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Common Allelic Variants of Exons 10, 12, and 33 of the Thyroglobulin Gene Are Not Associated with Autoimmune Thyroid Disease in the United Kingdom

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Thyroglobulin (Tg) is a major autoantigen for autoimmune thyroid disease (AITD). The Tg gene (*Tg*) has been mapped to chromosome 8q24, which has recently been linked in two independent studies to AITD. Association of specific alleles of microsatellite markers within *Tg* itself supports a role for *Tg* as a good candidate susceptibility locus for AITD. Resequencing of the *Tg* exons has led to the identification of a number of novel single nucleotide polymorphisms, four of which have been reported to be associated with AITD. Resequencing of *Tg* in Caucasian subjects in the United Kingdom (UK) has confirmed the presence of four single nucleotide polymorphisms in exons 10, 12, and 33. However, in the largest case-control association study to date with adequate power to detect the reported effect if present, we found no evidence for association of the *Tg* DNA variants with AITD in the UK. These data suggest that the recently identified single nucleotide polymorphisms do not have a causal role for AITD in the UK. At this stage, we cannot exclude the *Tg* region as harboring a susceptibility locus for AITD, and only large scale sequencing and fine mapping of the region, including neighboring genes, will allow us to identify any potential causal variants within this region. (*J Clin Endocrinol Metab* 89: 6336–6339, 2004)

THE AUTOIMMUNE THYROID diseases (AITDs), Graves' disease (GD) and autoimmune hypothyroidism (AH), are common complex diseases caused by a breakdown in immune tolerance to self thyroid antigens, such as thyroglobulin (Tg), thyroid peroxidase, and TSH receptor (TSH-R). The definitive etiology for this breakdown in immune tolerance remains elusive, although both genetic and environmental factors have been postulated (1–5). The only consistent associations with AITD, however, are with the human leukocyte antigen (HLA) class II region on chromosome 6p21 (specifically in the DR3 region) and the cytotoxic T lymphocyte-associated-4 region on chromosome 2q33 (2–4, 6, 7). Despite genome-wide linkage and candidate gene studies, the identification of a third locus has proved elusive, although a number of studies (8–11) have focused on chromosome 8q24, which contains the *Tg*. Two genome-wide scans found linkage between chromosome 8q24 (8, 9) and AITD, and two studies involving different alleles of *Tg* microsatellite markers have reported allelic association with AITD (9, 10). Most recently, resequencing of all exons of *Tg* has lead to the identification of novel single nucleotide polymorphisms (SNPs), four of which, in exons 10, 12, and 33, were reported to be associated with AITD in a U.S. study (11). The same study also found that disease risk increased further when combined with the known HLA susceptibility allele, DR3.

Here, we have resequenced the exons of *Tg*, confirming the presence of the novel SNPs of the U.S. study and have performed a case-control association study of AITD patients and control subjects to elucidate whether these SNPs are also associated with the development of AITD in a United Kingdom (UK) Caucasian population.

Abbreviations: AH, Autoimmune hypothyroidism; AITD, autoimmune thyroid disease; GD, Graves' disease; HLA, human leukocyte antigen; LD, linkage disequilibrium; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; Tg, thyroglobulin; TSH-R, TSH receptor.

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Subjects and Methods

Subjects

One thousand two hundred and fourteen Caucasian patients of UK origin with AITD (960 with GD and 254 with AH) were recruited from the thyroid clinics, as described previously (12, 13). Briefly, patients with GD were defined by the presence of biochemical hyperthyroidism together with two of the following criteria: diffuse goiter; significant titer of thyroid peroxidase, Tg, or TSH-R autoantibodies; or the presence of thyroid eye disease. Thyroid eye disease was classified using the NOSPECS classification, with disease being defined as positive features in any of classes 2–6 (14). AH was defined by the presence of positive thyroid autoantibodies (thyroid peroxidase and/or Tg) and biochemical evidence of hypothyroidism. Thyroid peroxidase and Tg antibodies were measured by gelatin particle agglutination (SERODIA-ATG, Fujirebio, Inc., Tokyo, Japan), and a titer of 1:100 was considered significant for both assays. TSH-R antibody status was determined by a radioinhibition method (RSR Ltd., Cardiff, UK), and a value of 10 U/liter or more was deemed significant after comparison with 50 control subjects obtained from the local blood transfusion service. Four hundred and eighty control subjects with no history of autoimmune disease had blood drawn at various sites, including the Blood Transfusion Service, Birmingham Heartlands Hospital, and the Queen Elizabeth Hospital, Birmingham. Control subjects were matched with patients for age, gender, and ethnicity, and all patients and control subjects had white

Caucasian grandparents of UK origin. The study was approved by the local research ethics committees, and all subjects gave informed, written consent.

DNA was extracted from the whole blood of subjects using the Nucleon Bacc II kit from Tepnel Life Sciences PLC (Manchester, UK).

Sequencing of the human Tg gene exons

Exonic sequences (accession nos. X06068, AH008122, and AF169662) were obtained from GenBank (accessed through www.ncbi.nlm.nih.gov/) and aligned to genomic clones of human *Tg* [accession nos. AF230667 (exons 10 and 12) and AF305872 (exon 33)] using a basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/) to enable primers to be designed approximately 100 bp upstream of the 5′ intron-exon junction and approximately 100 bp downstream of the 3′ intron-exon junction. Primers were designed using DNASTAR (DNASTAR Inc., Madison, WI; primer sequences shown in Table 1). Genomic DNA from 32 control subjects (16 males and 16 females) was amplified by PCR using these primers (Sigma-Genosys, Haverhill, UK; PCR product sizes shown in Table 1), and products were sequenced using an ABI 377 DNA sequencer (PE Applied Biosystems, Warrington, UK). The four SNPs in exons 10, 12, and 33, respectively (E10SNP24, E10SNP158, E12SNP, and E33SNP), were confirmed manually by comparison with *Tg* clone sequences (accession nos. AF230667 and AF305872).

Case-control association analyses

Genomic DNA from 960 GD patients (254 AH patients and 480 control subjects) was amplified using PCR (primer sequences in Table 1) for the SNPs E10SNP24, E10SNP158, E12SNP, and E33SNP. Annealing temperatures were 53 C (E10SNP24 and E10SNP158), 56 C, and 54 C, respectively. SNP genotyping was performed using restriction fragment length polymorphism (RFLP), where PCR products were digested using restriction enzymes (New England Biolabs, Hitchin, UK; Table 1) according to the manufacturer's guidelines. RFLP fragment sizes are given in Table 1, and all products were visualized on 3% agarose gels stained with ethidium bromide.

Statistical analyses

Analysis of the data were performed using the χ^2 test (MINITAB version 10.2), where $P < 0.05$ was considered significant.

Linkage disequilibrium (LD) was calculated among the four SNPs and between each of the SNP control subjects. LD was calculated using the COCAPHASE program (15, 16) to give D′ values between 0 and 1. A D′ value of 0 indicates no LD between markers, and a D′ value of 1 indicates complete LD given the allele frequencies at each of the loci.

Multiple logistic regression was performed on case-control data using STATA (www.stata.com) with software written by David Clayton for use within that package (www.gene.cimr.cam.ac.uk/clayton/software/stata) to model genotype effects of the four *Tg* SNPs with respect to HLA-DR3 genotypes. This was to establish whether *Tg* and HLA-DR3 were interacting to increase disease risk.

Results

PCR-RFLP

Allele frequencies and genotype distributions for the four *Tg* SNPs were similar in the AITD cases and control subjects (summarized in Table 2). There was no evidence for an independent association with either GD or AH (summarized in Table 3). All genotypes were in Hardy-Weinberg equilibrium. Power calculations showed that we had greater than 94% power to detect an effect if present, with an odds ratio of 1.5 and $P = 0.001$.

LD among SNPs

LD was calculated among the four SNPs (Table 4). Strong LD was only detected between the two exon 10 SNPs, E10SNP24 and E10SNP158 (D′ = 0.96).

Multiple logistic regression

Multiple logistic regression (using both multiple degrees of freedom and 1 degree of freedom) showed no evidence for an interaction of any of the four *Tg* SNPs with HLA-DR3 to either increase or decrease disease risk (Table 5).

TABLE 2. Allele and genotype frequencies of the *Tg* SNPs in patients with AITD and control subjects

SNP	Alleles/ genotypes	AITD[a] [no. (%)]	Control subjects[a] [no. (%)]	χ^2	*P* value
E10SNP24	T	1171 (52.09)	392 (50.52)		
	G	1077 (47.91)	384 (49.49)	0.573[b]	0.449
	TT	319 (28.38)	98 (25.26)		
	TG	533 (47.42)	196 (50.52)		
	GG	272 (24.20)	94 (24.23)	1.593[c]	0.451
E10SNP158	T	1157 (51.38)	388 (49.24)		
	C	1095 (48.62)	400 (50.76)	1.068[b]	0.301
	TT	302 (26.82)	91 (23.10)		
	TC	553 (49.11)	206 (52.28)		
	CC	271 (24.07)	97 (24.62)	2.191[c]	0.334
E12SNP	A	1201 (51.32)	462 (49.04)		
	G	1139 (48.68)	480 (50.96)	1.397[b]	0.237
	AA	302 (25.81)	106 (22.51)		
	AG	597 (51.03)	250 (53.08)		
	GG	271 (23.16)	115 (24.42)	1.975[c]	0.372
E33SNP	C	1210 (53.21)	481 (53.92)		
	T	1064 (46.79)	411 (46.08)	0.131[b]	0.717
	CC	333 (29.29)	139 (31.17)		
	CT	544 (47.85)	203 (45.52)		
	TT	260 (22.87)	104 (23.32)	0.776[c]	0.678

[a] Numbers indicate successful genotyping from a dataset of 1214 AITD patients (960 with GD and 254 with AH) and 480 control subjects.
[b] One degree of freedom.
[c] Two degrees of freedom.

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)
E10SNP24	Exon 10	T/G	F: AACTGCCACATTTCTGC	437	*Blp*I	TT: 437
			R: AGAACAAGGGCCAGGATC			GG: 248, 189
E10SNP158	Exon 10	T/C	F: AACTGCCACATTTCTGC	437	*Ava*I	TT: 437
			R: AGAACAAGGGCCAGGATC			CC: 382, 55
E12SNP	Exon 12	A/G	F: TGTAATACTGTGGGTTGAAATGTT	433	*Bsa*AI	AA: 359, 74
			R: ATTCCTTAGCTTCTCGGGCCTCCA			GG: 205, 154, 74
E33SNP	Exon 33	C/T	F: TCCCCAAAGCAAGAATGAC	400	*Hpy*CH4III	CC: 190, 115, 95
			R: TTCTACCTGGCCAAACTTCC			TT: 210, 190

[a] F, Forward primer sequence; R, reverse primer sequence.

TABLE 3. Summary of independent case-control analysis of *Tg* SNPs in GD and AH patients

SNP	Alleles χ^2	*P* value	Genotypes χ^2	*P* value	Power (%)[a]
GD					
E10SNP24	1.1	0.295	2.003	0.368	99.92
E10SNP158	1.373	0.242	2.207	0.332	99.93
E12SNP	1.85	0.173	2.100	0.350	99.98
E33SNP	0.25	0.617	0.579	0.749	99.97
AH					
E10SNP24	0.103	0.748	0.643	0.725	96.7
E10SNP158	0.069	0.793	1.219	0.544	96.69
E12SNP	0.036	0.85	1.889	0.389	97.47
E33SNP	0.024	0.878	1.219	0.544	97.49

[a] Power calculations for each SNP are based upon a predicted odds ratio of 1.5, $P < 0.05$ for both GD and AH.

TABLE 4. D′ values between *Tg* SNPs in control subjects

***Tg* SNP**	E10SNP158	**E12SNP**	**E33SNP**
E10SNP24	**0.96**	0.62	0.01
	E10SNP158	0.65	0.01
		E12SNP	0.05

No LD = 0; complete LD = 1; strong LD = 0.7–1.0 (value in *bold*).

TABLE 5. Interaction analyses between *Tg* gene SNPs and HLA-DR3

Loci	χ^2	df[a]	*P* value	χ^2	df[b]	*P* value
E10SNP24/DR3	5.06	6	0.5358	0.41	1	0.5221
E10SNP158/DR3	7.30	6	0.2944	0.46	1	0.4953
E12SNP/DR3	3.48	5	0.6259	1.29	1	0.2559
E33SNP/DR3	4.61	6	0.5944	0.37	1	0.5406

[a] Multiple degrees of freedom.
[b] One degree of freedom.

Discussion

Serum from most patients with AITD shows restricted epitope Tg autoantibody specificity compared with that from healthy subjects, which recognize Tg antigenic domains distinct from those recognized by AITD serum (Ref. 17; reviewed in Ref. 18). Tg immunization also induces autoimmune thyroiditis in mice in a major histocompatibility complex-dependent manner (19). Therefore, *Tg* is an excellent candidate for AITD.

Recent studies have investigated the role of *Tg* in AITD (8–11) and have led to the publication of independent reports of linkage and association of the *Tg* region with both GD and AH. Tomer *et al.* (9) initially found association of a microsatellite marker in the *Tg* region with AITD in a case-control dataset, although association in the family dataset was with different alleles. We subsequently reported weak association of a rare intron 27 *Tg* microsatellite allele with AITD (2.2% of AITD cases and 0.25% of control subjects; $\chi^2 = 24.97$; $P < 0.001$) in the same dataset as that used in the present study, although we explained that the finding could be the result of a random chance event (10). The latest study by Ban *et al.* (11), involving a comprehensive screening of all *Tg* exons and genotyping in 435 subjects, showed association of four novel *Tg* SNPs with AITD. In the present study examining the same SNPs as those studied by Ban *et al.* (11), we found no evidence of allelic association despite our study having greater than 94% power to detect an effect using conservative odds ratios smaller than those reported.

In our current study of 1214 AITD patients, we have not routinely measured Tg autoantibodies. However, of 380 subjects tested for Tg autoantibodies, 148 (38.9%) were found to have positive titers. The allele frequencies of the four candidate SNPs in this subgroup of patients with positive Tg autoantibody titers were not significantly different from those in subjects with negative Tg autoantibody titers. It is, therefore, unlikely that the *Tg* polymorphism is involved in the induction of Tg autoantibodies, although data from subgroup analysis with reduced numbers should be interpreted with caution. Furthermore, separate subgroup analysis of the four *Tg* SNPs in patients with GD and AH revealed no differences in allele frequencies compared with control subjects. Similarly, no significant differences in allele frequencies of the four *Tg* SNPs were observed between males and females when comparing AITD patients and control subjects (data not shown).

The most important factors underlying the inability to replicate reports of allelic association are publication bias (20), failure to attribute results to chance (20), and inadequate sample sizes (21). Adequately sized datasets are important to prevent the generation of false positive (type I errors) and false negative (type II errors) results (21). A meta-analysis by Lohmueller *et al.* (22) showed that only about one quarter of positive association studies actually represent real associations, which emphasizes the need for highly powered datasets. This combined with a number of studies that have failed to replicate associations with initial significances below the accepted threshold of $P < 0.05$ have led to the suggestion that the threshold should be lowered to 10^{-5} even with regions containing good candidate loci (23). The best evidence for association between *Tg* and AITD is seen in the study by Ban *et al.* (24), although with a $P < 10^{-3}$ this could still be a false positive result due to sample size (42% power, with an OR of 1.56 and $P = 0.001$). It is, of course, possible that *Tg* contributes to AITD susceptibility in the U.S. and not the UK, and this remains an important alternative explanation, as exemplified by studies showing racial differences in susceptibility to AITD (24).

Evidence was also provided in the study by Ban *et al.* (11) to support a strong interaction between the exon 33 SNP and HLA DR3. Such an effect would be attractive, because a cysteine-rich domain of invariant chain, a protein that facilitates folding of the major histocompatibility complex class II molecules, has remarkably high homology to the cysteine-rich repetitive elements in Tg (25). Koch *et al.* (25) suggested that these homologous domains may play a role in hormone formation or intracellular molecule transport. A subsequent study (26) showed that mutations in the antigen-binding pockets of HLA-DR3 hinder the release of class II-associated invariant chain peptides from DR3 molecules, which must take place before antigenic peptides can be bound. This together with the reports by Ban *et al.* (11) of interaction between *Tg* and HLA-DR3 to increase AITD susceptibility led us to investigate interaction of the four *Tg* SNPs with HLA-DR3. We found no evidence of statistical interaction between any of the *Tg* SNPs with HLA-DR3 when using multiple logistic regression with multiple or single degrees of free-

dom. Hence, the most consistently associated allele of the HLA region does not appear to interact in any way with the four *Tg* SNPs to increase susceptibility to disease over and above that already seen with HLA-DR3 in the UK.

Although this is the largest dataset to study the *Tg* in AITD, we still cannot exclude this gene region as harboring a causal variant for AITD in the UK. A more comprehensive analysis of chromosome 8q24, including a wider range of SNPs in *Tg* and neighboring genes in a large, well matched dataset, is required to determine whether a causal variant within any one of a number of candidates in this linked region is conferring susceptibility to AITD.

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