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ORIGINAL ARTICLE

Association of the TGrI29 microsatellite in thyroglobulin gene with autoimmune thyroiditis in a Argentinian population: a case–control study

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Abstract Autoimmune thyroid disease (AITD) is a multifactorial disorder that involves a putative association with thyroid autoantigen-specific and immune regulatory genes, as well as environmental factors. The thyroglobulin gene is the main identified thyroid autoantigen-specific gene associated to autoimmune thyroiditis. The aim of this work was to test for evidence of allelic association between autoimmune thyroiditis (AT) and thyroglobulin polymorphism markers in Argentinian patients. We studied six polymorphisms distributed throughout all the thyroglobulin gene: four microsatellites (Tgms1, Tgms2, TGrI29, and TGrI30), one insertion/deletion polymorphism (IndelTG-IVS18), and one exonic single nucleotide polymorphism (c.7589G>A) in 100 AT patients and 100 healthy control subjects. No differences in allele and genotype frequencies distribution were observed between autoimmune thyroiditis cases and controls for Tgms1, Tgms2, TGrI30, IndelTG-IVS18, and c.7589G>A. However, when we analyzed autoimmune thyroiditis patients with the TGrI29 microsatellite we found a significant association between the 197-bp allele and autoimmune thyroiditis (33.50% vs. 19.00% in control group) ($P = 0.001$). In addition, a significant major prevalence of the 197/201-bp genotype has been also seen in autoimmune thyroiditis subjects (59% vs. 24% in control group, $P < 0.0001$). In conclusion, our work showed the association between the thyroglobulin gene and autoimmune thyroiditis in Argentinian population and supports the described evidence of thyroglobulin as a thyroid-specific gene linked to AITD.

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

Abbreviations

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

Introduction

Autoimmune thyroid disease (AITD) is organ-specific T-cell-mediated disease in which T lymphocytes infiltration results in dysfunction of the thyroid cells. The two most common phenotypes of AITD are Graves' disease (GD; MIM 275000) and chronic autoimmune thyroiditis (AT) with its classical goitrous form (Hashimoto's thyroiditis;

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MIM 140300) and the atrophic variant. These two disorders affect up to 5% of the population [1], the female to male prevalence ratio ranges from 5:1 to 10:1 [2]. In GD, the thyroid is infiltrated by thyroid-specific T cells that activate B lymphocytes to secrete TSH receptor (TSHR)-stimulating antibodies [3]. These antibodies induce proliferation of thyrocytes and the secretion of excess thyroid hormones, resulting in classic hyperthyroid symptoms, diffuse goiter and specific ophthalmopathy. Whereas in AT, T lymphocytes are sensitized to thyroid antigens and the end result is apoptosis of thyroid follicular cells manifested clinically by hypothyroidism [4]. The etiology of the AITD is multifactorial. Environmental factors such as iodine supplementation and infection [5, 6] modulate the effect of susceptibility genes [7, 8]. Linkage studies on multiplex families with AITD have shown that several genes are responsible for a predisposition towards GD and AT, and that some are common to both diseases and some are unique [2, 9–13].

In the last decades, five candidate immune-regulator genes have been shown to contribute to the development of AITD: human leukocyte antigen (HLA) [14–16], CD40 [17–19], cytotoxic T lymphocyte antigen 4 (CTLA-4) [19–22], protein tyrosine phosphatase-22 (PTPN22) [19, 23–25], and interleukin 23 receptor (IL-23R) [26]. Increased frequency of HLA-DR3 and HLA-DQA1*0501 haplotypes has been demonstrated in GD patients and AT has been associated with HLA-DR3, HLA-DR5, and DQB1*0301 [15, 16]. However, others studies have shown no linkage between GD or AT and the HLA gene [27]. CD40 gene, one of the most extensively studied loci for autoimmunity, plays a fundamental role in B cell activation, inducing B cell proliferation and antibody secretion [19]. CD40 was identified as a susceptibility gene for GD. A C/T single nucleotide polymorphism (SNP) in the 5-untranslated region of the CD40 gene is associated with GD. The presence of CC genotype may be associated with increased risk [18]. Further studies demonstrated that the CC genotype increased the translational efficiency of CD40 [28]. CTLA-4, expressed on the surface of activated T lymphocytes, is a key inhibitor of T cell activation. CT60 polymorphism (c.6230G>A) of the CTLA-4 gene have also been associated with GD and AT [22]. The G allele increases the odds of both GD and AT. The lymphoid tyrosine phosphatase encoded by the PTPN22 gene is an inhibitor of T cells. The c.1858 C>T polymorphism (p.R620W) within the PTPN22 gene has been associated with GD [23] and AT [25]. Variants in the IL-23R gene are strongly associated with Graves' ophthalmopathy [26]. IL-23 enhances lymphocyte proliferation, induces interferon-γ production and promotes T-helper 1 cell differentiation by dendritic cells. However, none of the immune-regulator genes produced replicable association and consequently proved to be the major susceptibility genes for AITD.

Thyroglobulin (TG) along with thyroid peroxidase (TPO) and TSHR genes encodes the major specific thyroid autoantigens. The TG gene has been identified as the major susceptibility gene for AITD, by linkage and association analysis in Caucasians patients of American [29, 30], British [31] and Spanish [32] origin, and in Japanese [33], Taiwanese [34, 35] and Chinese [36] population using informative polymorphic markers. In contrast, analysis in Tunisian population [37] and a largest case–control association study in the United Kingdom [38] did not show association of the TG gene with AITD.

The purpose of the present work was to test whether TG gene plays a role in the development of AT in a Argentinian population, a case–control study was conducted on 100 unrelated AT women patients and 100 healthy control women subjects. We analyzed four microsatellites (Tgms1, Tgms2, TGrI29, and TGrI30) [29, 39], one insertion/deletion (Indel) polymorphism (IndelTG-IVS18) [40], and one exonic SNP (c.7589 G>A) [41] of the TG gene. Our genotype data showed significant increase in the incidence of 197-bp allele of the TGrI29 microsatellite in AT Argentinian patients.

Materials and methods

Subjects

A total of 100 consecutive unrelated Caucasian female AT patients (mean age 42 ± 15 years) from Argentinian population evaluated at the "Centro de Estudios Metabólicos y Endocrinos" of Buenos Aires were studied. AT was diagnosed on the basis of high levels of anti-TPO and/or anti-TG. The information of thyroid function tests are shown in Table 1. A whole of 65 patients had clinical (22 patients) or subclinical (43 patients) hypothyroidism, requiring thyroid hormone replacement therapy (mean dose of levothyroxine 90 ± 28 μg/day). None of the AT patients had been previously treated with amiodarone, interferon-alpha, or lithium.

A control group of 100 healthy Caucasian female volunteers (mean age 46 ± 15 years) from the same area, with similar food habits, without family history of autoimmune diseases including type 1 diabetes and with normal levels of anti-TPO and anti-TG antibodies were selected. They were in euthyroid state according to the laboratory tests (Table 1).

Serum TSH was determined by immunoradiometric assay (Immulite Siemens AG, Erlagen, Germany). Sera free T_4 and T_3 were determined by immunoradiometric analysis (Immulite Siemens AG, Erlagen, Germany) and anti-TPO and anti-TG levels were measured by

Table 1 Biochemical parameters of AT patients and controls

	Serum TSH mU/l	Serum TT_4 (μg/dl)	Serum TT_3 (ng/dl)	Serum FT_4 (ng/dl)	Anti-TPO (UI/ml)	Anti-TG (UI/ml)
AT	3.03 ± 4.15	7.97 ± 2.19	1.04 ± 0.24	1.25 ± 0.27	218.9 ± 485.08	256.25 ± 406.19
Control	2.28 ± 1.32	7.53 ± 1.69	1.10 ± 0.27	1.21 ± 0.21	20.75 ± 2.93	12.78 ± 5.47
Reference range	0.40–4.0	4.5–12.5	0.7–1.8	0.8–2.0	<40	<35
P	ns	ns	ns	ns	<0.005	<0.0001

ns not significant

chemiluminescent sequential immunometric assay (Immulite Siemens AG, Erlagen, Germany).

Written informed consent was obtained from all participants in this study and the research project was approved by the institutional review board.

Genomic DNA isolation

Genomic DNA was isolated from white blood cells by the sodium dodecyl sulfate (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 [29, 39]. 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 [40]. The amplified fragments were analyzed in a 2% agarose gel. The amplification generates two fragments of 374 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 [41]. Non TG-specific sequences (10 nucleotides long) have been incorporated at the 5′-end of the forward and reverse primers. The PCR products 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, 94-bp) in the G homozygous form and two fragments (94 and 107-bp) in the homozygous A form.

Statistical analysis

Allele or genotype carrier frequency was defined as the percentage of the individuals carrying the allele or genotype among the total of the individuals. χ^2 test or Fisher's exact test (Graph Pad Instat software) was used to determine whether intergroup differences in the genotype distributions of the polymorphisms were significant between cases and controls. Case–control association of an allele in particular was performed using Fisher's exact test. $P < 0.05$ was considered statistically significant. For the sake of simplicity, *P*-values were rounded to three decimals.

Results

The six polymorphisms genotyped in this study were chosen depending on their variability and location throughout the TG gene. Allele and genotype frequencies of each are shown in Tables 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13. To test the association between each locus and AT, we compared differences in allele and genotype frequency of each polymorphism between case and control subjects.

We found four alleles ranging from 303 to 311-bp for Tgms1. The 303 and 311-bp alleles were present only in

Table 2 Allele frequencies of Tgms1 system

Alleles (bp)	AT	Frequency	Control	Frequency
303	0	0.000	2	0.010
305	84	0.420	84	0.420
307	112	0.560	111	0.555
309	4	0.020	2	0.010
311	0	0.000	1	0.005

$\chi^2 = 3.671$, $P = 0.452$ (not significant), d.f. = 4; AT $n = 200$; control $n = 200$

Table 3 Genotype frequencies of Tgms1 system

Genotypes (bp)	AT	Frequency	Control	Frequency
303-307	0	0.000	2	0.002
305-305	20	0.200	22	0.220
305-307	44	0.440	40	0.400
305-309	2	0.002	0	0.000
307-307	32	0.320	33	0.330
307-309	2	0.002	2	0.002
307-311	0	0.000	1	0.001

$\chi^2 = 5.301$, $P = 0.506$ (not significant), d.f. = 6, AT $n = 100$; control $n = 100$

Table 4 Allele frequencies of Tgms2 system

Alleles (bp)	AT	Frequency	Control	Frequency
320	5	0.025	1	0.005
322	0	0.000	1	0.005
324	1	0.005	1	0.005
326	43	0.215	44	0.220
328	8	0.040	6	0.030
330	2	0.010	3	0.015
332	3	0.015	1	0.005
334	0	0.000	1	0.005
336	1	0.005	1	0.005
338	66	0.330	77	0.385
340	17	0.085	24	0.120
342	16	0.080	8	0.040
344	1	0.005	2	0.010
346	22	0.110	16	0.080
348	15	0.075	12	0.060
350	0	0.000	2	0.010

$\chi^2 = 14.486$, $P = 0.489$ (not significant), d.f. = 15, AT $n = 200$, control $n = 200$

controls although there was no statistically significant difference compared with the AT group (Table 2). The 305/305, 305/307, and 307/307-bp were the most frequent genotypes in both populations (Table 3).

Results of Tgms2 microsatellite showed a distribution from 320 to 350-bp showing the 326 and 338-bp alleles the highest frequencies in AT and control subjects (Table 4). The 322, 334, and 350-bp alleles were present only in controls. No differences in allele or genotype frequencies were observed between AT and control for this locus (Tables 4, 5). The genotype analysis has revealed a high heterogenous distribution in both groups (Table 5).

Four alleles (from 197 to 203-bp) were observed in AT and controls for TGrI29 microsatellite (Table 6). This marker was significantly associated with AT ($P = 0.003$).

Table 5 Genotype frequencies of Tgms2 system

Genotypes (bp)	AT	Frequency	Controls	Frequency
320-328	1	0.010	1	0.010
320-330	1	0.010	0	0.000
320-346	1	0.010	0	0.000
320-348	2	0.020	0	0.000
322-340	0	0.000	1	0.010
324-340	1	0.010	0	0.000
324-350	0	0.000	1	0.010
326-326	5	0.050	7	0.070
326-328	0	0.000	2	0.020
326-330	1	0.010	0	0.000
326-332	1	0.010	1	0.010
326-336	0	0.000	1	0.010
326-338	15	0.150	10	0.100
326-340	4	0.040	3	0.030
326-342	4	0.040	2	0.020
326-344	0	0.000	2	0.020
326-346	5	0.050	7	0.070
326-348	3	0.030	2	0.020
328-328	1	0.010	0	0.000
328-338	4	0.040	2	0.020
328-342	0	0.000	1	0.010
328-346	1	0.010	0	0.000
328-348	0	0.000	1	0.010
330-338	0	0.000	2	0.020
330-340	0	0.000	1	0.010
332-344	1	0.010	0	0.000
332-348	1	0.010	0	0.000
334-338	0	0.010	1	0.010
336-346	1	0.010	0	0.000
338-338	10	0.100	18	0.180
338-340	7	0.070	8	0.080
338-342	7	0.070	4	0.040
338-344	0	0.000	0	0.000
338-346	8	0.080	5	0.050
338-348	5	0.050	7	0.070
338-350	0	0.000	1	0.010
340-340	0	0.000	3	0.030
340-342	2	0.020	1	0.010
340-346	1	0.010	3	0.030
340-348	2	0.020	1	0.010
342-342	1	0.010	0	0.000
342-346	1	0.010	0	0.000
346-346	1	0.010	0	0.000
346-348	2	0.020	1	0.010

$\chi^2 = 40.539$, $P = 0.535$ (not significant), d.f. = 42, AT $n = 100$, control $n = 100$

Table 6 Allele frequencies of TGrI29 system

Alleles (bp)	AT	Frequency	Control	Frequency
197[a]	67	0.335	38	0.190
199[b]	39	0.195	45	0.225
201[c]	92	0.460	108	0.540
203[d]	2	0.010	9	0.045

$\chi^2 = 14.173$, $P = 0.003$ (significant), d.f. = 3, AT $n = 200$, control $n = 200$

[a] AT versus control $P = 0.001$ (significant)

[b] AT versus control $P = 0.539$ (not significant)

[c] AT versus control $P = 0.133$ (not significant)

[d] AT versus control $P = 0.062$ (not significant)

[a, b, c] and [d] by Fisher' exact test

Table 7 Genotype frequencies of TGrI29 system

Genotypes (bp)	AT	Frequency	Control	Frequency
197-197[a]	0	0.000	2	0.020
197-199[b]	8	0.080	10	0.100
197-201[c]	59	0.590	24	0.240
197-203[d]	0	0.000	1	0.010
199-199[e]	4	0.040	5	0.050
199-201[f]	21	0.210	22	0.220
199-203[g]	2	0.020	3	0.030
201-201[h]	6	0.060	30	0.300
201-203[i]	0	0.000	3	0.030

$\chi^2 = 37.316$, $P < 0.0001$ (significant), d.f. = 8, AT $n = 100$, control $n = 100$

[a] AT versus control $P = 0.497$ (not significant)

[b] AT versus control $P = 0.806$ (not significant)

[c] AT versus control $P < 0.0001$ (significant)

[d] AT versus control $P = 1.000$ (not significant)

[e] AT versus control $P = 1.000$ (not significant)

[f] AT versus control $P = 1.000$ (not significant)

[g] AT versus control $P = 1.000$ (not significant)

[h] AT versus control $P < 0.0001$ (significant)

[i] AT versus control $P = 0.246$ (not significant)

[a, b, c, d, e, f, g, h] and [i] by Fisher' exact test

We identified a statistically significant difference ($P < 0.001$) in the incidence of the 197-bp allele, being more prevalent in AT patients (33.5%) compared with control subjects (19.0%) (Table 6). We observed also a statistically significant increase ($P < 0.0001$) in the frequency of the 197/201-bp genotype in the AT (59.0% vs. 24.0% in control group) (Table 7). In contrast, when the 201/201-bp genotype was analyzed, the frequency was statistically lesser ($P < 0.0001$) in subjects with AT (6.0% vs. 30.0% in control group) (Table 7).

The products of the TGrI30 microsatellite were in a size ranging between 502 and 546-bp (Table 8). No differences

Table 8 Allele frequencies of TgrI30 system

Alleles (bp)	AT	Frequency	Control	Frequency
502	24	0.120	15	0.075
506	19	0.095	15	0.075
510	1	0.005	5	0.025
522	0	0.000	1	0.005
530	3	0.015	0	0.000
534	8	0.040	6	0.030
538	142	0.710	151	0.755
542	3	0.015	6	0.030
546	0	0.000	1	0.005

$\chi^2 = 11.776$, $P = 0.161$ (not significant), AT $n = 200$, control $n = 200$

Table 9 Genotype frequencies of TgrI30 system

Genotypes (bp)	AT	Frequency	Control	Frequency
502-502	1	0.010	0	0.000
502-506	0	0.000	1	0.010
502-534	1	0.010	0	0.000
502-538	21	0.210	14	0.140
506-510	1	0.010	0	0.000
506-534	3	0.030	1	0.010
506-538	15	0.150	11	0.110
506-542	0	0.000	1	0.010
506-546	0	0.000	1	0.010
510-538	0	0.000	5	0.050
522-538	0	0.000	1	0.010
530-538	3	0.030	0	0.000
534-538	4	0.040	5	0.050
538-538	48	0.480	55	0.550
538-542	3	0.030	5	0.050

$\chi^2 = 19.102$, $P = 0.161$ (not significant), AT $n = 100$, control $n = 100$

Table 10 Allele frequencies of IndelTG-IVS18 system

Alleles (bp)	AT	Frequency	Control	Frequency
374	83	0.415	67	0.335
541	117	0.585	133	0.665

$P = 0.121$ (not significant) Fisher' exact test, AT $n = 200$ control $n = 200$

Table 11 Genotype frequencies of IndelTG-IVS18 system

Genotypes (bp)	AT	Frequency	Control	Frequency
374-374	17	0.170	12	0.120
374-541	49	0.490	43	0.430
541-541	34	0.340	45	0.450

$\chi^2 = 2.785$, $P = 0.248$ (not significant), d.f. = 2, AT $n = 100$; control $n = 100$

Table 12 Allele frequencies of c.7589G>A system

Alleles	AT	Frequency	Control	Frequency
A	82	0.410	102	0.510
G	118	0.590	98	0.490

$P = 0.056$ (not significant) Fisher's exact test, AT $n = 200$, control $n = 200$

Table 13 Genotype frequencies of c.7589G>A system

Genotypes	AT	Frequency	Control	Frequency
AA	17	0.170	30	0.300
AG	48	0.480	42	0.420
GG	35	0.350	28	0.280

$\chi^2 = 4.774$, $P = 0.092$ (not significant); d.f. = 2, AT $n = 100$, Control $n = 100$

in allele or genotype frequencies were observed between AT and control for this marker (Tables 8, 9). The 538-bp allele has the higher frequency, 71.0% in AT patients and 75.5% in the control group. The 522 and 546-bp alleles were present only in controls, whereas the 530-bp was present only in AT group (Table 8). Besides, the 538/538-bp genotype was the most frequent in both groups (Table 9).

No significant differences were found for the allele and genotype frequencies of the IndelTG-IVS18 (Tables 10, 11) polymorphism and the c.7589G>A SNP (Tables 12, 13) between the cases and the controls.

Discussion

This study investigated the contribution of the TG gene in the genetic susceptibility of AITD in the Argentinian population. A case–control association analysis was performed by genotyping four microsatellites, one Indel polymorphism, and one exonic SNP.

Human TG is one of the main autoantigens in AITD and anti-TG antibodies are common in AITD. TG immunization induces experimental autoimmune thyroiditis in mice, major histocompatibility complex (MHC) dependent, implicating an interaction between the TG and the MHC glycoproteins in the induction of thyroiditis [30]. TG is a large homodimeric secretory glycoprotein that serves as a unique protein precursor for thyroid hormone formation [42]. It is coded by a single copy gene, 270 kb long (GenBank accession number NT_008046) which maps on chromosome 8q24 and contains 8.5 kb of coding sequence divided into 48 exons [42, 43]. 21 SNPs were identified and characterized in the coding sequence of the TG gene, 14 of them resulting in amino acid polymorphisms [42] suggesting that these alterations together with the interaction with immune regulatory genes as well as environmental factors may play a role in the pathogenesis of this autoimmune disorder. To date, there are no data on a putative functional role for the TG polymorphic changes.

The analysis obtained of several populations has provided controversial results due to the fact that the ethnic heterogeneity may influence the association of the AITD with the gene TG. Tomer et al. [29], Collins et al. [31], and Ban et al. [33] have reported previously a significant association between microsatellite Tgms2 and AITD in United States of America, United Kingdom, and Japanese populations, respectively. These studies support the idea that the TG is an important susceptibility gene for AITD. However, Collins et al. [31] reported the positive association by considering the whole number of rare alleles of the Tgms2, consequently as postulated by Collins et al. this positive association could be a random chance event. In contrast, genotyping of Tgms2 in multiplex families affected with AITD and a case–control study in patients affected with GD, in Tunisian population, did not show linkage or association [37]. Association studies of TG SNPs demonstrated that the exon 10, 12, and 33 SNPs were significantly associated with United States of America [30] and Chinese [36] AITD patients. Another study suggested that the exon 33 SNP is associated with relapse of the Graves' hyperthyroidism after antithyroid withdrawal in Taiwanese population [34]. However, Collins et al. [38], Ban et al. [33], and Belguith-Maalej et al. [37] did not find association of the variants of exons 10, 12, and 33 with AITD in the United Kingdom, Japanese and Tunisian population, respectively.

Our results do not show significant differences to the Tgms1, Tgms2, and TGrI30 microsatellites, and 7589G>A SNP between case and control subjects. No differences in allele and genotypes frequencies were also observed by us and by Ban et al. [30] using IndelTG-IVS18 marker. In contrast, TGrI29 microsatellite showed an association with AITD. We obtained a statistically significant increase in the frequency of the 197-bp allele in the AT population. This finding confirms our previous studies in Spanish populations using the TGrI29 marker, except for that the most frequent allele in these patients is the 199-bp suggesting an allelic heterogeneity [32]. The Argentinian population exhibits an ethnic diversity and includes an important component of Hispanic origin that would explain the similar results in both populations.

At this stage, the role that TG plays in the pathogenic mechanisms involved in AITD remains unknown. Though the TG gene can contribute in direct form to AITD development, we cannot exclude that the TG is only a marker for thyroid autoimmunity, not directly linked to its cause.

In conclusion, our results support an association between TG and AT in Argentinian population and confirmed a relationship between the AT and the TGrI29 microsatellite. This is the first analysis of the TG gene in AITD in the Argentinian population. The availability of informative polymorphic markers, as described here, is very suitable for use in laboratories engaged in the genetic susceptibility analysis of families with AT.

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