

Reduced Number of Thymoma CTLA4-Positive Cells Is Associated With a Higher Probability of Developing Myasthenia Gravis

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Abstract

Background and Objectives

Myasthenia gravis (MG) is an autoimmune disease associated with comorbid thymoma in 10%–15% of cases. Cytotoxic T lymphocyte–associated antigen 4 (CTLA4) expressed by T cells downregulates T-cell–mediated immune response. Polymorphisms in the CTLA4 gene have been associated with the development of MG. In this context, we aimed to determine whether CTLA4 expression in the thymoma differs between patients with and without MG and whether CTLA4 gene polymorphisms are associated with these differences.

Methods

This is a retrospective study of all patients, with and without MG, surgically treated at our institution for thymoma between January 2010 and December 2020. Ten samples were obtained from normal thymuses as controls. The number of CTLA4-positive cells in paraffin-embedded thymoma samples was determined by immunohistochemistry. The presence of follicular-center and regulatory T-cell lymphocytes was determined by immunohistochemistry (B-cell lymphoma [BCL]-6 expression) and double immunofluorescence–based staining of CD4-FOXP3, respectively. We evaluated the association between thymic expression of CTLA4 and the development of MG. We also determined the association between CTLA4 expression and various clinical and prognostic characteristics of MG. We sequenced the CTLA4 gene and evaluated possible associations between CTLA4 polymorphisms and thymic CTLA4 expression. Finally, we assessed the potential association between these polymorphisms and the risk of MG.

Results

Forty-one patients with thymoma were included. Of them, 23 had comorbid MG (56.1%). On average, patients with MG had fewer CTLA4-positive cells in the thymoma than non-MG patients: 69.3 cells/mm² (95% CIs: 39.6–99.1) vs 674.4 (276.0–1,024.0) cells/mm²; $p = 0.001$ and vs controls (200.74 [57.9–343.6] cells/mm²; $p = 0.02$). No between-group differences (MG vs non-MG) were observed in the number of cells positive for BCL6 or CD4-FOXP3. CTLA4 expression was not associated with differences in MG outcome or treatment refractoriness. Two polymorphisms were detected in the CTLA4 gene, rs231770 ($n = 30$ patients) and rs231775 ($n = 17$). MG was present in a similar proportion of patients for all genotypes. However, a nonsignificant trend toward a lower CTLA4-positive cell count was observed among carriers of the rs231775 polymorphism vs noncarriers: 77.9 cells/mm² (95% CI: –51.5 to 207.5) vs 343.3 cells/mm² (95% CI: 126.2–560.4).

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Glossary

AChR = acetylcholine receptors; **BCL** = B-cell lymphoma; **CTLA4** = cytotoxic T lymphocyte–associated antigen 4; **EDTA** = ethylene diamine tetraacetic acid; **GWAS** = genome-wide association study; **IgG** = immunoglobulin G; **MG** = myasthenia gravis; **MGFA-PIS** = MG Foundation of America postintervention status; **PBS** = phosphate buffer solution; **Treg** = T regulatory; **WHO** = World Health Organization.

Discussion

Reduced CTLA4 expression in thymoma may predispose to a higher risk of developing MG.

Myasthenia gravis (MG) is an autoimmune disease characterized by fatigability and weakness. In approximately 85% of cases, the disorder is associated with autoantibodies that bind to acetylcholine receptors (AChRs) in the postsynaptic membrane of the neuromuscular junction.¹

Thymomas are uncommon neoplasms derived from the epithelial cells of the thymus. Comorbid thymoma and MG is common: up to 10%–15% of patients with MG have a thymoma, and 30%–45% of patients with thymoma develop MG. The World Health Organization (WHO) classification system divides thymomas into 6 types according to epithelial cell morphology and the lymphocyte to epithelial cell ratio.² The thymomas most frequently associated with MG are those with the highest lymphocytic component (types AB, B1, and B2).³ Almost all patients with MG with thymoma have anti-AChR antibodies. Thymomas have also been associated with other autoimmune diseases, although the relationship is not as strong as with MG.⁴ The pathophysiologic mechanisms by which autoimmunity is triggered in patients with thymoma-associated MG are believed to differ from those of other MG subtypes; however, these mechanisms are not completely understood.⁵ At present, it is not clear why some patients with thymoma develop MG and others do not.⁶

Cytotoxic T lymphocyte–associated antigen 4 (CTLA4) is a CD28 antagonistic homolog receptor expressed by T cells. When CTLA4 interacts with CD80 or CD86 on antigen-presenting cells, it exerts a downregulatory effect on T-cell-mediated immune response, thus acting as an immune checkpoint. CTLA4 is a key player in the control of self-reactive T-cell generation in the thymus. CTLA4-deficient animals experience a lymphoproliferative disorder characterized by polyclonal T-cell proliferation. Anti-CTLA4 antibodies such as ipilimumab, which is standard of care in several cancer types, have been associated with the development of a broad spectrum of autoimmune diseases, including MG.⁷

CTLA4 gene polymorphisms have been shown to predispose to several autoimmune diseases.⁸ A genome-wide association study (GWAS) conducted in a North American population with AChR antibody–positive MG found that MG was associated with the rs231770 variant of the CTLA4 gene.⁹ Another study found that the CTLA4 49A/A genotype predisposed

patients with thymoma to develop paraneoplastic MG.¹⁰ The findings of those studies suggest a potential association between CTLA4 and the development of MG. Although this association seems to be especially relevant in patients with thymoma, the underlying mechanisms are still unclear.¹¹

The major cell type expressing CTLA-4 are T regulatory (Treg) cells,¹² which are CD4⁺ cells that specifically express the FoxP3 transcription factor and play a key role in the maintenance of immunologic self-tolerance.¹³ The functional activity of Tregs seems to be inhibited in patients with MG, although the underlying molecular basis remains unknown.¹⁴

In this context, we postulate that differential expression of CTLA4 in the thymi of patients with MG would confer an increased risk of MG. We conducted this study to analyze differences in the number of CTLA4-positive thymic cells between patients with and without thymoma-associated MG. We then sought to determine whether CTLA4 polymorphisms were responsible for the differential expression of CTLA4 in T lymphocytes inside the thymoma.

Methods

Patients

The study population consisted of all consecutive patients aged 18 years or older who underwent surgery for thymoma between January 2010 and December 2020 at our center (Santa Creu i Sant Pau Hospital, Barcelona, Spain). We excluded patients who underwent preoperative chemotherapy or radiotherapy and those diagnosed with thymic carcinoma. For controls, 10 normal thymi were obtained from donors without autoimmune diseases (including MG) who had undergone cardiac or parathyroid surgery.

Standard Protocol Approvals, Registrations, and Patient Consents

All patients provided informed consent. The study was approved by the Institutional Ethics Committees at the Hospital de la Santa Creu i Sant Pau.

Clinical Variables

Patients with thymoma were classified into 2 subgroups according to the presence or not of comorbid MG. MG was

Table 1 Clinical Characteristics of Patients With Thymoma With and Without Associated Myasthenia Gravis

	No MG (n = 18), n (%) ^a	MG (n = 23), n (%) ^a	p Value
Sex, male	12 (66.7%)	10 (43.5%)	0.14
Mean age (SD) at thymectomy, y	66.9 (11.4)	53.4 (2.9)	0.002
Immunosuppressant treatment before thymectomy	2 (11.1%)	16 (70.0%)	<0.001
Thymoma histologic subtype (WHO)			0.003
A/AB	14 (77.8%)	7 (30.4%)	
B1/B2/B3	4 (22.2%)	16 (69.6%)	
Thymoma stage (Masaoka)			0.14
Localized (I & II)	17 (94.4%)	18 (78.3%)	
Invasive (III & IV)	1 (5.56%)	5 (21.7%)	
Other autoimmune diseases	6 (33.3%)	5 (21.7%)	0.41

Abbreviations: MG = myasthenia gravis.

^a All data are given as n (%) unless otherwise indicated.

defined as the presence of the usual symptoms of MG and a positive test for anti-AChR antibodies (>0.4 nmol/L by radioimmunoassay). All patients with thymoma were followed up preoperatively and postoperatively for a minimum period of a year by a neurologist specialized in MG. Anti-AChR antibodies and neurologic examination was performed in all patients to ensure they did not develop MG.

The following variables were recorded and assessed: demographic characteristics (sex, age at onset, and date of diagnosis); follow-up time; anti-AChR titer at thymectomy (by radioimmunoassay); distribution of muscle weakness (ocular, bulbar, or limb predominance); thymoma histology (WHO classification)²; thymoma stage (Masaoka staging system)¹⁵; frequency of myasthenic crisis; mortality and cause of death; treatment required; clinical outcome at last visit according to MG Foundation of America postintervention status (MGFA-PIS)¹⁶; and treatment refractoriness, which was defined as either unchanged or worse MGFA-PIS after treatment with corticosteroids and ≥ 2 other immunosuppressive agents at adequate dose levels for a sufficient duration.¹⁷

Treatment outcomes were classified as good or poor. A good outcome was defined as an MGFA-PIS score of minimal manifestations or better, which indicates an asymptomatic patient with a normal or only minimally altered physical examination. Poor outcome was defined as MGFA-PIS worse than minimal manifestations status.

Immunohistochemistry

Formaldehyde-fixed paraffin-embedded tissues were routinely obtained from all surgically removed thymomas. Sections measuring 2–4 μm were collected from the thymoma tissues and processed. Samples were dewaxed, hydrated through a graded ethanol series into water, and washed in double-distilled water. Antigen retrieval was performed with

ethylene diamine tetraacetic acid (EDTA). Goat nonimmune serum was used to block the tissue for 1 hour. The sections were incubated with the following monoclonal antibodies: mouse antihuman CTLA-4 (clone BNI3, isotype immunoglobulin G [IgG]2a, 1:100; Novus Biologicals, Minneapolis, MN), mouse antihuman B-cell lymphoma (BCL)-6 (clone GI191E, provided by Dr. Roncador CNIO, isotype IgG1, 1:350), mouse antihuman CD20 (clone L26, isotype IgG2a, nondiluted; Dako, Glostrup, Denmark), and mouse antihuman CD8 (clone C8/144 B, isotype IgG1, non-diluted; Dako). After 3 washes with phosphate buffer solution (PBS), a peroxidase-labeled goat antimouse secondary antibody was applied (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were developed with DAB (3,3'-Diaminobenzidine) staining (Vector Laboratories, Burlingame, CA). To label the Treg cells, samples were permeabilized with 0.5% Tween 20 (Sigma Aldrich, Saint Louis, MO), and then, double immunofluorescence was performed with rabbit antihuman CD4 (clone SP35, isotype IgG, 1:50; Abcam, Cambridge, UK) and mouse antihuman FOXP3 (clone 236A, isotype IgG1, 1:10; Abcam). After 3 washes with PBS, secondary antibodies were added: goat antirabbit IgG-AlexaFluor 594

Table 2 Autoimmune Diseases Present in Patients Diagnosed With Thymoma With and Without Myasthenia Gravis

No comorbid MG (6/18)	Comorbid MG (5/23)
CIDP (n = 2)	Multiple sclerosis (n = 1)
Erythroblastopenia (n = 1)	Bone marrow aplasia (n = 1)
Goods syndrome (n = 1)	Sjögren disease (n = 1)
Aplastic anemia (n = 1)	Graves' disease (n = 1)
Bullous pemphigoid (n = 1)	Pernicious anemia (n = 1)

Abbreviations: CIDP = chronic idiopathic demyelinating polyneuropathy; MG = myasthenia gravis.

Table 3 CTLA4-Positive Cell Count in Thymomas of Patients With and Without Myasthenia Gravis, Stratified by Sex, Histologic Subtype, and Previous Immunosuppressive Treatment

	No MG, cells/mm ²	MG, cells/mm ²	p Value
Sex			
Female	888.9	83.2	0.009
Male	557.4	51.3	0.02
Thymoma histologic subtype (WHO)			
A/AB	824.9	62.5	0.03
B1/B2/B3	185.5	72.3	0.03
Patients without immunosuppressive treatment before thymectomy	649.33	48.1	0.04

Abbreviations: MG = myasthenia gravis; WHO = World Health Organization.

(Invitrogen, Waltham, MA) and goat antimouse IgG-Alexa Fluor 488 (Invitrogen).

All samples were evaluated by a pathologist specialized in chest pathology. Five random fields of each tissue specimen were analyzed under $\times 10$ magnification using the Microscope Zeiss Axioskop 2 plus. Quantitative analysis of immunohistochemical staining was conducted using the image J imaging analysis software. A customized algorithm for each marker was used to determine the number of positive cells per field. The average value of the 5 fields was taken. Pathologist classifying thymomas and investigators performing immunohistologic analysis were blinded to whether the patient had MG or not.

DNA Extraction and Genotyping

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood. All coding exons and rs231770 were analyzed by Sanger sequencing using the Big-Dye Terminator sequencing kit (v. 3.1, Applied Biosystems), run on an ABI 3730xl DNA analyzer, and analyzed with Sequencer software (v. 4.2; Gene Codes). Primers are listed in eTable 1, links.lww.com/NXI/A795, in supplementary material.

Statistical Analysis

Data are summarized as percentages for categorical variables and mean values with SD for continuous variables. Differences between groups were assessed with the χ^2 test for categorical variables and the 2-sided *t* test for continuous variables (or Wilcoxon test in cases of non-normal distribution). Allele and genotype frequencies were compared between groups using the Fisher exact test. We performed an receiver operating characteristic analysis to determine the best CTLA4+ cell count cutoff that allows to predict MG occurrence with optimal sensitivity and specificity. CIs of odd ratios (ORs) were calculated using the Woolf method. Pairwise deletion was used for treating missing values. All statistical analyses were performed using STATA 15.

Data Availability

The anonymized data that support the findings of this study are available on request from the corresponding author.

Results

Forty-one patients with thymoma met the inclusion criteria and agreed to participate in the study; of them, 23 (56.1%) had MG. Of the 41 patients, 22 (53.7%) were female. The mean (SD) age at thymectomy was 59.3 (14.3) years. In general, patients with MG were younger, and a higher proportion was receiving immunosuppressants, had B-type thymomas (WHO classification), and more advanced thymoma (Masaoka staging system). Table 1 summarizes the clinical characteristics of patients with and without thymoma. Eleven patients (27%) had another autoimmune disease, 6 in the non-MG group and 5 in the MG group (Table 2). No differences in the prevalence of other autoimmune diseases were observed in patients with and without MG.

CTLA4-Positive Cell Count

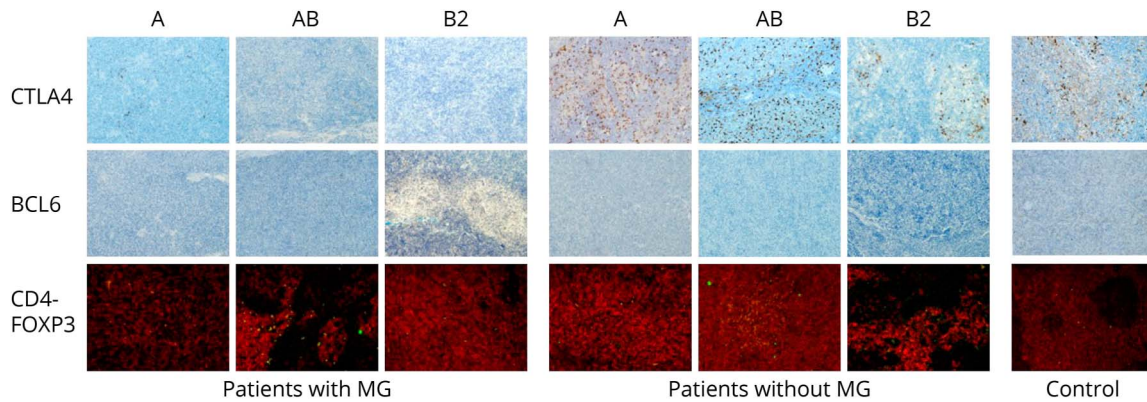
The number of thymic cells expressing CTLA4 was significantly lower in patients with MG vs those without MG, with a mean CTLA4-positive cell count of 69.3 cells/mm² (95% CI: 39.6–99.1) vs 674.4 cells/mm² (95% CI: 276.0–1,024.0), respectively (*p* = 0.001). Patients with thymoma also had fewer thymic CTLA4-positive cells than controls without thymoma (mean 1,702.7 cells/mm² [95% CI: 1,062.6–2,340.7]; *p* = 0.01). CTLA4 was more frequently expressed by medullar than cortical lymphocytes of control thymuses (3,330.0 cells/mm²

Table 4 Differences in the Number of CD4⁺, CD4-FOXP3⁺ and BCL6⁺ Cells in the Thymomas of Patients With and Without Associated Myasthenia Gravis

	No MG Cells/mm ² (95% CI)	MG	p Value
Total CD4⁺ cells	245.1 (221.1–269.1)	252.7 (213.0–292.5)	0.75
CD4-FOXP3⁺ cells	556.3 (185.1–927.6)	556.3 (350.2–762.5)	1
BCL6⁺ cells	107.82 (6.80–208.8)	498.94 (0–1,328.6)	0.38

Abbreviations: MG = myasthenia gravis.

Figure 1 Thymoma Sections of A, AB, and B2 Thymomas of 3 Patients With Myasthenia Gravis (MG), 3 Patients Without MG, and 1 Normal Thymus



Sections were processed for CTLA4 and BCL6 by immunohistochemistry and FOXP3 (green)-CD4 (red) using double immunofluorescence ($\times 10$ magnification). CTLA4 = Cytotoxic T lymphocyte-associated antigen 4.

[95% CI: 2,434.7–4,224.3] vs 761.6 [95% CI: 418.5–1,104.8]; $p = 0.02$). No differences in CTLA4-positive cell counts were observed between patients with thymoma who presented with comorbid autoimmune disease (other than MG) and patients without any comorbid autoimmune disease, which are as follows: 660.1 cells/mm² (95% CI: 182.1–1,138.0) vs 708.9 cells/mm² (95% CI: 90.8–1,279.0) ($p = 0.9$).

Stratified by sex, thymoma histologic subtype, and previous immunosuppressive treatment, the differences in the number of cells expressing CTLA4 between patients with and without MG remained significant (Table 3).

We performed a receiver operating characteristic analysis to assess the validity of the CTLA4-positive cell count to predict MG occurrence in patients with thymoma. We obtained an area under the curve of 0.85 (95% CI: 0.71–0.94) and a value of 170 cells/mm² below which MG occurs with Se of 87% and Sp of 78%.

No association was observed between the number of CTLA4-positive cells and the number of either CD4-FOXP3-positive or BCL6-positive cells ($r_2 = 0.011$ and $r_2 = 0.0015$, respectively). Similarly, no differences in the number of positive CD4, CD4-FOXP3, or BCL6 cells were observed between patients with and without MG (Table 4). Figure 1 shows examples of CTLA4, BCL6, and CD4-FOXP3 expression in the thymomas of patients with and without MG.

No association was found between CTLA4-positive cell count and anti-AChR antibody titers in patients with MG during thymectomy ($r_2 = 0.042$). No association was found between CTLA4-positive cell count and MG outcomes (MGFA-PIS) at 1 year post-thymectomy or at the final follow-up. At 1 year, patients with a good outcome had a mean of 52.8 cells/mm² (95% CI: 12–93.52) vs 78.4 (95% CI: 28.83–127.9) in patients with a poor outcome ($p = 0.38$). At the final follow-up,

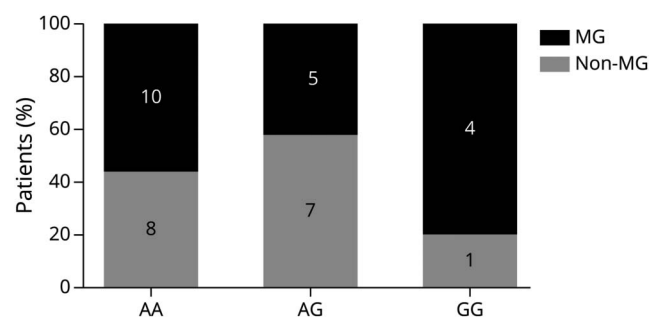
the corresponding values were 69.2 (95% CI: 24.40–114) vs 69.5 (95% CI: 22.81–116.22), $p = 0.99$. No association was detected between CTLA4-positive cell count and treatment refractoriness in patients with MG, which are as follows: no treatment vs treatment refractoriness: 62.54 cells/mm² (95% CI: 29.13–95.96) vs 101.61 (95% CI: 0–205.39), $p = 0.31$.

CTLA4 Gene Sequencing

We genotyped all coding exons and the single nucleotide polymorphism rs231770 in a noncoding region in patients with thymoma with ($n = 19$) and without ($n = 16$) associated MG.

The rs231770 polymorphism was present in 30 of these 35 patients (85.7%), and a second polymorphism, rs231775, was present in 17 of these patients (48.6%). The genotype distribution of CTLA4 polymorphisms in patients with thymoma with and without MG was in accordance with the Hardy-Weinberg equilibrium ($p > 0.05$). In patients with thymoma, the prevalence of paraneoplastic MG did not differ significantly

Figure 2 Prevalence of Paraneoplastic Myasthenia Gravis (MG) in Patients With Thymoma With Different CTLA4 rs231775 Genotypes



CTLA4 = Cytotoxic T lymphocyte-associated antigen 4.

Table 5 Differences in the Prevalence of Myasthenia Gravis Between Patients With Thymoma According to CTLA4 rs231770 Genotypes and Alleles

rs231770	MG (+) thymoma (n = 19)	MG (-) thymoma (n = 16)	OR (95% CI)	p Value
Genotype				
TT	3 (60%)	2 (40%)	1.31 (0.13–17.7)	0.78
TC	6 (43%)	8 (57%)	0.46 (0.09–2.23)	0.27
CC	10 (63%)	6 (37%)	1.85 (0.40–8.93)	0.37
Allele				
T	12 (50%)	12 (50%)	0.77 (0.29–2.07)	0.60
C	26 (57%)	20 (43%)	1.3 (0.48–3.50)	

Abbreviations: MG = myasthenia gravis; OR = odds ratio.

among the 3 rs231770 genotypes. In addition, no differences in the prevalence of MG were found among patients with the 3 rs231775 genotypes. However, a trend toward a higher prevalence of MG was found in patients with the G/G genotype compared with those with the A/A or A/G phenotype (OR: 4; 95% CI: 0.4–40.1). See Figure 2 and Tables 5 and 6.

None of the polymorphisms were associated with differences in the number of CTLA4-positive cells within the thymoma, although a trend was observed in patients carrying the rs231775 variant toward a lower cell count compared with noncarriers: 77.9 cells/mm² (95% CI: –51.5 to 207.5) vs 343.3 cells/mm² (95% CI: 126.2–560.4).

Discussion

In this study, the number of thymoma cells expressing CTLA4 were markedly reduced in patients with MG compared with those without MG (69.3 vs 674.4 cells/mm², $p = 0.001$), and these differences were independent of sex, age, histologic subtype, and previous immunosuppressive treatment. None

of the CTLA4 gene polymorphisms were associated with a higher probability of developing MG nor with a difference in the number of CTLA4-positive cells in the thymoma. However, carriers of the rs231775 variant showed a trend toward lower CTLA4 expression. CTLA4 is constitutively expressed by Treg cells and after T-cell receptor–mediated activation in a subset of CD4⁺ helper T cells. However, in contrast to previous reports,¹⁸ the number of Treg cells did not differ between patients with and without MG, suggesting that the lower number of CTLA4-positive cells in patients with MG is due to the reduced expression of the protein and not to a decrease in the number of Treg cells. We hypothesize that a dysfunction of Treg lymphocytes that would lead to a lower expression of CTLA4 in some patients with thymoma would confer an increased risk of developing MG. Indeed, an inducible deletion of CTLA-4 on Tfr cells results in decreased suppressive function in vitro and in vivo.¹⁹

We found a higher expression of CTLA4 in the medulla of control thymuses compared with that in the cortex. Likewise, we found a higher expression of CTLA4 in type A/AB thymomas (medullary or mixed component) compared with that

Table 6 Differences in the Prevalence of Myasthenia Gravis Between Patients With Thymoma With Different CTLA4 rs231775 Genotypes and Alleles

rs231775	MG (+) thymoma (n = 19)	MG (-) thymoma (n = 16)	OR (95% CI)	p Value
Genotype				
AA	10 (56%)	8 (44%)	1.1 (0.29–4.20)	1
AG	5 (42%)	7 (58%)	0.46 (0.11–1.9)	0.23
GG	4 (80%)	1 (20%)	4 (0.4–40.1)	0.23
Allele				
A	25 (52%)	23 (48%)	0.75 (0.27–2.1)	0.58
G	13 (59%)	9 (41%)	1.33 (0.48–3.69)	

Abbreviations: GG = guanine guanine; MG = myasthenia gravis; OR = odds ratio.

in type B thymomas (cortical component) of patients without MG. However, this finding is not replicated in patients MG in whom the expression of CTLA4-positive cells is equally low in both histologic subtypes. In the stratified study, the differences between CTLA4 cells between patients with and without MG remain significant regardless of the histologic subtype.

CTLA4 knock out results in increased T helper differentiation, increased germinal center B cells, and higher serum immunoglobulin levels in mice.¹⁹ We did not find any differences in the number of follicular center cells (BCL6-positive cells) in the thymomas of patients with and without MG. Similarly, we found no association between the number of CTLA4-positive cells and the number of follicular cells. Although the thymus is considered the site of induction for loss of self-tolerance in patients with MG, altered peripheral immunoregulatory mechanisms such as defective immunosuppressive function of Tregs may help to maintain the AChR-specific autoimmune response. In this regard, it would be valuable to conduct a study to evaluate CTLA4 and BCL6 expression at the periphery to better understand the role of CTLA4 in the pathogenesis of thymoma-associated MG.

A previous study shows an increase in germinal center number in adjacent thymic tissue but not in the thymomas of patients with thymoma-associated MG compared with nonmyasthenic thymoma patients.²⁰ In addition, recently, Song et al.²¹ demonstrated that thymoma-associated patients with MG have a higher percentage of BCL6-positive cells in adjacent thymic tissue around thymoma than non-MG thymoma patients or controls. Because we analyzed exclusively thymoma tissue and not adjacent tissues, this could explain the lack of differences in BCL6 expression between patients with and without MG.

Our data show that the number of thymic CTLA4-positive cells does not seem to influence MG prognosis. However, given the small sample size of our study, we cannot rule out this potential association. In addition, several different factors—such as age, immunosuppressive treatment, and/or histologic subtype—can act as confounders between the number of CTLA4-positive cells and prognosis.

Patients with loss-of-function mutations in the CTLA4 gene develop inflammatory lesions containing activated T cells and macrophages affecting multiple organs. Polymorphisms in the CTLA4 gene are also associated with several autoimmune diseases in humans.²² After a complete sequencing of all CTLA4 coding regions, we found the presence of a previously described polymorphism (rs231775) in exon 1 of the CTLA4 gene in a significant proportion of the patients. This A/G polymorphism at position +49 results in an amino acid change (p.Thr17Ala) in the leader sequence. The rs231775 polymorphism leads to inefficient processing in the endoplasmic reticulum, a differential glycosylation pattern, and decreased trafficking to the cell surface of CTLA4.²³ The guanine guanine (GG) genotype in the CTLA4 rs231775 polymorphism has been associated with an increased risk of several conditions, including Graves' disease,

primary biliary cirrhosis,²⁴ type 1 diabetes,²⁵ and other autoimmune diseases. A previous study found that the GG genotype was unexpectedly protective against paraneoplastic MG in patients with thymoma, in contrast to the significant association between this genotype and all other non-MG autoimmune diseases.¹⁰ We also sequenced the rs231770 polymorphism, which was associated with MG in a GWAS involving 1,032 patients with MG in North America.⁹ Another study found that this polymorphism was associated with a lower expression of CTLA4.²⁶

Although we found no increased risk of developing MG in patients carrying the rs231770 or rs231775 polymorphisms, we did observe a trend toward a higher risk in patients with the +49 GG genotype. However, this genotype was not associated with the number of CTLA4+ cells, although the lack of statistical association could be due to an insufficient sample size. In addition, other variants (apart from those evaluated in this study) in the noncoding region of the CTLA4 gene could also be implicated in the lower number of CTLA4+ cells in the thymomas of patients with MG. Finally, nongenetic factors could also explain these differences, with a lower CTLA4 expression in patients with MG being an epiphenomenon and not the primary cause of the loss of immune self-tolerance.

A better understanding of the mechanisms by which autoimmunity is triggered in patients with MG is essential to develop specific therapies designed to minimize or even prevent the frequent side effects of current immunosuppressants. Confirmation of CTLA4 involvement in MG would represent a potential new therapeutic target.

In conclusion, in this study, the thymomas of patients with MG had fewer CTLA4-positive cells than those without MG. However, no clear association was found between CTLA4 gene polymorphisms and the number of positive cells or the development of MG in patients with thymoma. Larger studies are warranted to clarify this potential association.

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosure.

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Oriol Dols-Icardo, PhD	Memory Unit, Neurology Department and Sant Pau Biomedical Research Institute, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona; Network Center for Biomedical Research in Neurodegenerative Diseases (CIBERNED), Madrid, Spain	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; and analysis or interpretation of data
Shaima El Bounasri	Memory Unit, Neurology Department and Sant Pau Biomedical Research Institute, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona; Network Center for Biomedical Research in Neurodegenerative Diseases (CIBERNED), Madrid, Spain	Major role in the acquisition of data
Laura López-Vilaró	Department of Pathology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; and analysis or interpretation of data
Juan Carlos Trujillo, MD	Department of Thoracic Surgery, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain	Major role in the acquisition of data
David Reyes-Leiva, MD	Neuromuscular Diseases Unit, Department of Neurology, Hospital de la Santa Creu i Sant Pau, Barcelona; Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain	Major role in the acquisition of data; analysis or interpretation of data
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Appendix (continued)

Name	Location	Contribution
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