

24. Dalkara T, Nozari A, Moskowitz MA. Migraine aura pathophysiology: the role of blood vessels and microembolisation. *Lancet Neurol* 2010;9:309–317.
25. Ferrari M. In: Silberstein SD, Lipton RB, Dodick DW, eds. *Wolff's headache and other head pain*. 8th ed. Oxford, UK: Oxford University Press, 2008:133–149.

Autoantibodies to Low-Density Lipoprotein Receptor–Related Protein 4 in Myasthenia Gravis

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Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction, where acetylcholine receptor (AChR), muscle-specific kinase (MuSK), and low-density lipoprotein (LDL) receptor-related protein 4 (Lrp4) are essential. About 80% and 0% to 10% of patients with generalized MG have autoantibodies to AChR and MuSK, respectively, but pathogenic factors are elusive in others. Here we show that a proportion of AChR antibody-negative patients have autoantibodies to Lrp4. These antibodies inhibit binding of Lrp4 to its ligand and predominantly belong to the immunoglobulin G1 (IgG1) subclass, a complement activator. These findings together indicate the involvement of Lrp4 antibodies in the pathogenesis of AChR antibody-negative MG.

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The neuromuscular junction (NMJ) is a synapse between the motor nerve terminal and the skeletal muscle endplate.¹ Postsynaptic clustering of the neurotransmitter receptor acetylcholine receptor (AChR) is controlled by muscle-specific kinase (MuSK) and low-density lipoprotein (LDL) receptor-related protein 4 (Lrp4), which form an essential postsynaptic receptor complex for its ligand, neural agrin.^{2–4} Myasthenia gravis (MG) is an autoimmune disease of the NMJ.⁵ About 80% of patients with generalized MG have AChR antibodies, which is a causative factor for the disease, and a variable proportion of the remaining patients (0–50% throughout the world) have MuSK antibodies.^{6–12} However, diagnosis and clinical management remain complicated for patients who are negative for MuSK and AChR antibodies, giving rise to a need for unveiling the hidden causative factors in MG. Given the essential role and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. In this study, we developed a simple technique termed luciferase-reporter immunoprecipitation (LUCIP), which takes advantage of the

strong luminescence of *Gaussia* luciferase (GL) to detect protein-protein interactions with high sensitivity. Using this method, we evaluated the titers and pathogenic properties of serum antibodies to the extracellular portion of Lrp4 in patients with AChR antibody-negative MG.

Materials and Methods

We studied serum samples from 300 patients with AChR antibody-negative MG diagnosed by typical clinical features, the edrophonium test, and/or the repetitive nerve stimulation test. Sera from 100 healthy volunteers, 100 patients with AChR antibody-positive MG, and 101 patients with Lambert-Eaton myasthenic syndrome (LEMS) were also studied. All MG sera were tested for AChR and MuSK antibodies using the standard radioimmunoprecipitation assay (RIA).^{7,13}

For the Lrp4-LUCIP assay, HEK293 cells were transfected with expression plasmid for Lrp4-GL, in which the entire extracellular portion of Lrp4 was fused to GL,¹⁴ and the fusion protein was purified from the culture supernatant (Supporting Information Methods). The specific luciferase activities of Lrp4-GL were 1.25×10^8 relative light units [RLU]/pmol using the BioLux *Gaussia* luciferase assay kit (BioLabs) and a Lumat LB 9507 luminometer (Berthold Technologies).

To titrate Lrp4 antibodies, 5 μ l serum was added to 24 fmol Lrp4-GL in 700 μ l phosphate-buffered saline with 0.05% Tween 20 and 3% bovine serum albumin for overnight incubation at 4°C. Immunoglobulin G (IgG)-bound Lrp4-GL was precipitated with 15 μ l protein G-Sepharose (GE Healthcare). The precipitates were washed and their luciferase activities were determined to calculate the amount of Lrp4-GL protein, whose value was used to represent the titer of Lrp4 antibodies.

For subclass-specific titration of IgG antibodies to Lrp4, 1 μ l serum was incubated with Lrp4-GL as described above in the presence of 10, 5, 2, and 2 μ g biotinylated anti-human IgG1, IgG2, IgG3, and IgG4 antibodies (Binding Site), respectively.¹⁰ Immune complexes comprised of these subclass-specific antibodies, serum IgGs to Lrp4, and Lrp4-GL were precipitated with 15 μ l NeutrAvidin-Agarose (Thermo Scientific). The precipitates were washed and their luciferase activities were determined to calculate subclass-specific antibody titers.

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To evaluate agrin-Lrp4 interaction, HEK293 cells were transfected with expression plasmid for 3×FLAG-agrin in Opti-modified Eagle medium (Opti-MEM) and the culture supernatant containing secreted 3×FLAG-agrin was harvested. The culture supernatant containing 11fmol 3×FLAG-agrin was incubated with 6fmol Lrp4-GL, and then 3×FLAG-agrin was immunoprecipitated with anti-FLAG antibody-conjugated agarose (Sigma). The precipitates were washed and luciferase activities were determined to calculate the amount of Lrp4-GL bound to 3×FLAG-agrin (see Supporting Information Methods for details). To further investigate the effects of serum or serum IgGs on agrin-Lrp4 interaction, 11fmol 3×FLAG-agrin and different volumes of serum or corresponding amounts of IgGs were subjected to this assay. Serum IgGs were purified using the IgG Purification Kit-G (Dojindo), or IgGs were depleted from serum using an excess amount of protein G-Sepharose.

To validate the significance of the observed differences, we analyzed simple pairwise comparisons with the Student *t* test (2-tailed distribution with 2-sample equal variance).

Results

We tested for Lrp4 autoantibodies in sera from patients with AChR antibody-negative MG by the Lrp4-LUCIP assay, which uses a fusion protein of the entire extracellular portion of Lrp4 and GL (Lrp4-GL) as a reporter (Supporting Information Data; Supporting Information Figs 1 and 2). From a cohort of 300 patients, 9 patients were positive for antibodies to the extracellular portion of Lrp4, where the cutoff value (0.015nM) was determined based on the mean + 4 standard deviations (SDs) obtained with 100 healthy control sera (Fig 1A). The control GL-LUCIP assay confirmed that these patients were negative for serum antibodies to GL (Supporting Information Table 1). Titers of Lrp4 antibodies in the 9 patient sera ranged from 0.019nM to 2.07nM (median, 0.65nM; Table). These titers are statistically significant because the minimum titer value (0.019nM) in the Lrp4 antibody-positive sera occurs with a probability of less than 6.312×10^{-10} under the null hypothesis that the AChR antibody-negative MG data follow the same distribution as healthy controls.

Next, we tested sera from 100 MG patients positive for AChR antibodies and all were negative for the Lrp4 antibodies, suggesting that the autoantibodies are mutually exclusive in MG (see Fig 1A). Furthermore, Lrp4-LUCIP testing of sera from 101 patients with LEMS, a different form of NMJ autoimmune disease,¹⁶ revealed that the patients were negative for Lrp4 antibodies aside from one who showed weak positivity (see Fig 1A). In addition, among 28 patients with MuSK antibody-positive MG in the cohort of 300 AChR antibody-negative patients, 3 patients were also positive for Lrp4 antibodies (index case nos. 6, 8, and 9; see Table).

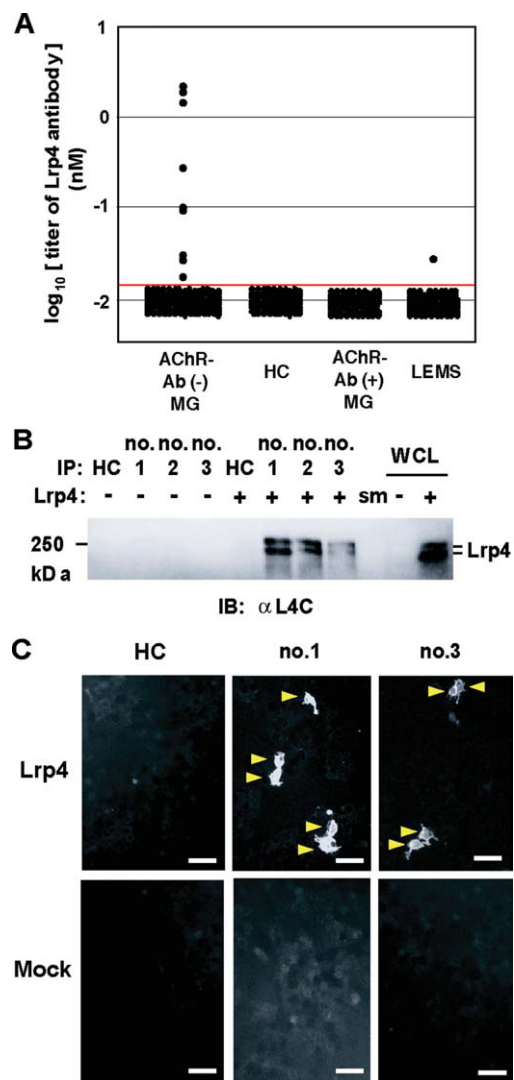


FIGURE 1: Serum autoantibodies to the extracellular portion of Lrp4 were found in patients with AChR-Ab– MG and recognized native Lrp4. (A) Scatter plot for the calculated titers (nM) of Lrp4 autoantibodies in sera from patients and the HC as indicated. The red line indicates the cutoff value, calculated as the mean + 4 SDs of the healthy control values. (B) IP of full-length Lrp4 protein with sera from patients (index cases nos. 1–3). Immunoprecipitates or WCL of HEK293 cells that had been transfected with expression plasmid for Lrp4 (+) or the empty vector (–) were subjected to IB with rat antiserum to the cytoplasmic portion of Lrp4 (α L4C) (Supporting Information Methods). The blank lane contained size markers (sm). (C) Immunostaining of cell surface Lrp4 with sera from patients (index case nos. 1 and 3). Intact (nonpermeabilized) HEK293 cells that had been transfected with expression plasmid for Lrp4 or the empty vector (mock) were stained with sera from patients or the HC (see Supporting Information Methods). Anti-human IgG-Alexa488 was used as a secondary antibody to visualize cell surface Lrp4 (arrowheads). Scale bars 50 μ m. AChR = acetylcholine receptor; AChR-Ab+ = AChR antibody-positive; AChR-Ab– = AChR antibody-negative; HC = healthy control; IB = immunoblotting; IgG = immunoglobulin G; IP = immunoprecipitation; Lrp4 = low-density lipoprotein receptor-related protein 4; LEMS = Lambert-Eaton myasthenic syndrome; MG = myasthenia gravis; SD = standard deviation; WCL = whole cell lysates. [Color figure can be viewed in the online issue, which is available at annalsofneurology.org.]

TABLE: Clinical Features of Lrp4 Antibody-Positive Patients with Generalized MG

Index Case No.	Lrp4 Antibody (nM) ^a	MuSK Antibody (nM) ^b	Sex	Age Examined	Thymoma	MGFA Classification ^c
1	2.07	0.02	Female	44	no	3b
2	1.79	0.001	Male	26	no	3a
3	1.42	0.003	Female	72	no	3a
4	0.27	0.006	Female	70	no	5
5	0.097	0.004	Female	68	no	2a
6	0.091	11.38	Male	74	no	5
7	0.031	0.005	Female	28	no	3b
8	0.027	6.25	Male	58	no	2b
9	0.019	10.59	Male	72	no	5

^aData from the Lrp4-LUCIP assay.
^bData from the MuSK-RIA assay.
^cDisease severity was graded according to the MGFA classification as described.¹⁵
Lrp4 = low-density lipoprotein receptor-related protein 4; LUCIP = luciferase-reporter immunoprecipitation; MG = myasthenia gravis; MGFA = Myasthenia Gravis Foundation of America; MuSK = muscle-specific kinase; RIA = radioimmunoprecipitation assay.

We next examined whether the serum antibodies to Lrp4 present in these MG patients recognize the native form of Lrp4. HEK293 cells transfected with full-length Lrp4 expression plasmid and C2C12 myotubes expressing endogenous Lrp4 were subjected to immunoprecipitation with Lrp4 antibody-positive sera (index case nos. 1–3) or healthy control sera (see Fig 1B and Supporting Information Fig 3A). In Supporting Information Figure 3A, index case no. 2 was excluded due to paucity. Sera from these patients but not serum from the healthy control precipitated Lrp4 proteins. Likewise, cell surface Lrp4 ectopically expressed in HEK293 cells could be visualized by immunostaining of intact cells with sera from these patients, but not the healthy control or antiserum to the cytoplasmic portion of Lrp4 (see Fig 1C and Supporting Information Figs 4 and 5), demonstrating that the serum antibodies to Lrp4 can recognize its native form. However, sera from the remaining patients (index case nos. 4–9) failed to visualize cell surface Lrp4 (data not shown), and these sera, aside from index case no. 4, also failed to immunoprecipitate Lrp4 (see Supporting Information Fig 3B). Unlike the healthy control, however, sera from these patients reacted with the LA domain of the extracellular portion of Lrp4 in immunoblots (see Supporting Information Fig 3C).

The clinical features of 9 patients with Lrp4 antibody-positive MG (index case nos. 1–9) are summarized in Table. Generalized MG was diagnosed in these patients, who showed severe limb muscle weakness or progressive bulbar palsy or both. Thymoma was not observed in any of these patients, unlike the situation in patients with AChR antibody-positive MG.¹⁷

Because Lrp4 is the agrin-binding subunit of the Lrp4:MuSK receptor complex,^{3,4} and serum antibodies to Lrp4 in MG patients bound to the molecule's extracellular portion, we speculated that those antibodies might compete with agrin for binding with Lrp4. Indeed, sera from Lrp4 antibody-positive patients, but not the healthy control, inhibited interaction of Lrp4-GL with neural agrin (Fig 2A). We confirmed that serum and IgGs prepared from the same patient (index case no. 3) showed comparable inhibition (see Fig 2B). Conversely, when IgGs were depleted from the patient's serum, it lost its inhibitory activity (Supporting Information Fig 6). Thus, autoantibodies to Lrp4 could exert pathogenicity through their potential to inhibit agrin and Lrp4:MuSK signaling required for NMJs. Furthermore, to assess potential involvement of the complement system in Lrp4 antibody-positive MG, we investigated the IgG subclass composition of Lrp4 antibodies in patients (see Fig 2C and Supporting Information Fig 7). The Lrp4-LUCIP assay in combination with subclass-specific immunoprecipitation of IgGs revealed that Lrp4 autoantibodies were predominantly comprised of IgG1, a complement activator, in each patient, suggesting the potential for these antibodies to cause complement-mediated impairment of NMJs.

Discussion

We identified the novel antigen Lrp4 as a target for autoantibodies in AChR antibody-negative MG. These antibodies are mainly IgG1 and have the potential to inhibit interaction between neural agrin and the extracellular

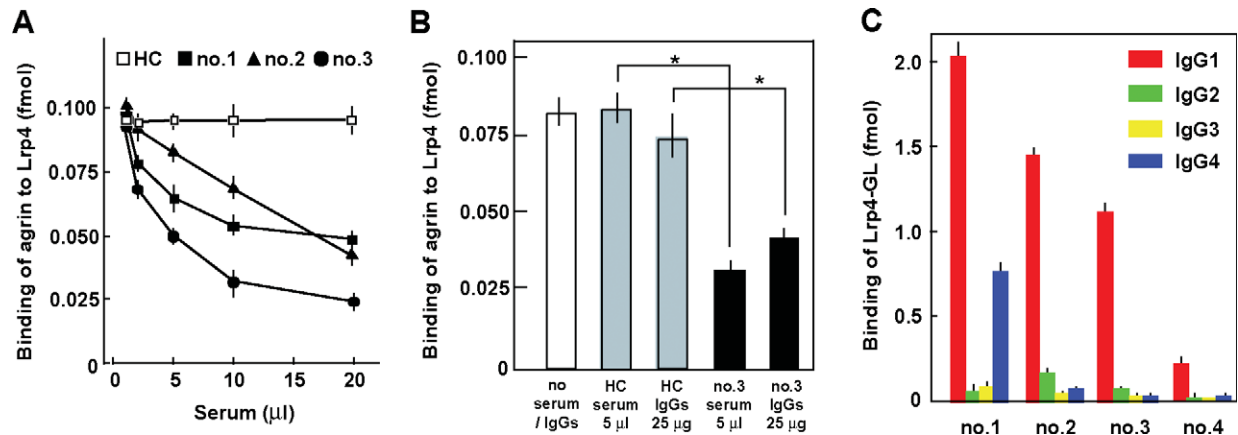


FIGURE 2: Autoantibodies to Lrp4 in MG patients have pathogenic properties. (A, B) Inhibition of the interaction between neural agrin and Lrp4 by serum autoantibodies to Lrp4. (A) Binding of neural agrin (3×FLAG-agrin) to Lrp4-GL was inhibited by sera from patients (index case nos. 1–3), but not by the HC, in a dose-dependent manner. (B) Interaction between 3×FLAG-agrin and Lrp4-GL was comparably inhibited by serum and the corresponding amount of serum IgGs purified from a patient (index case no. 3), but not by the HC. Student *t* test, **p* < 0.01. (C) Determination of IgG subclasses composing Lrp4 autoantibodies. Biotinylated anti-human IgG subclass (G1–G4) antibodies were used instead of protein G in the Lrp4-LUCIP assay to evaluate the subclass-specific titer of serum antibodies from patients (index case nos. 1–4) to the extracellular portion of Lrp4. Data are means ± SDs, *n* = 3 for each experimental group. HC = healthy control; IgG = immunoglobulin G; Lrp4 = low-density lipoprotein receptor-related protein 4; LUCIP = luciferase-reporter immunoprecipitation; MG = myasthenia gravis; SD = standard deviation. [Color figure can be viewed in the online issue, which is available at annalsofneurology.org.]

portion of Lrp4. Therefore, these findings suggest pathogenic involvement of the complement system and reduced agrin:Lrp4:MuSK signaling in Lrp4 antibody-positive MG. However, it is important to carefully evaluate contributions of these antibodies to myasthenia, especially those of the antibodies with lower titers (index case nos. 4–9; see Table), which failed to visualize cell surface Lrp4 likely due to their lower titers. Interestingly, Lrp4 antibodies were found in 3 of 28 patients with MuSK antibody-positive MG and 1 of 101 patients with LEMS. Because MuSK antibodies predominantly belong to the IgG4 subclass, which does not activate complement, and Lrp4 is a postsynaptic protein, antibodies to Lrp4 might contribute differently to pathogenesis than antibodies to MuSK or the P/Q-type presynaptic Ca²⁺ channel, a target for autoantibodies in LEMS.¹⁶ However, since MuSK antibodies, though predominantly IgG4, are partially IgG1 subclass capable of activating complement,¹⁸ MuSK and Lrp4 antibodies might also contribute similarly to pathogenesis in a complement-dependent manner. Given that titers of Lrp4 antibodies were relatively low in sera from patients with MuSK antibody-positive MG or LEMS, again contributions of Lrp4 antibodies to each myasthenia must be carefully evaluated. The LUCIP assay developed in this study is a simple *in vitro* system using no radioisotope. Moreover, the MuSK-LUCIP assay, in which MuSK-GL was used as a reporter, showed roughly a 50-fold lower cutoff value than that determined with the conventional RIA for MuSK autoantibodies, indicating greater sensitivity for the LUCIP assay (Supporting Information Fig 8). Therefore, this assay system could be

used for routine diagnosis and clinical management of various autoimmune disorders, including MG.

It should be noted that the proportion of MuSK antibody-positive patients within an AChR antibody-negative MG cohort varies from 0% to 50% throughout the world.¹² Given the number and narrow ethnic origins of patients in the current study, further clinical and experimental data on greater numbers of patients worldwide are required to fully understand the etiology and pathology of Lrp4 antibody-positive MG.

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The study was approved by the ethics committees of the Graduate School of Biomedical Sciences, Nagasaki University (no. 09031864), and the Institute of Medical Science, the University of Tokyo (no. 20-60-210403).

Potential Conflicts of Interest

Nothing to report.

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References

- Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* 2001; 2:791–805.
- Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. *Development* 2006;133:4993–5000.
- Kim N, Stiegler AL, Cameron TO, et al. Lrp4 is a receptor for agrin and forms a complex with MuSK. *Cell* 2008;135:334–342.
- Zhang B, Luo S, Wang Q, et al. LRP4 serves as a coreceptor of agrin. *Neuron* 2008;60:285–297.
- Vincent A. Unravelling the pathogenesis of myasthenia gravis. *Nat Rev Immunol* 2002;2:797–804.
- Almon RR, Andrew CG, Appel SH. Serum globulin in myasthenia gravis: inhibition of alpha-bungarotoxin binding to acetylcholine receptors. *Science* 1974;186:55–57.
- Lindstrom JM, Seybold ME, Lennon VA, et al. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. *Neurology* 1976;26:1054–1059.
- Toyka KV, Brachman DB, Pestronk A, et al. Myasthenia gravis: passive transfer from man to mouse. *Science* 1975;190:397–399.
- Engel AG, Lambert EH, Howard FM. Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc* 1977;52:267–280.
- Hoch W, McConville J, Helms S, et al. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med* 2001;7:365–368.
- McConville J, Farrugia ME, Beeson D, et al. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann Neurol* 2004;55:580–584.
- Vincent A, Leite MI. Neuromuscular junction autoimmune disease: muscle specific kinase antibodies and treatments for myasthenia gravis. *Curr Opin Neurol* 2005;18:519–525.
- Matthews I, Chen S, Hewer R, et al. Muscle-specific receptor tyrosine kinase autoantibodies—a new immunoprecipitation assay. *Clin Chim Acta* 2004;348:95–99.
- Tannous BA, Kim DE, Fernandez JL, et al. Codon-optimized *Gussia luciferase* cDNA for mammalian gene expression in culture and in vivo. *Mol Ther* 2005;11:435–443.
- Jaretzki A III, Barohn RJ, Ernstoff RM, et al. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. *Neurology* 2000;55:16–23.
- Newsom-Davis J. Lambert-Eaton myasthenic syndrome. *Rev Neurol (Paris)* 2004;160:177–180.
- Vernino S, Lennon VA. Autoantibody profiles and neurological correlations of thymoma. *Clin Cancer Res* 2004;10:7270–7275.
- Leite MI, Jacob S, Viegas S, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain* 2008;131:1940–1952.