-4*), to examine its relationship with the susceptibility to GD endowed by *TSHR* gene polymorphisms.
Methods: We used *TSHR*-SNP-rs2268458, located in intron 1 of the *TSHR* gene, measured using standard PCR-RFLP procedures, as our marker for the *TSHR* gene association. We genotyped 200 patients with GD, 83 patients with Hashimoto's thyroiditis (HT), and 118 healthy controls (all female Caucasians).
Results: The allele and genotype frequencies from GD patients, but not HT patients, were significantly different from controls. The frequency of the combined genotype (allele) CC + TC was significantly higher in GD patients versus controls, suggesting that the C-containing genotype increased the risk for GD in a dominant manner ($p = 0.018$, odds ratio [OR] = 1.8). When compared with *CTLA-4* $(A/G)_{49}$ single-nucleotide polymorphism (SNP), we were unable to demonstrate additive risk in patients with established AITD. Further, subsetting the patients ($n = 120$) into those with clinically significant Graves' ophthalmopathy (GO) showed no association with the *TSHR* SNP.
Conclusions: These results demonstrated that the intronic *TSHR*-SNP-rs2268458 was associated with GD, but not with HT, thus indicating that the *TSHR* gene has the potential to increase susceptibility to GD. However, we were not able to demonstrate any additive risk with the *CTLA-4* $(A/G)_{49}$ SNP, which is, therefore, an independent risk factor for AITD. This suggested that, within the limits of the study population, each of these two genes provided a small contribution to GD susceptibility and that neither was essential. In addition, there was no evidence for the *TSHR* gene association adding to the risk of developing GO. Direct functional analyses are now needed to help explain the mechanisms of this *TSHR* gene susceptibility to GD.

Introduction

The thyroid-stimulating hormone receptor (TSHR) is a 7-transmembrane domain G protein–coupled receptor, which is the master switch in the regulation of the thyroid gland, and also a major autoantigen in autoimmune thyroid diseases (AITDs), especially in Graves' disease (GD) (1). The hallmark of GD is the generation of stimulating TSHR autoantibodies and the presence of T cells reactive to TSHR antigen. Hence, the *TSHR* gene, on chromosome 14q, has long been thought of as a likely disease-specific susceptibility gene for GD.

Studies from this laboratory, and those of others, using microsatellite markers and exonic single-nucleotide polymorphisms (SNPs) were unable to directly demonstrate significant linkage or association of the *TSHR* gene with GD (2–7). Association studies using three common *TSHR* exonic and nonsynonymous SNPs (in the extracellular and intracellular domain of the receptor) were inconsistent (5,6,8–12). However, in two linkage studies we described a GD-specific chromosome 14 locus of ~25 cM, designated GD-1 (4,7,13), in the center of which was the *TSHR* (between markers D14S258 and D14S1054). GD-1 was consistently linked with GD and not with Hashimoto's thyroiditis (HT) (7).

Subsequently, the study of intronic polymorphisms has been entertained because we now know that intronic DNA may be responsible for regulatory small RNAs as well as providing and/or influencing different start sites for TSHR

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mRNA generation (14,15). Indeed, we and others have also shown previously that the thyroid cell expresses a variety of TSHR mRNA splice variants [for review see Graves and Davies (16)], indicating that SNPs or small RNAs in this intronic DNA may be important in the generation of different receptor forms and/or their control. Recently, a study from Singapore demonstrated an association of a *TSHR* intron 1 SNP with GD (17). SNPs in intron 7 of the *TSHR* were also found to be associated with GD in Japanese (18), and SNPs in intron 1 of the *TSHR* were reported to be associated with GD in Caucasians (19).

To further examine the *TSHR* gene as a susceptibility gene in GD and evaluate the additive role of *TSHR* intronic gene polymorphisms in GD compared to other more established susceptibility genes, we examined the association of GD with one of the reported highly associated intronic *TSHR* gene SNPs (rs2268458) in a series of well-characterized Caucasian female patients with AITD.

Materials and Methods

Subjects

Peripheral blood DNA was obtained from Caucasian females with a diagnosis of GD ($n = 200$), including 75 with severe Graves' ophthalmopathy (GO) defined as requiring orbital decompression, and 45 with milder clinical GO, female patients with HT ($n = 83$), and healthy controls ($n = 118$) (Table 1). GD was defined as the presence of chemical hyperthyroidism and a normal or increased diffuse thyroid radioiodine uptake and/or the presence of TSHR antibodies. HT was defined as increased TSH levels in the presence of thyroid antibodies requiring treatment with thyroid hormone replacement therapy.

SNP analysis by PCR-RFLP methodologies

TSHR SNP. Based on the report of Dechairo *et al.* (19), we examined *TSHR*-SNP-rs2268458, located in intron 1, as measured using standard PCR-RFLP procedures. This intronic SNP was the most strongly associated of 40 SNPs examined for association with GD. Human genomic DNA (~25 ng) was amplified by PCR and 415-bp products were generated. Primer 1 was 5′-CCAGCAGAGGGAGCACAA-3′, and primer 2 was 5′-TAGAGAATAGAGCAGCAAGAGACT-3′.

These primers flank the DNA fragment in *TSHR* gene intron 1. The PCR reaction (25 μL) containing 1 U Platinum Taq polymerase was carried out as directed by the manufacturer (Invitrogen, Carlsbad, CA). The PCR parameters were as follows: 95°C for 5 minutes, 35 cycles (95°C/1 min, 52°C/1 min, 72°C/1 min) followed by 72°C for 10 minutes. Then 8 μL of PCR products was digested for 2 hours in 10 μL total volume with the restriction endonuclease Alu I according to the manufacturer's instructions (New England BioLabs, Beverly, MA) along with a nondigested DNA control. After digestion, the digested fragments were mixed with gel loading buffer, separated on a 3% agarose gel, visualized by ethidium bromide and UV light, documented with AlphaImager2200 (Alpha Innotech, San Leandro, CA), and analyzed by the genotype patterns. Since Alu I digestion determines AGTT versus AGCT, this allowed the determination of each individual hetero- or homogenotype. The patterns were recorded as TT with one 333-bp DNA fragment, TC with one 333-bp and one 275-bp fragments, and CC with one 275-bp fragment.

Table 1. Groups Studied on Caucasians

Diagnosis	*Number*	*Sex*	*Age in years (mean)*	*Age at onset (mean years)*	*Ethnicity*
Graves' disease	200	Female	48	39	Caucasian
Hashimoto's thyroiditis	83	Female	43	33	Caucasian
Healthy controls	118	Female	41	–	Caucasian

The numbers represent individual subjects.

Cytotoxic T-lymphocyte antigen 4 SNP. The cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) exon 1, $(A/G)_{49}$ SNP was assayed by the same method as described above, but using a different primer pair and the *Bbv*I restriction enzyme (New England BioLabs) (20). The forward primer was 5′-GCTCTACTTCCTGAAGACCT-3′, and the reverse primer was 5′-AGTCTCACTCACCTTTGCAG-3′.

The A allele resulted in an undigested PCR product of 162 bp; the G allele resulted in a digested PCR product, showing 90- and 72-bp fragments viewed as one bold band on the agarose gel.

Statistical analyses

We used the chi-square test (χ^2) and Fisher's exact test (where appropriate) to compare the frequencies of alleles and genotypes between patients and controls. All tests were two tailed, and $p \leq 0.05$ was considered as significant. Since we were testing one SNP in the *TSHR* gene, no correction for multiple testing was performed. To assess the power of the association studies, we used the Centers for Disease Control (CDC) simulation software (Epi Info v.3.4.3.), and for the multiplicative interaction analyses we used the method of Gauderman (21) available as the Quanta program. This gave us, for the association studies, 80% power with 91% confidence to detect an odds ratio (OR) of >2.0 at an assumed susceptibility allele frequency of 22% from the controls, whereas for the interaction studies we had 80% power to detect an OR of >2.0. Such power assessments, of course, are particularly important for the interpretation of negative results.

Results

Comparison of AITD and controls by TSHR allelic frequencies

The C allele was significantly more frequent in the GD patients compared to controls ($p = 0.048$, OR = 1.5, 95% CI = 1.0–2.1) (Table 2). In contrast, no statistically positive differences in allelic frequency were observed between HT patients and controls (Table 2).

Comparison of TSHR genotypic frequencies in AITD patients and controls

The genotype frequencies were also statistically different between GD and controls ($p = 0.045$, $\chi^2 = 6.2$; 2 degrees of

Table 2. Allele and Genotype Frequencies for the rs2268458 SNP in the *TSHR* Gene

Allele/genotype	*Controls* (n = *118*)	*AITD* (n = *283*)	*AITD vs. controls* p-*value (OR)*	*GD* (n = *200*)	*GD vs. controls* p-*value (OR)*	*HT* (n = *83*)	*HT vs. controls* p-*value (OR)*
T	185 (78.4%)	410 (72.4%)	0.079 (1.4)	285 (71.3%)	0.048 (1.5)	125 (75.3%)	0.166 (1.5)
C	51 (21.6%)	156 (27.6%)		115 (28.8%)		41 (24.7%)	
TT	78 (66.1%)	153 (54.1%)	0.055	105 (52.5%)	0.045	48 (57.8%)	0.193
TC	29 (24.6%)	104 (36.7%)		75 (37.5%)		29 (35.0%)	
CC	11 (9.3%)	26 (9.2%)		20 (10.0%)		6 (7.2%)	
TT vs. CC + TC			0.026 (1.7)		0.018 (1.8)		0.092 (1.8)
CC vs. TT + TC			0.966 (1.0)		0.844 (1.1)		0.997 (1.0)

The numbers represent individual subjects or alleles, with the percentage in parentheses.
SNP, single-nucleotide polymorphism; TSHR, thyroid-stimulating hormone receptor; AITD, autoimmune thyroid disease; OR, odds ratio; GD, Graves' disease; HT, Hashimoto's thyroiditis.

freedom), but not for HT (Table 2). In addition, the frequency of the combined genotypes CC + TC was significantly higher in GD patients versus controls, suggesting that the C-containing genotype increased the risk for GD in a dominant manner ($p = 0.018$, OR = 1.8, 95% CI = 1.1 − 2.8).

GO and the TSHR *gene*

One hundred and twenty patients had the GO phenotype including 75 with severe disease. The *TSHR* SNP genotype frequencies for the GO patients were not statistically different from the controls ($p = 0.166$, $\chi^2 = 3.6$) (Table 3). In addition, the frequency of the combined genotypes CC + TC was not significantly higher in GO patients versus controls ($p = 0.105$). Similarly, the C allele was no more frequent in the GO patients compared to controls ($p = 0.236$) (Table 3). When the 75 most severe GO patients were analyzed separately, there was still no statistically significant association with the *TSHR* SNP. However, these groups were small in size, and with 80% power would only detect an OR of >2.3.

Relationship between the TSHR *and the* CTLA-4 *susceptibility genes*

To study if the *TSHR* gene association with GD was additive to another AITD susceptibility gene, we examined its interaction with the $(A/G)_{49}$ SNP in exon 1 of the *CTLA-4* gene, which had an OR of 1.2 in this study (data not illustrated), and which has been consistently shown to be associated with AITD (22–25). However, there was no evidence for any additive risk between *TSHR*-SNP-rs2268458 and *CTLA-4* SNP $(A/G)_{49}$ in the GD patients (Table 4). Indeed, the combined OR for the *CTLA-4* G allele with the *TSHR*-SNP C allele gave an OR of only 1.3 (Table 4). These data suggested that the *TSHR* gene polymorphism assigned more susceptibility to GD than that assigned by *CTLA-4*. A power analysis indicated that we could only exclude interactions giving ORs of >2.0, but a significant interaction appears very unlikely.

Discussion

AITD is now recognized to be secondary to a combination of genetic and environmental susceptibility factors. A variety of genes have been reported to contribute to AITD susceptibility, many of which await careful confirmation, and their multiplicity indicates that each contributes only a small amount of genetic susceptibility. Major candidates include *HLA*, *CTLA-4*, *PTPN22*, thyroglobulin, the *TSHR*, *CD40*, the *IL23-R*, and others (26–28). In contrast, a genetic susceptibility to the ophthalmic changes of Graves' disease (GO), separate and distinct from the thyroid disease, remains controversial (26,29). In this study we have focused on the *TSHR* and *CTLA-4* in GD and GO.

The human *TSHR* gene, first cloned in 1989 (30), occupies 191 kb of DNA and is located at chromosome 14q31 within the site of our first GD-linked chromosomal locus designated GD-1 (4,31). Because of its obvious role in the pathogenesis of GD, many laboratories have tried to demonstrate that the *TSHR* is an important susceptibility gene for GD. Hence, much effort was focused on linkage and association studies with nearby microsatellite marker and exonic SNP analyses of the receptor over the past 12 years. Our original description of GD-1 (4) and its further delineation (7,13), about 10.8 Mb from the *TSHR* gene, has now been further refined using *TSHR* intronic SNPs (17,18,19). These data suggest that it may

Table 3. Allele and Genotype Frequencies for the rs2268458 SNP of *TSHR* Gene in GO Patients

Allele/genotype	*Controls* (n = *118*)	*GO* (n = *120*)	*GO vs. controls* p-*value (OR)*	*Non-GO* (n = *80*)	*Non-GO vs. controls* p-*value (OR)*
T	185 (78.4%)	177 (73.8%)	0.236 (1.3)	108 (67.5%)	0.015 (1.7)
C	51 (21.6%)	63 (26.3%)		52 (32.5%)	
TT	78 (66.1%)	67 (55.8%)	0.166	38 (47.5%)	0.031
TC	29 (24.6%)	43 (35.8%)		32 (40.0%)	
CC	11 (9.3%)	10 (8.3%)		10 (12.5%)	
TT vs. CC + TC			0.105 (1.5)		0.009 (2.2)
CC vs. TT + TC			0.788 (1.1)		0.476 (1.4)

The numbers represent individual subjects or alleles, with the percentage in parentheses.
GO, Graves' ophthalmopathy; OR, odds ratio.

Table 4. *TSHR*-rs2268458 SNP and CTLA-4 $(A/G)_{49}$ ($N = 200$)

	TSHR-SNP genotype				
CTLA-4 genotype	*Presence of C allele (%)*	*Absence of C allele (%)*	χ^2-*test*	p-*Value*	*OR*
Presence of G	56 (58.9)	68 (64.8)	0.716	0.398	1.3
Absence of G	39 (41.1)	37 (35.2)			

The numbers represent individual genotype carriers, with the percentage in parentheses.

TSHR, thyroid-stimulating hormone receptor; SNP, single-nucleotide polymorphism; CTLA-4, cytotoxic T-lymphocyte antigen 4; OR, odds ratio.

indeed be the *TSHR* gene within the GD-1 locus that is responsible for GD susceptibility.

Introns are the noncoding DNA sections, located between coding regions of the gene termed exons, and they are spliced out from mRNA before translation. Exons only compose about 1.5–2% of the human genome, and intronic and intergenic DNAs make up the remaining human genome space. The total length of introns comprises about 37% of the human genome. Despite this, introns were generally thought of as junk DNA regions with no function after their recognition (32). However, recent evidence has indicated that introns contain important gene regulatory sequences that have a variety of functional roles, such as alternative intronic promoters/enhancers, noncoding RNAs, RNA editing, nested genes, and transacting elements. Sometimes, intronic mutations may even lead to diseases due to the modification of the mRNA splicing process (33,34).

It is also well known that intron 1 of many genes contains certain regulatory *cis*-elements (transcription factor binding sites) (35). Although alternative promoters can be found in other introns, they appear to be preferentially present in intron 1. In our current study and in accordance with previous reports, the *TSHR* gene intron 1 region was highly associated with GD but not HT, giving an OR of up to 1.8 with *TSHR*-SNP-rs2268458. This association was similar to that observed in the literature for the *CTLA-4* gene (which has averaged an OR of 1.5) (23,24), indicating a modest influence on genetic susceptibility. However, examining *TSHR* SNP and *CTLA-4* susceptibility alleles failed to demonstrate a significant interaction between them although this was restricted to ruling out an OR >2.0. Another possibility was that the *TSHR* SNP was more strongly associated with a particular clinical phenotype. In the first instance, we were able to examine its relationship with severe GO patients but again found no evidence for a preferential association although more patients with severe GO are need to confirm this.

The TSHR intron 1 is about 106 kb and occupies roughly 56% of the *TSHR* gene sequence. Whether this long length of intronic DNA contains any functional elements related to regulation of transcription or translation is unknown. Bioinformatic data suggest that there is one alternative promoter located inside intron 1 of the *TSHR*. In addition, TSHR mRNAs containing intron 1 fragments have also been found in EST sequences (such as EST DB134081, DB115428, and DA946337). The *TSHR* is known to transcribe a variety of mRNA transcripts of variable size (36). For example, previous studies from this laboratory have identified and cloned a 1.3-kb TSHR transcript variant (37) of uncertain functional activity. These different lengths of mRNA suggest that alternative splicing may play an important physiological role in TSHR function and perhaps susceptibility to GD. Moreover, recent reports suggest that intronic small RNAs may be involved in the regulation of mRNA splicing, and gene expression including the control of the immune response (38) and may also potentially arise from intron 1.

In summary, our study demonstrated that the intronic *TSHR*-SNP-rs2268458 was associated with GD, but not with HT, thus indicating that the *TSHR* gene has the potential to increase the risk for GD. *TSHR* gene susceptibility did not appear to interact strongly with the immune regulatory gene *CTLA-4*, suggesting these are independent risk factors. Hence, the *TSHR*-SNP-rs2268458, or another with which it is in linkage disequilibrium, must be directly involved in the mechanism of this genetic association. This may generate alternative mRNA splicing or small regulatory RNAs that influence wild-type *TSHR* expression or function or produce different thyroid autoantigens involved in the thyroid autoimmune response. Although bioinformatic mining has suggested to us that intron 1 of the *TSHR* gene may contain functional elements and explain this potential physiologic role, experimental confirmation with functional analyses are needed to help explain the mechanisms of the *TSHR* gene-related susceptibility to GD.

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