



## Association of *CTLA4* Gene Polymorphism with Ophthalmopathy of Graves' Disease in a Spanish Population

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### ARTICLE INFO

#### Article type:

Original Article

#### Article history:

Received: 05 Feb 2011

Revised: 29 Mar 2011

Accepted: 30 Apr 2011

#### Keywords:

Graves' Disease

Polymorphism

Ophthalmopathy

*CTLA4*

### ABSTRACT

**Background:** Graves' disease (GD) is an autoimmune disease that develops as a result of a complex interaction between genetic and environmental factors. Numerous studies have demonstrated the important role of *CTLA4* gene polymorphisms in the susceptibility to this disease. The *CTLA4* gene is located on chromosome 2q33 and codes for the T-cell receptor, which negatively modulates the immune response by disabling T cells.

**Objectives:** The aim of the present work was to determine whether A/G dimorphism at position +49 of exon 1 in the *CTLA4* gene contributes to the severity and clinical manifestations of GD.

**Patients and Methods:** We performed clinical and genetic studies on 100 Graves' patients and 50 healthy controls. We determined the subjects' genotypes for the +49 A/G polymorphism of the *CTLA4* gene by PCR and an enzyme restriction test. Comparison of individual clinical and laboratory variables between genotypes was performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

**Results:** We found a statistically significant relationship between *CTLA4* gene polymorphism and ophthalmopathy in Graves' patients.

**Conclusions:** The +49A/G SNP of the *CTLA4* gene is related to the development of Graves' disease; however, more studies are necessary to clarify the role of the *CTLA4* gene in influencing GD susceptibility and to explore other potential costimulation pathways in this disorder.

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#### ► Implication for health policy/practice/research/medical education:

Evaluation whether the A/G +49 SNP marker of the *CTLA* gene is associated with GD in a Spanish population, and associations between the *CTLA4* genotype and clinical characteristics of Grave patients could permit choosing the more appropriate treatment regimen and predict the post-treatment outcome of these patients.

#### ► Please cite this paper as:

Alvarez-Vazquez P, Constenla L, Garcia-Mayor RV, Larranaga A, Valverde D. Association of *CTLA4* Gene Polymorphism With Ophthalmopathy of Graves' Disease in a Spanish Population: 397-402. DOI: 10.5812/Kowsar.1726913X.2613

## 1. Background

Autoimmune thyroid diseases (AITD) encompass a number of conditions that have common cellular and

humoral immune responses targeted to the thyroid gland. Classical AITD includes Graves' disease (GD), which is a heterogeneous autoimmune disorder affecting the thyroid, eyes, and skin (1). As with some other autoimmune endocrine disorders, it is generally accepted that GD has a genetic basis. The majority of autoimmune endocrinopathies are inherited as complex genetic traits, with multiple genetic factors interacting with each other and with environmental factors, which ultimately confer disease susceptibility. Recently, efforts have been made

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DOI: 10.5812/Kowsar.1726913X.2613

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to define these genetics factors, and several loci have emerged as contributors to the genetic susceptibility to these autoimmune diseases (2).

Several studies have shown an association between the cytotoxic T lymphocyte-associated antigen-4 (*CTLA-4*) gene and AITD (3). A number of investigations have provided evidence in support of a relationship between the *CTLA-4* gene and features of autoimmune thyroid disease, such as the production of thyroid autoantibodies (Tab) (4-6). *CTLA-4* protein is also implicated in autoimmune thyroid disease, and can downregulate T cell responses by two separate mechanisms. The first of these mechanisms is *CTLA-4*-mediated negative signaling in response to T-cell receptor activation (7); this requires the cytoplasmic tail of the *CTLA-4* protein and can occur in the early stages of an immune response when expression of *CTLA-4* and B7 is limited (8). The second mechanism operates through cell surface competition between *CTLA-4* and CD28 for B7 binding, depends on the levels of surface expression of *CTLA-4*, and can occur in late stages of the immune response when there is increased expression of B7 and *CTLA-4*. Binding of B7 to *CTLA-4* leads to termination of the immune response via limitation of CD28-mediated signaling, T cell anergy, and T cell apoptosis (8).

Three sequence markers located in the *CTLA-4* gene have been demonstrated to be associated with GD (9-11). The first marker is located in the 3' untranslated region (UTR) of the *CTLA-4* gene, whereas the other two are SNP's (single nucleotide polymorphisms). One SNP is located in the promoter region (-318 C/T), and the other is an A/G transition at position 49 of the coding region, resulting in an alanine/threonine polymorphism. The association between GD and the *CTLA-4* 3' UTR microsatellite and A/G +49 SNP (rs 231775) has been described in several populations with different ethnic backgrounds, including Caucasians, Japanese, Chinese, and Koreans (12-14).

Association studies using the -C/T 318 polymorphism have been inconsistent and have produced controversial results, with some studies reporting a disease association and others suggesting no association (14). However, the G allele in the *CTLA-4* marker A/G +49 SNP has been associated with more severe manifestations of several different autoimmune disorders, including GD, and is a general predisposing allele for both the presence and severity of autoimmunity. The effect of this SNP on the expression and function of the *CTLA-4* protein remains unclear, although several authors have found different levels of surface expression of *CTLA-4*, and its intracellular distribution is correlated with the genotype at position 49. The sequence where this SNP is located serves as a signal peptide that directs the secreted protein to the endoplasmic reticulum. Importantly, it is unclear whether this SNP can affect the conformation of the leader peptide, leading to an altered intracellular trafficking destination, or whether this change may be due to the effects of another *CTLA-4* polymorphism that is in linkage disequilibrium with the A/G +49 SNP (15). Several authors

have noted an association of this SNP with long-term remission after antithyroid therapy in distinct populations with different ethnic backgrounds (16-18).

## 2. Objectives

The purpose of this study was to evaluate whether the A/G +49 SNP marker of the *CTLA* gene is associated with GD in a Spanish population, and to search for putative associations between the *CTLA4* genotype and clinical characteristics of the patients. In the case of find relationship between the mentioned genetic marker with some clinical variables, it could permit us choose the more appropriate treatment regimen and predict the post-treatment outcome of these patients.

## 3. Patients and Methods

### 3.1 Patients and Controls

One hundred Graves' disease patients (84 female, 16 male; aged  $40 \pm 18$  years, range 18-79) were included in this study, which was carried out at the Endocrine Department of the University Hospital of Vigo, Spain. Patients were diagnosed according to the following criteria: symptoms and signs (presence of orbitopathy) of hyperthyroidism, elevated values of serum-free thyroxine (FT<sub>4</sub>) or FT<sub>3</sub> and decreased values of thyroid-stimulating hormone (TSH), diffuse thyroid uptake of <sup>99</sup>Tc, and TSH receptor antibody (TRAb) values over the reference values, as previously described (19). We also included 50 normal control subjects from the local population (hospital employees) who had neither a familial history of thyroid disease nor thyroid dysfunction or serum-positive thyroid antibodies.

Patients were considered in remission when FT<sub>4</sub>, FT<sub>3</sub>, TSH, and TRAb values were within the normal range after discontinuing antithyroid drugs.

### 3.2. Evaluation of Patients

We collected information on the *CTLA-4* genotype and the following clinical and laboratory variables at diagnosis (before initiation of treatment): age, gender, family history of thyroid disease, smoking history, previous stress levels, presence and classification of eye disease, presence and size of diffuse goiters, and the percentage of patients in early remission after cessation of treatment. The size of the goiter at diagnosis was determined by palpation and categorized according to the Delange *et al.* classification (20). Eye disease was defined by the presence of eye manifestations in categories 2-6 according to the presence or absence of signs or symptoms, soft-tissue involvement, proptosis, extraocular muscle involvement, corneal involvement and sight loss (NOSPECS) classification (21).

### 3.3. Hormone Tests

Serum concentrations of FT<sub>4</sub> (reference values, 0.93-1.71

ng/dl), FT3 (reference values, 2.55–4.33 pg/mL), and TSH (reference values, 0.30–4.50  $\mu$ U/mL) were determined by immunochemiluminiscent assay (ICMA), using IMMULITE (Diagnostic Products Corp., Los Angeles, CA, USA). The intra- and inter-assay coefficients of variations (CV) for FT4, FT3, and TSH were 4.4% and 4.8%, 5.7% and 8.1%, and 3.8% and 4.6%, respectively. TRAbs (reference values, 0–10 U/l) were measured using a radioreceptor assay (TRAK-assay; Henning, Berlin, Germany). The intra- and inter-assay CVs were 5% and 7.5%, respectively.

### 3.4. Treatment and Follow-up

Patients were treated with an initial methimazole dosage of 30 mg daily, which was reduced gradually as serum thyroid hormone concentrations declined. Patients completed a treatment course of at least 1 year and were followed after drug withdrawal. Early relapse was defined as the presence of clinical manifestations, elevated FT4 and/or FT3, and suppressed TSH levels during the first year after cessation of drug therapy.

### 3.5. CTLA-4 Exon 1 Polymorphism Analysis

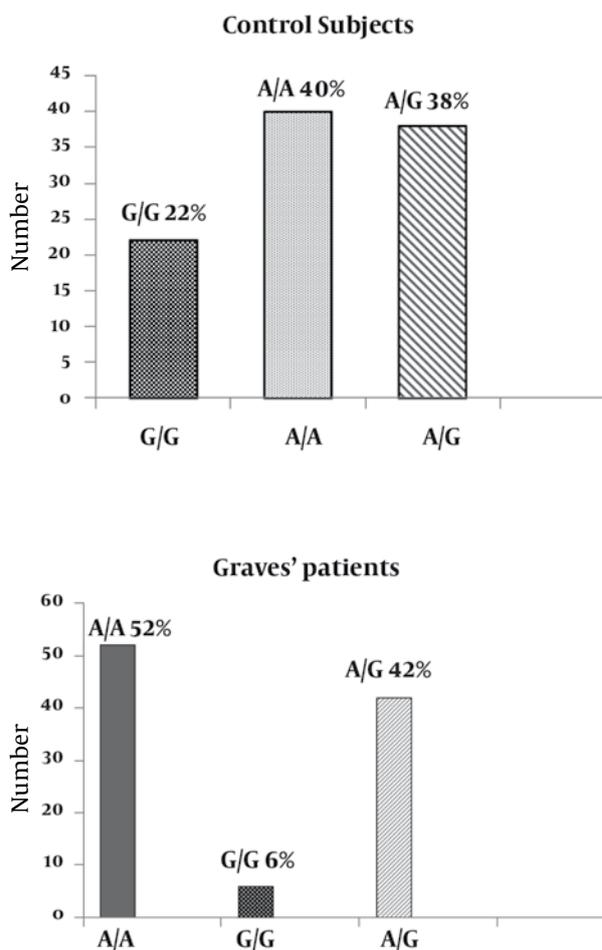
DNA was extracted from whole blood using a DNA Isolation Kit for Mammalian Blood (Roche Applied Science). The CTLA-4 exon 1 A/G +49 SNP was genotyped by PCR-restriction fragment length polymorphism (RFLP) analysis. The primers used for amplification were described previously by Vaidya *et al.* (22). PCR reactions were performed as follows in a final volume of 25  $\mu$ l: 5-min denaturation at 95°C, 30 cycles of 0.3 min at 95°C, 0.3 min at 58°C, and a final 5-min extension at 72°C. The 323-base pair (bp) PCR product was incubated with the restriction enzyme *Bse*XI (Fermentas International Inc.) at 65°C overnight in a final volume of 20  $\mu$ l. After digestion, the fragments were resolved on a 2% agarose gel containing 10 mg/mL ethidium bromide, visualized under UV light, and compared with a molecular weight marker (Ecoladder IV, 1000 bp). The presence of the A allele results in an undigested PCR fragment, whereas the presence of the G allele results in a digested PCR product with two fragments of 258 bp and 65 bp. We confirmed the correct designation of the allele for the CTLA4 +49A/G polymorphism by sequencing.

### 3.6. Statistical Analysis

The genotypes and alleles of patients and controls were compared using the  $\chi^2$  test or Fisher's exact test. Odds ratios (OR) were calculated using Wol's method. Comparison of individual clinical and laboratory variables between genotypes were assessed with a one-way ANOVA for normally distributed continuous variables and with the Mann-Whitney *U*-test for not normally distributed continuous variables. Categorical variables were analyzed using the  $\chi^2$  test. A two-tailed *P* value < 0.05 was considered statistically significant. These analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA) for Windows XP.

## 4. Results

Among the 100 GD patients analyzed, 14 were men and 86 were women. The control group comprised 100 chromosomes from the local population. The genotype frequencies for the two groups analyzed are shown in Figure 1. The frequencies of the genotypes described were signif-



**Figure 1.** Frequencies of Three Genotypes Observed in the two Groups Studied

**Table 1.** Frequencies of A and G Alleles in Graves' Patients and Control Subjects

A49G Mutation	Graves (n = 100)	Controls (n = 50)
Genotype distribution, No. (%)		
A/A	39 (39)	26 (52)
A/G	39 (39)	21 (41)
G/G	22 (22)	3 (6)
Allele frequency		
A, %	61%	73%
G, %	39%	27%
$\chi^2$ , No.	6.04	-
p, No.	0.0140	-
OR, No.	1.9	-

**Table 2.** Comparison of Various Clinical and Laboratory Variables in Graves' Patients with G/G, G/A, and A/A Genotypes

	G/G	G/A	A/A	P
Age, y, Mean $\pm$ SD	38.2 $\pm$ 12.3	39.54 $\pm$ 13.7	43.15 $\pm$ 15.17	N.S.
Patient, No.				
Men	0	9	5	0.038
Female	22	29	34	0.038
Initial T4, $\mu$ g/dL, Mean $\pm$ SD	6.19 $\pm$ 5.04	4.62 $\pm$ 1.23	6.42 $\pm$ 6.29	N.S.
Goiter, %	22	37.23	40.4	N.S.
Dermopathy	0	0	3	-
Antithyroid treatment (MTZ/CBZ) <sup>a</sup>	13/8	23/10	26/10	N.S.
Duration of therapy, Mean $\pm$ SD	1.59 $\pm$ 0.59	1.88 $\pm$ 0.9	2.25 $\pm$ 1.48	N.S.
Remission, %	10	20	18	N.S.
Ophthalmopathy, %	23.8	59.5	44.7	0.024
Cigarette smoking, %	100	84	87	N.S.

<sup>a</sup> Abbreviations: MTZ, methimazole; CBZ, carbimazol

**Table 3.** Frequencies of G/G, G/A, and A/A Genotypes in Graves' Patients and Control Subjects in Distinct Populations

Reference	Population	A49G Genotype Distribution	GG, %	GA, %	AA, %
Vaidya, et al. 1999	Caucasian	Graves	21	48	31
		Controls	12	38	50
Donner, et al. 1997	Caucasian	G	21	53	26
		C	13	46	42
Kinjo, et al. 2002	Japanese	G	34.7	43.1	22.2
		C	23.6	41.8	34.6
Sahin, et al. 2005	Turkish	G	19.5	42.9	37.7
		C	7.1	49	43.9

icantly different between Graves' patients and controls; the frequency of GG was higher in Graves' patients than in the controls, as 6% of the controls were homozygous for the G allele, whereas in the patient group 22% were homozygotes. We also calculated the frequencies of A and G alleles in Graves' patients and controls. As shown in *Table 1*, a significant increase in the frequency of the G allele was seen in Graves' patients compared to the controls ( $p = 0.0140$ ). The mean age for the diagnosis of Graves' disease was  $40.45 \pm 15.65$  years. A referred family history of thyroid disease was reported in 39.4% of the patients, and 43.8% of the patients had a smoking history, with a mean of  $18.6 \pm 14.2$  cigarettes smoked per day. Ophthalmopathy affected 46.4% of the patients, whereas only 3% of the patients had dermatopathy. Previous stress was reported in 40% of the patients, and goiter was present in 93.6% of the patients. Remission of hyperthyroidism was seen in 48% of patients at a 12-month follow-up after antithyroid drugs had been discontinued.

A comparison of clinical data [age, gender, goiter, frequency of ophthalmopathy, dermatopathy, smoking habit, initial FT4 values, type of antithyroid drug, and frequency of remission among the three different genotypes (G/G, A/G, A/A)] is shown in *Table 2*. We found a statistical association among the A allele, gender, and oph-

thalmopathy in the patient group.

## 5. Discussion

Over the last decade, several authors have reported an association between the A/G +49 SNP marker and Graves' disease in different populations with distinct ethnic backgrounds (23). Our results indicate a statistically significant association between the G allele of this dimorphism and Graves disease in Spain ( $P = 0.0140$ ). The frequency of the G allele was higher in the group of Graves' patients than in the controls. The frequency of the GG genotype was also significantly higher in GD patients than in the controls. This observed association is in agreement with other studies that show similar values in the Caucasian population, as shown in *Table 3*.

The genotype AA is more prevalent in control Caucasian populations, which is in contrast with the Asian population, which has an increased frequency of the GG genotype and a decreased frequency of the AA genotype. The incidence of the GG genotype is lower in South European populations than in populations from Central Europe. Importantly, significant associations have been demonstrated between the G allele of this SNP and Graves's patients in all the ethnic groups studied. While

analyzing clinical parameters, we observed a significant association with gender and ophthalmopathy. This significant association with gender could be due to the fact that Graves' disease occurs predominantly in females. The prevalence of GD is 10 times lower in men in several countries, relative to women with a GD diagnosis (15).

We found significant associations between the A allele and the development of ophthalmopathy in the Graves' group of patients ( $p = 0.024$ ). This is in contrast to Vaidya *et al.* and Buzzetti *et al.*, who describe an association between ophthalmopathy and the G allele in GD patients (22, 24). Interestingly, we obtained controversial results from our set of patients, as the A allele is significantly related to the presence of ophthalmopathy. It is possible that there is linkage disequilibrium with other alleles of other candidate immune regulatory genes for thyroid autoimmunity in the same region of *CTLA4* (2q33), as has been described by Tomer and Davies (15). The 2q33 region contains several genes that have been shown to be related to the genetic susceptibility to AITD, such as CD28 or the inducible costimulator *ICOS*. It was unclear whether the *CTLA4* gene itself or another immune regulatory gene in the region was involved in the genetic susceptibility to autoimmune diseases described in previous expression analyses. Xu and colleagues found no difference in the expression of *CTLA4* or in the inhibitory function of *CTLA4* when T cells were transfected with A- or G-allele *CTLA4* cDNA, suggesting that the A or G alleles of *CTLA4* did not directly influence its function (24). However, other studies have demonstrated a relationship between the G allele and decreased T cell proliferation upon *CTLA4* inhibition, when compared to the AA genotype (25). Recently, an association between the A allele and a predisposition to paraneoplastic myasthenia gravis in thymoma patients has been described (26). This finding underscores the importance of other costimulation pathways in this disorder.

In our pool of Graves' patients, all of those who presented with dermopathy (3 patients) had an AA genotype. As described by Bahn, a diffuse infiltration of lymphocytes is observed within the orbital adipose tissues of patients with ophthalmopathy (27). A similar, if more sparse, cellular infiltrate is present in the interstitial tissues of the extraocular muscles. The majority of the cells are T lymphocytes; this could be related to the increase in T cell proliferation observed in the AA genotype. We did not find any significant association between genotype and treatment duration or between genotype and remission 1 year after discontinuation of treatment. It is possible that an evaluation of long-term follow-ups may reveal a relationship between these variables and genotype, as the remission rate is slightly inferior (data not shown) in the group of patients with a GG genotype. The influence of the GG genotype in remission after antithyroid drug treatment was described by Kinjo *et al.* in a cohort of 144 Japanese patients, and by Sahin *et al.* in a cohort of 77 Turkish patients (16,17). In both of these studies, the

patients required an extended treatment time.

In conclusion, the +49 A/G SNP of the *CTLA4* gene is related to the development of Graves' disease; however, further studies are necessary to clarify the role of the *CTLA4* gene in influencing GD susceptibility and to explore other potential costimulation pathways in this disorder.

## Acknowledgments

None declared.

## Financial Disclosure

None declared.

## Funding/Support

None declared.

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