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Review

# Cortactin autoantibodies in myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) is an autoimmune disease characterized by muscle weakness, fatigability, and autoantibodies against protein antigens of the muscle endplate. Antibodies against acetylcholine receptor (AChR), and less frequently against muscle-Specific Kinase (MuSK) or lipoprotein related protein 4 (LRP4) occur in patients with seropositive MG (SPMG). However, about 10% of patients do not have detectable autoantibodies despite evidence suggesting that the disorder is immune mediated; this disorder is known as seronegative MG (SNMG). Using a protein array approach we identified cortactin (a protein that acts downstream from agrin/MuSK promoting AChR clustering) as potential new target antigen in SNMG. We set up an ELISA assay and screened sera from patients with SPMG, SNMG, other autoimmune diseases and controls. Results were validated by immunoblot. We found that 19.7% of patients with SNMG had antibodies against cortactin whereas only 4.8% of patients with SPMG were positive. Cortactin antibodies were also found in 12.5% of patients with other autoimmune disorders but only in 5.2% of healthy controls. We conclude that the finding of cortactin antibodies in patients with SNMG, suggests an underlying autoimmune mechanism, supporting the use of immune therapy.



**Contents**



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## 1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction (NMJ), where approximately 80% of patients have antibodies against the nicotinic acetylcholine receptor (AChR) [1,2]. These antibodies block acetylcholine binding to the receptor, induce complement-mediated damage to the muscle fiber, and reduce the number of functional AChRs by increasing their degradation and turnover [3–5]. In up to 50% of MG patients without antibodies to AChR, antibodies against the muscle specific tyrosine kinase (MuSK) are detected [6,7]. These patients often experience bulbar, facial and respiratory muscle weakness. Patients' IgG4 autoantibodies have been shown to be the crucial pathogenic factor of MuSK + MG, causing combined pre- and postsynaptic functional NMJ defects with the absence of an adequate synaptic homeostatic response, contributing to muscle weakness [8]. MuSK antibody titers correlate with disease severity and response to immunotherapy [9,10]. The finding of these different antibodies indicates that MG is, immunologically, a heterogeneous disease.

In approximately 10–15% of patients with generalized MG no antibodies against AChR o MuSK can be detected by routine methods, this group is known as seronegative myasthenia (SNMG) [11]. There is evidence that in SNMG the muscle weakness is also caused by pathogenic autoantibodies: patients benefit from plasma exchange, myasthenic weakness has been passed from an affected mother to baby transplacentally, and passive immunization of mice with patient serum induced defects in neuromuscular transmission [12–14]. Furthermore, patients with SNMG respond to immunotherapies [15]. The target antigen in SNMG is unclear. Clinically and demographically SNMG is very similar to AChR + MG, but diagnosis and clinical management remain complicated giving rise to a need for a biomarker.

Leite et al. reported that up to 66% of patients with SNMG have low-affinity IgG1 antibodies against the AChR, that are only detectable in a cellular assay using HEK cells cotransfected with human AChR subunits and rapsyn to induce AChR clustering on the cell surface [16]. Later on, the same authors reported that up to 50% of patients with SNMG, generalized or ocular, have antibodies that recognize clustered AChR and their levels correlate with the electrophysiologic features. The pathogenic mechanism in that case seems similar to the typical AChR + MG [17]. Recently, antibodies against low-density lipoprotein receptor-related protein 4 (LRP4) have been identified in variable proportions of SNMG patients (2% to 50%) [18–20], and it is proposed that they can inhibit the interaction of agrin-LRP4-MuSK and disrupt AChR aggregation. The role of these antibodies in causing myasthenic symptoms in vivo has not been completely elucidated [21].

In our study, serum from a SNMG patient was probed with a human protein array, pointing cortactin as a new candidate autoantigen. Cortactin is concentrated at the NMJ and acts downstream from agrin/MuSK promoting AChR clustering. Given its role regulating actin polymerization and aggregation of AChR in response to synaptogenic stimulation [22–24], we investigated the presence of cortactin autoantibodies in a large series of patients with MG. We detected cortactin autoantibodies in 19.7% of patients with SNMG indicating that these antibodies could be used to reinforce an immune process in seronegative myasthenia.

## 2. Methods

### 2.1. Patients

The study included sera of 91 patients with SNMG and 103 patients with SPMG (69 AChR + and 34 MuSK+), followed in Hospital Santa Creu i Sant Pau or Leiden University Medical Centre. Controls included 30 patients with LEMS (19 VGCC antibodies and 11 seronegative), 9 patients with other immune-mediated neurological diseases (6 chronic inflammatory demyelinating polyneuropathy (CIDP), 3 multiple sclerosis (MS)), 31 other autoimmune diseases (12 lupus, 8 rheumatoid arthritis (RA), 3 mixed connective tissue disease, 3 polymyositis, 2 Raynaud's syndrome, 1 acrocyanosis and 2 sclerodermia), and 19 healthy controls.

Clinical information and informed consent were obtained by the referring physicians and the study was approved by the medical ethical committee of Hospital de la Santa Creu i Sant Pau and Leiden University Medical Centre.

### 2.2. Human protein array

A protein array (ProtoArray® v5.0 Invitrogen, Carlsbad, California, USA) was used as a method to detect potential autoantigen biomarkers. This microarray contains more than 9000 human proteins, similar to the mammalian cells with respect to protein folding and post-translational modifications. This allows protein interaction detection at a functional level. Each human open reading frame (ORF) is expressed as an N-terminal GST fusion protein using a baculovirus expression system, purified from insect cells, and printed in duplicate on a nitrocellulose-coated glass slide. Built-in controls are printed on each array to control for background and detection. After the analysis of the results, the software generates a list of human proteins showing significant interactions with the sample.

Six SNMG serum samples, were mixed in 2 different tubes and diluted (1:500) in washing buffer. The microarray was blocked with blocking buffer following manufacturer's instructions. Serum samples were probed with the array and detected using Alexa 647 goat anti-human IgG (Molecular Probes) on a fluorescence microarray scanner. Acquired array images were analyzed using the recommended software. Analysis of normalized data pointed Cortactin (CTTN) transcript variant 2 as one candidate autoantigen.

### 2.3. ELISA

Briefly, 96-well ELISA plates (NUNC, Kamstrup,Denmark) were coated with 100 ng/well of purified recombinant cortactin (OriGene, Rockville, Maryland, USA) diluted in phosphate-buffered saline (PBS) and left to stand overnight at 4 °C. Wells were incubated for 1 h at room temperature (RT) with blocking buffer (10% non-fat dry milk in PBS). Plates were then washed (HRP Wash, INOVA Diagnostic Inc., San Diego, California, USA), human serum samples diluted 1:100 in blocking buffer were added in duplicate and plates were incubated at RT for 1 h. After washing, horseradish peroxidase-labeled goat anti-human IgG antibody (INOVA Diagnostic Inc.) was added to each well, and plates were incubated for 1 h at RT and washed again. Color development used TMB chromogen reagent for peroxidase (INOVA Diagnostic Inc., SA), and absorbance at 450 nm was determined and expressed as optical density units. The same high and low positive serum samples were used as references in each assay.

### 2.4. Immunoblot

Purified recombinant protein cortactin (5 μg) (OriGene, Rockville, Maryland, USA) was run on 4–12% polyacrylamide-sodium dodecyl sulfate minigels with 3-(N-morpholino) propanesulfonic acid running buffer, and Western blotting was performed using the Invitrogen NuPAGE (Carlsbad, California, USA) electrophoresis system 14 over a nitrocellulose membrane. Cortactin-transferred nitrocellulose was vertically cut into strips and incubated for 1 h at RT in PBS with 0.05% Tween containing 3% non-fat dry milk (blocking buffer). Each strip was then incubated with a human serum sample diluted 1:100 in blocking buffer for 1 h at RT. After washing, phosphatase alkaline-labeled goat anti-human IgG antibody (1:2000; Dako, Glostrup, Denmark) was added to each strip and strips were incubated for 1 h at RT. Color development used phosphatase reagent (BCIP/NBT, Sigma-Aldrich, St Louis, Missouri, USA).