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ORIGINAL ARTICLE

Analysis of thyroglobulin gene polymorphisms in patients with autoimmune thyroiditis

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Abstract The autoimmune thyroid disease is a complex disorder caused by a combination of genetic susceptibility and environmental factors, which are believed to initiate the autoimmune response to thyroid antigens. Identification of the susceptibility genes has found that unique and diverse genetic factors are in association with Graves' disease and autoimmune thyroiditis. The thyroglobulin gene is an identified thyroid-specific gene associated to autoimmune thyroid disease and, principally, with autoimmune thyroiditis. The aim of this work was to test for evidence of allelic association between autoimmune thyroiditis and thyroglobulin polymorphism markers. We studied six polymorphisms distributed throughout all the thyroglobulin gene: four microsatellites (Tgms1, Tgms2, TGrI29 and TGrI30), one insertion/deletion (Indel) polymorphism (IndelTG-IVS18) and one exonic single nucleotide polymorphism (SNP) (c.7589G>A) in 122 patients with autoimmune thyroiditis compared with 100 non-related normal subjects. No differences in allele and genotype distribution were observed between autoimmune thyroiditis cases and controls for Tgms1, Tgms2, TGrI30, IndelTG-IVS18 and c.7589G>A. However, when we analyzed the patients with the TGrI29 microsatellite we found a significant association between the 199-bp allele and AT (33.7% vs. 24.5% in control group) ($P = 0.0372$). In addition, a higher prevalence of the 201-bp allele has been observed in control subjects (47.5% vs. 38.1% in patients group), although not statistically significant ($P = 0.0536$). Our work shows the association between the thyroglobulin gene and autoimmune thyroiditis and reinforce that thyroglobulin is a thyroid-specific susceptibility gene for this disease.

Keywords Thyroglobulin gene · Autoimmune thyroid disease · Autoimmune thyroiditis · Genotype analysis · Polymorphism

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Abbreviations

AITD	Autoimmune thyroid diseases
AT	Autoimmune thyroiditis
GD	Graves' disease
TG	Thyroglobulin
TPO	Thyroid peroxidase
TSH-R	Thyroid-stimulating hormone receptor
HLA	Human leukocyte antigen
CTLA4	Cytotoxic T lymphocyte-associated-4
SNP	Single nucleotide polymorphism
Indel	Insertion/deletion
SDS	Sodium dodecyl sulphate

Introduction

The autoimmune thyroid disease (AITD) is a complex disorder caused by an interaction between genetic

susceptibility and environmental factors with a prevalence up to 5% of the general population in Western countries, being more frequent in women [1]. Several entities are included under this denomination, being the more relevant Graves' disease (GD) and autoimmune thyroiditis (AT), one of the most frequent human autoimmune disease [2]. GD and AT are characterized by loss of immunological self-tolerance to thyroid antigens, such as thyroglobulin (TG), thyroid peroxidase (TPO) and thyroid-stimulating hormone receptor (TSH-R) [2]. AT is characterized by lymphocytic infiltration of the thyroid gland with thyroid cell death and subsequent hypothyroidism, whereas in GD the production of stimulating autoantibodies against the TSH-R is the direct cause of hyperthyroidism. About 10% of general population and 25% of subjects above 60 years old carry antithyroid antibodies [3].

It is assumed that AT is a disease caused by interactions between exogenous factors (such as iodine intake among other unknown factors) and susceptibility genes [4, 5]. Several lines of evidence strongly suggest a genetic predisposition to AT, including familial clustering and a highest concordance rate in monozygotic twins when compared with dizygotic twins. Moreover, up to 30% of subjects with AT refer that other family members also suffer AIDT.

A whole-genome screening of 102 families revealed that multiple genes may predispose to GD and AT and that some may be common to both disorders [6, 7]. However, it was also found loci that were unique to each disease, implying that genetic factors contribute to the distinct GD and AT phenotypes.

Identification of the susceptibility genes in AITD has relied upon the investigation of candidate genes in population-based association studies or linkage analysis in families. Underpowered studies, genetic heterogeneity and type of statistic are probably the causes of the controversial results [8]. Basically, there have been identified genes involved in the immune system and thyroid-specific genes.

The primary susceptibility genes have been associated to the human leukocyte antigen (HLA) class II region on chromosome 6p21 (DR3 region) by diverse studies with different ethnic groups [9–14]. Another gene extensively studied is the cytotoxic T lymphocyte-associated-4 (CTLA4) region on chromosome 2q33. The CTLA4 molecule has been shown to provide a negative signal to the T cell, thus limiting immune responses [15] and it has been associated with diabetes [16], sclerosis multiple [17], rheumatoid arthritis [18] and AITD [19]. Case–control studies from several ethnic groups have shown an association between this gene and GD or AT [20–23]. However, none of the immune-regulator genes produced replicable association and consequently proved to be a major susceptibility gene for AITD.

Antibodies against TPO and TG are common markers for AITD and these specific genes are great candidates for association to AITD, mainly to AT. Previous studies have examined in Caucasians TPO [24, 25] and TSH-R [26, 27] genes, but they did not find linkage. TG is the major protein product synthesized in the thyroid gland, it is expressed specifically in this gland and there is solid evidence that it plays an important role in the pathogenesis of AT: antithyroglobulin antibodies are detected in almost all patients with AT [28], immunization with TG or TG fragments can induce experimental autoimmune thyroiditis in susceptible mice [29] and some sequence changes could enhance degradation in endosomes, playing a key role in the development of autoimmunity [30].

Two whole genome screens have shown strong evidence for linkage of AITD with chromosome 8q24 region, which contains the TG gene [31, 32]. There are several polymorphisms distributed throughout the TG gene. Some of them have been associated with AITD [32–39] and experimental autoimmune thyroiditis in different strains of mice [35].

The aim of this work was to test for evidence of allelic association between patients with AT and TG polymorphic markers. We have described previously two microsatellites (TGrI29 and TGrI30) [40], one insertion/deletion (Indel) polymorphism (IndelTG-IVS18) [41] and one exonic single nucleotide polymorphism (SNP) (c.7589 G>A) [42] of the TG gene, which are analyzed in this work. We also have studied microsatellites Tgms1 and Tgms2 previously reported by Tomer et al. [32]. Our genotype data showed significant increase in the incidence of 199-bp allele of the TGrI29 microsatellite in AT patients.

Materials and methods

One hundred and twenty-two unrelated Caucasian patients (mean age 53 ± 16 years) controlled at the Endocrinology Department of the University Hospital of Salamanca, diagnosed as suffering autoimmune thyroiditis based on clinical and laboratory findings were studied. All patients had clinical or subclinical hypothyroidism, requiring thyroid hormone replacement therapy, and showed very high levels of autoantibodies against TPO and TG.

A control group of 100 healthy subjects (mean age 45 ± 12) from the same area without family history of autoimmune diseases and with normal levels of antiTPO and antiTG antibodies and with similar food habits was selected.

Serum TSH was determined by immunoradiometric assay (CISbio Int., France). Sera free T_4 and T_3 were determined by immunoradiometric analysis (DPC, USA)

and antiTPO and antiTG levels were measured by radioimmunoassay (Medipan, Germany). Levels of anti-TPO were considered to be normal below 50 U/ml and of antiTG below 100 U/ml. All patients showed levels of antiTPO and of antiTG antibodies above 1500 and 500 U/ml, respectively.

Written informed consent was obtained from the individuals involved in this study and the research project was approved by the Ethical Committee of the Hospital.

Genomic DNA isolation

Genomic DNA was isolated from white blood cells by the sodium dodecyl sulphate (SDS)-proteinase K method.

Microsatellite genotyping

The Tgms1, Tgms2, TGrI29 and TGrI30 microsatellites, localized in introns 10, 27, 29 and 30 of the human TG gene, respectively, were typed as reported elsewhere [32, 40, 43]. PCR products were resolved by electrophoresis in 6% polyacrylamide denaturing gels.

IndelTG-IVS18 polymorphism genotyping

The large Indel polymorphism of 1464 bp (IndelTG-IVS18) localized in intron 18 of the human TG gene was analyzed by multiplex PCR, using the primers and PCR conditions described previously [41, 43]. The amplified fragments were analyzed in a 2% agarose gel. The amplification generates two fragments of 374-bp and 541-bp, indicating the exclusion or inclusion of the indel polymorphic region, respectively.

c.7589G>A single nucleotide polymorphism (SNP) genotyping

*Taq*I endonuclease was used to screen for the presence of the c.7589G>A SNP in exon 44 (p.R2511Q). The primers and PCR conditions were described previously [42, 43]. Non TG-specific sequences (10 nucleotides long) have been incorporated at the 5′ end of the forward and reverse primers. The samples were cleaved with *Taq*I restriction endonuclease, according to the specifications of the manufacturer (Fermentas Inc, Hanover, MD, USA) and analyzed by electrophoresis in 12% polyacrylamide gel. The 201-bp amplified products (181-bp correspond to TG sequences) contains two *Taq*I sites (positions 7587 polymorphic and 7667 not polymorphic). *Taq*I restriction showed three fragments (27, 80, and 94-bp) in the G homozygous form and two fragments (94 and 107-bp) in the homozygous A form.

Statistical analysis

The statistical difference of the genotypes distribution was analyzed by Chi-square test for trend. Case–control association of an allele in particular were performed using Fisher's exact; $P < 0.05$ was considered significant (Graph Pad Instat software). Test for Hardy–Weinberg equilibrium was done with GDA software [44].

Results

We have performed an association study to AT with several polymorphisms throughout the TG gene. All the polymorphisms were tested for Hardy–Weinberg equilibrium in cases and control groups. No difference with the expected result was found, indicating that they were on Hardy–Weinberg equilibrium.

With respect to Tgms1, we found four alleles ranging from 303-bp to 309-bp. The 305-bp/307-bp was the most frequent genotype in both populations (Table 1).

Results of Tgms2 microsatellite showed a distribution from 320-bp to 350-bp, being the most frequent alleles the 326-bp and 338-bp in AT and control subjects (Table 2). No differences in allele or genotype frequencies were observed between AT and control for this locus.

Four individual alleles (from 197-bp to 203-bp) were observed in AT and controls for TGrI29 microsatellite (Table 3). We identified a statistically significant difference in the incidence of the 199-bp allele, being more prevalent in AT patients (33.7%) compared with control subjects (24.5%). This marker was significantly associated with AT ($P = 0.0372$) (Table 3). We observed also an

Table 1 Allele and genotype frequencies of the Tgms1 system

	AT	Frequency	Control	Frequency
Alleles[a]				
303	3	0.012	0	0.000
305	109	0.447	92	0.460
307	130	0.533	106	0.530
309	2	0.008	2	0.010
Genotypes[b]				
303–305	2	0.016	0	0.000
303–307	1	0.007	0	0.000
305–305	26	0.213	20	0.200
305–307	55	0.452	52	0.520
305–309	0	0.000	1	0.010
307–307	36	0.296	26	0.260
307–309	2	0.016	1	0.010

[a] χ^2 2.54, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] χ^2 4.67, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

Table 2 Allele and genotype frequencies of the Tgms2 system

	AT	Frequency	Control	Frequency
Alleles[a]				
320	5	0.020	3	0.015
322	1	0.004	2	0.010
324	5	0.020	1	0.005
326	66	0.270	52	0.260
328	14	0.057	4	0.020
330	4	0.016	4	0.020
332	4	0.016	1	0.005
336	0	0.000	1	0.005
338	74	0.303	68	0.340
340	13	0.053	14	0.070
342	18	0.075	18	0.090
344	2	0.008	3	0.015
346	18	0.075	22	0.110
348	18	0.075	6	0.030
350	2	0.008	1	0.005
Genotypes[b]				
320–326	1	0.008	2	0.020
324–338	3	0.025	0	0.000
326–326	10	0.082	6	0.060
326–328	2	0.016	0	0.000
326–330	2	0.016	0	0.000
326–332	1	0.008	0	0.000
326–338	17	0.139	17	0.170
326–340	5	0.041	5	0.050
326–342	3	0.025	3	0.030
326–346	3	0.025	7	0.070
326–348	8	0.066	4	0.040
328–328	1	0.008	0	0.000
328–338	6	0.049	1	0.010
328–340	0	0.000	1	0.010
328–348	3	0.025	1	0.010
330–338	1	0.008	3	0.030
332–338	2	0.016	1	0.010
338–338	11	0.090	12	0.120
338–340	2	0.016	3	0.030
338–342	4	0.034	9	0.090
338–346	11	0.090	7	0.070
338–348	4	0.034	0	0.000
340–346	1	0.008	1	0.010
342–342	2	0.016	0	0.000
342–344	1	0.008	2	0.020
342–346	2	0.016	1	0.010
342–348	2	0.016	1	0.010
346–346	0	0.000	2	0.020
Others	14	0.115	11	0.110

[a] χ^2 16.54, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] χ^2 30.71, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

Table 3 Allele and genotype frequencies of the TGrI29 system

	AT	Frequency	Control	Frequency
Alleles[a]				
197	64	0.262	54	0.270
199[b]	82	0.337	49	0.245
201[c]	93	0.381	95	0.475
203	5	0.020	2	0.010
Genotypes[d]				
197–197	6	0.049	6	0.060
197–199	19	0.156	14	0.140
197–201	32	0.262	28	0.280
197–203	1	0.008	0	0.000
199–199	16	0.131	7	0.070
199–201	28	0.230	20	0.200
199–203	3	0.025	1	0.010
201–201	16	0.131	23	0.230
201–203	1	0.008	1	0.010

[a] χ^2 6.17, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] AT vs. control $P = 0.0372$ (significant)

[c] AT vs. control $P = 0.0536$ (not significant)

[d] χ^2 7.02, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

increase in the frequency of the 201-bp allele in the control population (47.5% vs. 38.1% in AT group), but the difference was not statistically significant ($P = 0.0536$).

The products of the TGrI30 microsatellite were in a size ranging between 502-bp and 542-bp (Table 4). No differences in allele or genotype frequencies were observed between AT and control for this marker. The most frequent allele was the 538-bp with a prevalence of 68.5% in AT patients and 70% in the normal group. Besides, the 538-bp/538-bp genotype was the most frequent (Table 4).

The results of the IndelTG-IVS18 polymorphism (Table 5) and the c.7589G>A SNP (Table 6) have revealed a homogenous distribution in the AT patients. We also noted this in the control group, being the frequencies of the different alleles around 50% for both populations. No association with either of the polymorphisms has been found in the studied population.

Discussion

It is well known that Tg gene is linked to AT. Human TG is also one of the main antigens in AITD and it is very common the presence of antiTg antibodies in this disorder. Linkage studies in different genome screens have shown strong evidence that the Tg gene region is linked with AITD [31, 32].

Moreover, association studies of SNPs showed a significant association of the SNP cluster exons 10–12 and the

Table 4 Allele and genotype frequencies of the TgrI30 system

	AT	Frequency	Control	Frequency
Alleles[a]				
502	28	0.115	30	0.150
506	23	0.094	15	0.075
510	4	0.016	1	0.005
522	1	0.004	0	0.000
530	2	0.008	2	0.010
534	13	0.053	11	0.055
538	167	0.685	140	0.700
542	6	0.025	1	0.005
Genotypes[b]				
502–502	2	0.016	3	0.030
502–506	0	0.000	1	0.010
502–534	1	0.008	2	0.020
502–538	22	0.180	21	0.210
502–542	1	0.008	0	0.000
506–506	1	0.008	0	0.000
506–510	1	0.008	0	0.000
506–534	1	0.008	2	0.020
506–538	18	0.149	12	0.120
506–542	1	0.008	0	0.000
510–538	3	0.025	1	0.010
522–538	1	0.008	0	0.000
530–534	1	0.008	0	0.000
530–538	1	0.008	2	0.020
534–534	0	0.000	1	0.010
534–538	10	0.082	5	0.050
538–538	54	0.443	49	0.490
538–542	4	0.033	1	0.010

[a] χ^2 6.37, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] χ^2 13.08, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

Table 5 Allele and genotype frequencies of the IndelTG-IVS18 system

	AT	Frequency	Control	Frequency
Alleles[a]				
541	129	0.529	118	0.590
374	115	0.471	82	0.410
Genotypes[b]				
541–541	34	0.279	36	0.360
541–374	61	0.500	46	0.460
374–374	27	0.221	18	0.180

[a] χ^2 0.21, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] χ^2 1.80, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

exon 33 SNP with AITD [35–39]. The exon 33 SNP showed evidence for interaction with HLA-DR3 in conferring susceptibility to Graves' disease. In contrast, Collins et al. [45] did not found association of the variants

Table 6 Allele and genotype frequencies of the c.7589G>A system

	AT	Frequency	Control	Frequency
Alleles[a]				
A	114	0.467	99	0.495
G	130	0.533	101	0.505
Genotypes[b]				
A–A	25	0.205	22	0.220
A–G	64	0.525	55	0.550
G–G	33	0.270	23	0.230

[a] χ^2 0.57, $P > 0.05$ (not significant); AT $n = 244$; control $n = 200$

[b] χ^2 0.48, $P > 0.05$ (not significant); AT $n = 122$; control $n = 100$

of exons 10, 12 and 33 with AITD in the United Kingdom population and similar results have been reported by Belguith-Maalej et al. in a Tunisian population [38]. The SNP cluster exons 10–12 were also associated to susceptibility to thyroiditis in mice [35]. Our results do not showed significant differences to the 7589 G>A SNP between case and control subjects. We did not observe any difference in allele and genotype frequency using IndelTG-IVS18 marker. This result is similar to that reported by Ban et al. [35].

Tomer et al. [32], Collins et al. [33], and Ban et al. [34] have reported previously a significant association between Tgms2 and AITD in United States of America, United Kingdom and Japanese populations, respectively. Thus, a microsatellite inside the TG gene linked and associated with AITD support the idea of TG in the etiology of AITD.

We have observed no association for any particular allele neither Tgms1 nor Tgms2. Ban et al. [34] found a significant association between the 330-bp/352-bp genotype and AITD in Japanese population, whereas Tomer et al. [32] found that the 336-bp allele was present in 30% of the patients versus the 23% of the matched Caucasian controls. Because of the low frequency of this allele in the studied population (we only found 1/444), we were unable to perform meaningful replication analysis. Similar results were described by Collins et al. [33] analyzing the alleles with a reported frequency lower than 10%. When we performed this analysis in our series, we did not find any association with AT. The discrepancies could be explained because Tomer et al. [32] and Collins et al. [33] studied more than 80% of GD patients within AITD, whereas we have included exclusively patients with AT. Several reports showed evidence for genetic heterogeneity and gene interactions for Graves and Hashimoto diseases [7, 23].

The analysis of Tgms1 do not differ from Ban et al. [34], who did not found differences in allele frequencies for this locus when comparing all AITD patients to controls in Japanese population. Tomer et al. [32] found an equivalent data in Caucasian population. He described five alleles, two

with low frequency, whereas we obtained four alleles being the more represented: 305-bp and 307-bp.

Our analysis of TGrI30 microsatellite shows fragments ranging between 502-bp and 542-bp, as described by Rivolta et al. [40]. In spite of the variability of this microsatellite, the common allele was the 538-bp with a prevalence of 68.5% in AT patients and 70% in the normal group. The allelic and the genotypic frequencies were in agreement with previous reports [40]. No association was found for this locus and AITD. However, our analysis of TGrI29, a complex microsatellite with a repeat region containing a mixture of dinucleotide tandem repeats (TC)n and (TG)n, showed an association with AITD. The frequency of the different alleles in the normal group was distributed in a similar way as described previously [40]. We obtained a statistically significant increase in the frequency of the 199-bp allele in the AT population, indicating a significant association with this disease. Nevertheless, we cannot discard that this single significant difference may be due to Type 1 error. Further analysis in larger series is necessary to confirm our results. The development of AT is a multi-step process and the pathogenesis is not well known. There are sufficient epidemiological data to support an important genetic contribution to the development of AITD combined with environmental effects. Unlike the other putative AITD immune regulators susceptibility genes, such as HLA and CTLA4, there is strong evidence to support the TG gene as a thyroid-specific AITD susceptibility gene. Identification of additional AITD susceptibility genes would help to understand molecular mechanism by which they induce thyroid autoimmunity.

In conclusion, our results suggest that TG is a susceptibility gene for AT and confirm previous reports that associated polymorphisms within the TG gene and this autoimmune disease.

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