Clinical Characteristics of Patients With Double-Seronegative Myasthenia Gravis and Antibodies to Cortactin

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IMPORTANCE Double-seronegative myasthenia gravis (dSNMG) includes patients with myasthenia gravis (MG) without detectable antibodies to the nicotinic acetylcholine receptor (AChR) or to muscle-specific tyrosine kinase (MuSK). The lack of a biomarker hinders the diagnosis and clinical management in these patients. Cortactin, a protein acting downstream from agrin/low-density lipoprotein receptor–related protein 4 (LRP4)/MuSK, has been described as an antigen in dSNMG.

OBJECTIVE To describe the frequency and clinical features of patients with dSNMG who have cortactin antibodies.

DESIGN, SETTING, AND PARTICIPANTS A retrospective cross-sectional study was conducted at Hospital de la Santa Creu i Sant Pau, an institutional practice referral center in Barcelona, Spain, between May 1, 2015, and November 30, 2015. We included 250 patients with a definitive diagnosis of MG with available serum samples at the time of diagnosis. Descriptive and comparative data analyses were performed.

EXPOSURES Cortactin antibodies were measured by enzyme-linked immunosorbent assay and Western blot; AChR, MuSK, and anti–striated muscle antibodies were detected using a standard method; and LRP4 antibodies were tested using a cell-based assay.

MAIN OUTCOMES AND MEASURES The primary outcome was the frequency of patients with dSNMG who have cortactin antibodies. Secondary outcomes were demographic, clinical, neurophysiological, and laboratory data.

RESULTS Of 250 patients (mean [SD] age at onset, 49.7 [21.2] years; 56% female), 38 (15.2%) had dSNMG, 201 (80.4%) had MG with AChR antibodies, and 11 (4.4%) had MG with MuSK antibodies. Cortactin antibodies were identified in 28 patients with MG: 9 of 38 (23.7%) who had dSNMG, 19 of 201 (9.5%) who had MG with AChR antibodies (significantly lower than those with dSNMG: 9.5% vs 23.7%; \( P = .02 \)), and 0 of 11 who had MG with MuSK antibodies; 0 of 29 controls had cortactin antibodies. At onset, among the 9 patients with dSNMG and cortactin antibodies, 6 had ocular MG and 3 had Myasthenia Gravis Foundation of America clinical classification IIA. Two patients with ocular MG developed generalized MG. The group with dSNMG and cortactin antibodies, compared with those who had MG with AChR antibodies, more frequently had mild forms at onset (100.0% vs 62.7%; \( P = .03 \)), had fewer bulbar signs at maximal worsening (0% vs 41.3%; \( P = .01 \)), and were younger at onset (median [interquartile range], 34.9 [9.5] vs 53.9 [38.5] years; \( P = .03 \)); the group with dSNMG and cortactin antibodies also more frequently had ocular MG at onset than those with MG and AChR antibodies, although the difference was not statistically significant (66.7% vs 40.8%; \( P = .17 \)). Of 17 patients with ocular dSNMG, 4 (23.5%) had antibodies to cortactin.

CONCLUSIONS AND RELEVANCE In this study, patients with cortactin antibodies and dSNMG had an ocular or mild generalized phenotype of MG. Including the detection of cortactin antibodies in the routine diagnosis of dSNMG may be helpful in ocular MG.

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Myasthenia gravis (MG) is an autoimmune antibody-mediated disease of the neuromuscular junction characterized by fatigable muscle weakness. Autoantibodies against the nicotinic acetylcholine receptor (AChR) are detectable in up to 80% of cases of generalized MG and are present in only 50% of patients with ocular-restricted MG. Antibodies to muscle-specific tyrosine kinase (MuSK) are detected in around 5% of patients with generalized MG. In these patients, the disorder may be clinically different from MG with AChR antibodies, being characterized by early facial, bulbar, neck, and respiratory weakness.

In around 15% of patients with generalized MG and in up to 50% of patients with ocular forms of the disease, no antibodies against AChR and MuSK are detected. These cases are identified as double-seronegative MG (dSNMG). The clinical presentation of this subgroup of MG is similar to MG with AChR antibodies in terms of muscle weakness distribution, disease severity, and response to immunotherapy and plasma exchange, suggesting that it is an immune-mediated disorder of the neuromuscular junction. The lack of a detectable pathogenic antibody or an autoimmune biomarker in dSNMG has hindered the diagnosis and clinical management in these patients, especially in ocular forms of the disease.

Double-seronegative MG encompasses a heterogeneous group of patients with antibodies to different neuromuscular junction proteins. Antibodies against low-density lipoprotein receptor-related protein 4 (LRP4) has been described in subgroups of patients with dSNMG (2%-27%). Low-affinity IgG1 AChR antibodies binding clustered AChRs have been found in 38% of patients with dSNMG in a cellular assay using human embryonic kidney cells. The finding of these autoantibodies, regardless of their pathogenic role in vivo, indicates an ongoing immune process and supports the initiation of immunotherapy. However, a subgroup of patients still has unidentified antibodies to an antigen that is as yet unknown.

Our group recently described a new target antigen, cortactin, in 19.7% of patients with dSNMG. Cortactin was found using a human protein array approach. It was considered a good candidate because it is concentrated at the neuromuscular junction and acts downstream from agrin/LRP4/MuSK, promoting AChR clustering. Simultaneously, cortactin antibodies were discovered using mass spectrometry analysis and were found by enzyme-linked immunosorbent assay in 20% of patients with polymyositis. Overall, these antibodies were reported in around 10% of patients with other autoimmune diseases.

The aim of this study is to describe the results of testing the initial serum sample (the serum sample obtained at the first visit) for cortactin antibodies in our series of patients with a definitive diagnosis of MG. We report the MG phenotype of patients with these autoantibodies and discuss its usefulness in the clinical setting.

Methods

Patients

This was a retrospective cross-sectional study conducted between May 1, 2015, and November 30, 2015. We selected patients from the neuromuscular unit at Hospital de la Santa Creu i Sant Pau, an institutional practice referral center Barcelona, Spain, who fulfilled the criteria for definitive MG and had available serum samples at the time of diagnosis. Serum samples from healthy controls were also included in the cortactin antibody assays.

Myasthenia gravis was diagnosed by a consultant neurologist based on compatible clinical features together with 1 or more of the following criteria: (1) positive results on an AChR or MuSK antibody assay; (2) electrophysiological study; (3) positive results on an AChR or MuSK antibody assay; and (4) a response to cholinesterase inhibitors. In dSNMG, the diagnosis was always confirmed by abnormal findings on a neurophysiological study.

Written informed consent was obtained from all patients. The study was approved by the ethics committee at Hospital de la Santa Creu i Sant Pau.

Clinical Evaluation

The main clinical features of the disease were reviewed: weakness distribution and severity according to the Myasthenia Gravis Foundation of America clinical classification; presence of thymoma by thoracic computed tomography or thymus pathology in patients undergoing thymectomy; and treatment required (no treatment or cholinesterase inhibitors, and immunosuppressive therapy). We also recorded demographic data (sex, date of birth, age at onset) as well as positive results on assays for AChR, MuSK, cortactin, LRP4, and anti–striated muscle antibodies. Testing for low-affinity anti-AChR antibodies was not available.

Autoantibody Assay

Antibodies against AChR and MuSK were tested according to the manufacturer’s instructions by radioimmunounassay in serum samples collected from all patients at diagnosis. Cortactin antibodies were measured using the in-house enzyme-linked immunosorbent assay, and the results were confirmed by Western blot using a purified recombinant protein cortactin (Origene) as previously described. A positive control and a negative control were prediluted from 200 to 6.25 standard units and were used as calibrators. A sample was considered positive when the value was at least 20 standard units. Values from 12.5 to 20 standard units were considered moderately positive and were always analyzed by Western blot to confirm positive results.

Key Points

Question What are the frequency and clinical features of patients who have double-seronegative myasthenia gravis (dSNMG) with cortactin antibodies?

Findings In this cross-sectional study of 250 patients, cortactin antibodies were identified in 9 of 38 patients with dSNMG (23.7%). These patients had an ocular or mild generalized phenotype of myasthenia gravis.

Meaning Including the detection of cortactin antibodies may be helpful in the routine diagnosis of dSNMG.
confirm positivity. Serum samples were tested for antibodies against the nonrelated recombinant protein TIF1γ (Origene) to confirm the specificity of the assay. Antibodies against LRP4 were tested using a transfect ed human embryonic kidney 293 cell-based test as previously described. Anti-striated muscle antibodies were detected using standard immunofluorescence on skeletal muscle (primate) slides (Inova Diagnostics Inc) following the manufacturer’s instructions.

Statistical Analysis
A descriptive data analysis was performed. The frequencies of symptoms are reported as percentages. Demographic characteristics are reported as medians and interquartile ranges. Differences in baseline characteristics between patient subgroups were evaluated using Fisher exact test when comparing categorical variables and Mann-Whitney U test when comparing quantitative variables. Data analysis was carried out using Stata for Windows version 13.0 statistical software (StataCorp LP).

Results
We studied 250 patients (mean [SD] age at onset, 49.7 [21.2] years; 56% female) who fulfilled the inclusion criteria, including 38 (15.2%) with dSNMG, 201 (80.4%) with MG and AChR antibodies, and 11 (4.4%) with MG and MuSK antibodies (Figure). Serum samples from 29 healthy individuals were analyzed as controls.

We identified a total of 28 patients with MG who had cortactin antibodies. Patients with dSNMG had cortactin antibodies with a frequency of 23.7% (9 of 38 patients). No LRP4 and anti-striated muscle antibodies were detectable in this group of patients. No TIF1γ antibodies were detected. Cortactin antibodies were detected in 19 of 201 patients who had MG with AChR antibodies, a significantly lower rate than in patients with dSNMG (9.5% vs 23.7%; P = .02). Of 11 patients who had MG with MuSK antibodies, none had cortactin antibodies (Figure).

No healthy controls had antibodies against cortactin (P = .004). Of 17 patients with ocular dSNMG, 4 (23.5%) had antibodies to cortactin.

Patients with dSNMG and cortactin antibodies (n = 9) had a median age at onset of 34.9 years (interquartile range, 9.5 years), and 77.8% were female. Of these patients, 6 presented with restricted ocular forms and 3 presented with mild generalized forms (Myasthenia Gravis Foundation of America clinical classification IIA) at onset. During the follow-up, 2 of the 6 patients with ocular MG developed a generalized disease. No patients presented with bulbar signs; severe forms and admission at the intensive care unit; or thymoma. Immunosuppressive therapy was required in 55.6% of the patients.

Patients with dSNMG and cortactin antibodies, compared with patients who had MG with AChR antibodies, more frequently had mild forms (I and IIA) at onset (100.0% vs 62.7%; P = .03), less frequently had bulbar signs at maximal worsening (0% vs 41.3%; P = .01), and were significantly younger at onset (median [interquartile range], 34.9 [9.5] vs 53.9 [38.5] years; P = .03). They also more frequently had restricted ocular forms at onset, although this difference was not statistically significant (66.7% vs 40.8%; P = .17). Although not statistically significant, the group of patients with dSNMG and cortactin antibodies, compared with those who had MG with AChR antibodies, had lower rates of immunosuppressive therapy required (55.6% vs 77.1%; P = .22), generalized disease development (33.3% vs 53.7%; P = .42), and presence of thymoma (0% vs 14.4%; P = .62) (Table 1). When adjusting for age and comparing patients younger than 50 years in both groups, patients with dSNMG and cortactin antibodies, compared with those who had MG and AChR antibodies, had a higher frequency of ocular MG (at onset: 75.0% vs 30.4%; P = .02; at maximal worsening: 50.0% vs 13.0%; P = .02) and fewer bulbar symptoms at maximal worsening (0% vs 34.8%; P = .05) (Table 2).

Among patients with dSNMG, the subgroup with cortactin antibodies compared with the subgroup without them were younger (median [interquartile range], 34.9 [29.7–39.2] vs 49.4 [35.2–61.7] years; P = .03). Although not statistically significant, the subgroup with cortactin antibodies had milder forms at onset (9 of 9 patients [100%] vs 22 of 29 patients [75.9%]; P = .16) and less bulbar involvement (0 of 9 patients vs 10 of 29 patients [34.5%]; P = .08). No differences were found in treatment required (5 of 9 patients [55.6%] vs 18 of 29 patients [62.1%]; P > .99).

When comparing the subgroup of patients with MG who had both AChR and cortactin antibodies with patients who had...
MG and AChR antibodies alone, we did not find differences in sex (female: 11 of 19 patients [57.9%] vs 94 of 182 patients [51.6%]; \( P = .64 \)), age at onset (median [interquartile range], 50.6 [22.6-73.2] vs 54.1 [31.2-69.3] years; \( P = .75 \)), restricted ocular forms (6 of 19 patients [31.6%] vs 76 of 182 patients [41.8%]; \( P = .47 \)), mild forms (I and IIA) (10 of 19 patients [52.6%] vs 116 of 182 patients [63.7%]; \( P = .46 \)), bulbar symptoms at maximal worsening (7 of 19 patients [36.8%] vs 76 of 182 patients [41.8%]; \( P = .81 \)), presence of thymoma (2 of 19 patients [10.5%] vs 27 of 182 patients [14.8%]; \( P = .99 \)), or treatment required (14 of 19 patients [73.7%] vs 141 of 182 patients [77.5%]; \( P = .78 \)).

### Discussion

Cortactin antibodies were detected in a subgroup of patients with dSNMG. These patients were younger than those who had MG and AChR antibodies.
MG with AChR antibodies and those who had dSNMG without cortactin antibodies, and they had a predominance of ocular or mild generalized clinical forms and no bulbar signs. The MuSK, LRP4, and anti-striated muscle antibodies were not associated with cortactin antibodies. Some patients with MG and AChR antibodies had cortactin antibodies, but at a significantly lower rate than those with dSNMG (P = .02). No healthy controls had cortactin antibodies.

Double-seronegative MG has classically been considered similar to MG with AChR antibodies in terms of clinical features and response to treatment. Classifying patients with MG into subgroups according to serological criteria has implications in diagnosis, prognosis, and therapy. The finding of MuSK antibodies among AChR-seronegative patients with MG defined a subgroup with predominant involvement of cranial and bulbar muscles, with an excellent and maintained response to prednisone and other immune therapies, including rituximab. Among patients with dSNMG, those with LRP4 antibodies have a female preponderance and predominating ocular or mild generalized MG. These antibodies have a low incidence, and they could coexist with MuSK antibodies. The clinical characteristics of patients with low-affinity antibodies to clustered AChR have recently been described. Patients have early onset and milder disease with predominantly isolated ocular symptoms and a low generalization rate, but 25% present with mild bulbar symptoms. A commercial test to measure these antibodies is not yet available, so these subgroups can be identified in only a few centers. Our results confirm that patients with dSNMG and cortactin antibodies present with predominantly ocular or mild clinical forms of the disease, and interestingly, we did not find bulbar signs initially or during follow-up. These findings suggest that cortactin antibodies may imply good prognosis in patients with dSNMG.

It is difficult to diagnose the ocular forms of MG at an immunological level because, in contrast with the generalized forms, up to 50% of patients with ocular MG are seronegative for AChR antibodies. In our series, as up to 24% of patients with ocular dSNMG had antibodies to cortactin, the detection of these antibodies constituted a useful biomarker especially in this group of patients. A biomarker of disease in ocular MG is especially relevant because the differential diagnosis of ptosis and diplopia includes several diseases, the pharmacological test may be confusing, and results on electrophysiological studies may be borderline or normal in some patients.

Cortactin antibodies were found in 9.5% of patients with MG and AChR antibodies, but the clinical features of this subgroup of patients were similar to those found in patients with MG and only AChR antibodies. This suggests that AChR antibodies dominate when found in coexistence with cortactin antibodies. In this context, the good prognostic value of cortactin antibodies would not be applicable. We did not test the serum samples of these patients for low-affinity IgG1 AChR antibodies, but we would expect to find them at similar rates to those found for high-affinity AChR antibodies. We found that MuSK, LRP4, and anti-striated muscle antibodies were not associated with cortactin antibodies in our series.

Cortactin is a postsynaptic neuromuscular junction intracellular protein that acts downstream from agrin/LRP4/MuSK, promoting clustering of AChR. Because of this important role in neuromuscular transmission, cortactin has been considered as a potential antigen in the pathogenesis of MG. While the pathogenicity of antibodies to AChR was long ago proven and the pathogenic role has also been shown for MuSK antibodies, it remains to be determined how antibodies to cortactin are generated and whether they are pathogenic. The fact that most patients had no other autoantibodies suggests that the cortactin antibodies did not arise as a consequence of epitope spreading or neuromuscular junction damage. However, the presence of cortactin antibodies has been described in other neuromuscular autoimmune disorders and also in around 5% of healthy controls. This suggests that they may not be involved in the pathogenesis of MG, but they should be considered a good biomarker in dSNMG.

In this study, we confirm that cortactin antibodies are significantly prevalent in dSNMG, a clinical entity in which the absence of a diagnostic marker may hinder the clinical management of patients. For this reason, although its pathogenic role has not yet been proven, the finding of cortactin antibodies in patients with dSNMG is a useful clinical tool and supports the use of immunosuppressive therapies.

The main limitation of the study is that although we tested a wide sample of serum samples from patients with MG, the group of patients with dSNMG and cortactin antibodies is small. In a larger sample of patients with dSNMG, we might be able to reach statistical significance in terms of sex, treatment required, generalized disease development, or presence of thymoma, as we found clear differences in these variables between both groups.

**Conclusions**

We consider that cortactin antibodies are a biomarker in a significant proportion of patients with dSNMG, especially those with ocular dSNMG. In addition, the presence of cortactin antibodies supports the diagnosis of autoimmune MG and, if clinically required, treatment with immunosuppressive therapies.
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Seronegative Myasthenia Gravis—A Vanishing Disorder?

Henry J. Kaminski, MD

With the initial identification in 1976 of antibodies directed toward the acetylcholine receptor (AChR) in the serum of patients with myasthenia gravis (MG),¹ it became clear that upwards of 20% of patients with clinical and electrophysiological evidence of a neuromuscular disorder lacked such antibodies. Was this because of the presence of low-affinity antibodies that were capable of disease induction but not detectable by the standard radioimmunoassay? This answer appears to be partially the case. Using cell-based assays that present the complex pentameric, membrane-bound AChR in a much more native state, AChR antibodies can be found among seronegative patients.² However, Lindstrom et al³ suggested another possibility that antibodies may be directed toward other target antigens and produce a similar clinical and electrophysiological disorder.

In 2000, this hypothesis was confirmed with the detection of muscle-specific kinase (MuSK) antibodies among a subgroup of patients seronegative for AChR autoantibodies; however, some individuals remained double seronegative.³ The discovery of MuSK antibodies has been beneficial for diagnosis not only in patients with a myasthenic phenotype but also in some patients who had previously defined diagnosis of a chronic disorder marked by muscle atrophy and primarily bulbar and ocular involvement. Strong evidence supports that the MuSK antibodies act by interference with AChR clustering through the activity of IgG4 autoantibodies, rather than the predominant complement-mediated damage induced by AChR antibodies.⁴ Further clinical definition of the MuSK antibody phenotype indicates that such patients have a differential response to treatment, with a poor to intolerant response to cholinesterase inhibition and better response to plasma exchange than patients with AChR antibodies.¹ In addition, basic biology has benefited from identification of the MuSK antibody, allowing more detailed understanding of mechanisms that regulate neuromuscular junction stability at the mature synapse.⁶ More recently, antibodies against low-density lipoprotein 4 (LRP4) were identified by several groups (reviewed by Gilhus et al⁷). Cell-based assays and animal models strongly suggest a pathogenic role.⁴ The clinical characteristics of patients with LRP4 antibodies are beginning to be defined.⁷

In this issue of JAMA Neurology, Cortés-Vicente et al⁸ describe antibodies against cortactin in serum samples of patients without antibodies to AChR or MuSK. Why would the investigators look for cortactin antibodies? Cortactin has a critical role in the development of the neuromuscular junction. Nerve-derived agrin binds to LRP4, which activates MuSK; phosphorylation of cortactin then occurs, which is a required mediator of AChR clustering.⁹ The group investigated serum samples from 38 patients with clinically and electrophysiologically defined MG who were negative for AChR and MuSK antibodies. Nine of these patients had antibodies directed toward cortactin, and these patients had ocular or mild generalized MG. Among the 17 patients with ocular MG in the double-seronegative MG group, 4 (23.5%) had antibodies to cortactin. A few other individuals had antibodies to both AChR and cortactin. Several caveats need to be appreciated. The study population was small and the control group consisted of healthy participants, not potential mimics of generalized or ocular MG. Cortactin antibodies are found among patients with other autoimmune disorders and healthy individuals.¹⁰,¹¹ Therefore, much remains to be done to assess the positive and negative predictive value of the cortactin antibody assessment prior to use in clinical practice.

Whether a putative autoantigen is pathogenic requires rigorous confirmation by strict criteria: (1) antibodies are identified at the site of pathology; (2) immunoglobulin from patients or the target antigen induces pathology consistent with the disease; (3) immunization with antigen reproduces the disease; and (4) removal of antibodies improves disease manifestations.¹² Striational antibodies are found among patients. They bind several muscle antigens, but their pathogenic role is not established; however, they are found in elderly individuals and those with thymoma more commonly, providing a somewhat useful biomarker.⁴ Among patients with MG, antibodies to several self-antigens, including agrin, potassium channels, and the ryanodine receptor, have been detected.⁶ One can now add cortactin to this list. The pathogenic nature of these antibodies has not been assessed, and their value may be limited to enhanced diagnosis and perhaps more focused therapeutic approaches.

A major limitation for the field of MG is the lack of biomarkers that associate with treatment response or are of prognostic value. The serological examination for autoantibodies and single-fiber electromyography are good diagnostic markers, but they do not correlate with disease activity and are not adequate to assess treatment responsiveness among individual patients.¹³,¹⁴ The situation complicates design of clinical trials and routine clinical care. Clinical trials in rare disorders face challenges owing to limited understanding of natural history, difficulties in selection of appropriate outcome measures, study design issues, and small sample sizes that affect power. Markers that are predictive of treatment outcome will improve clinical decision making, including counseling of patients, and sensitive and reliable biomarkers for early treatment scanning will facilitate drug trials and speed regulatory


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For MG, one can add disease heterogeneity as evidenced by the growing number of autoantibodies associated with a myasthenic phenotype, variation in thymic pathology, and age-related differences. As such, the identification of reliable biomarkers becomes a high priority to enhance patient care.

ARTICLE INFORMATION

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