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Development and Application of a Cell-Based Assay for LRP4 Antibody Associated With Myasthenia Gravis

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Background and Purpose Among patients with double-seronegative myasthenia gravis (dSN-MG) who do not have detectable antibodies against acetylcholine receptor or muscle-specific tyrosine kinase, autoantibodies against low-density lipoprotein receptor-related protein 4 (LRP4-Ab) have been detected recently. The purpose of this study was to develop an in-house cell-based assay (CBA) to detect LRP4-Ab and to apply it to samples from patients with MG.

Methods The complementary DNA of LRP4 fused into a vector plasmid containing GFP was transfected into human embryonic kidney 293 (HEK293) cells. LRP4 expression in the transfected HEK293 cells was assessed using the reverse-transcription polymerase chain reaction (RT-PCR), Western blotting, and immunocytochemistry. The CBA included 252 sera collected from 202 patients with MG and 38 with other neuromuscular diseases, and 12 healthy controls. The transfected HEK293 cells were incubated using sera and antihuman immunoglobulin G antibodies conjugated with Alexa Fluor 594. The presence of LRP4-Ab was determined based on the fluorescence intensity and the localization in fluorescence microscopy.

Results The expressions of the mRNA and protein of LRP4 in the transfected HEK293 cells were confirmed using RT-PCR and Western blotting, respectively. Immunocytochemistry indicated LPR4 expression on the cell membrane. Among 202 patients with MG including 53 with dSN-MG, LRP4-Ab were positive in 3 patients who were all double seronegative. LRP4-Ab were not detected in the patients with other neuromuscular diseases or the healthy controls.

Conclusions A CBA for detecting LRP4-Ab associated with MG has been developed, and was used to find LRP4-Ab in the sera of patients with MG.

Keywords myasthenia gravis; autoimmune disorders; low-density lipoprotein receptor-related protein 4; cell-based assay; diagnosis.

INTRODUCTION

Myasthenia gravis (MG) is an autoantibody-mediated autoimmune disorder of the neuromuscular junction (NMJ) characterized by fatigability and muscle weakness. Detecting autoantibodies is important in diagnosing MG. Approximately 80% of patients with MG have autoantibodies against the acetylcholine receptor (AChR-Ab) and 40%–70% of patients with MG without AChR-Ab have antibodies against muscle-specific tyrosine kinase (MuSK-Ab).[1,2] The relationships among the pathophysiology of MG and these antibodies have been well established. AChR-Ab and MuSK-Ab are not detected in about 10% of patients with MG; these patients are defined as having double-seronegative MG (dSN-MG).[3] Autoantibodies against low-density lipoprotein receptor-related protein 4 (LRP4-Ab) have recently been detected in patients with dSN-MG at frequencies of 2%–50%.[4-8] Although the pathomechanism of LRP4-Ab in MG has not been fully defined, LRP4-Ab disrupt the interaction between LRP4 and agrin, leading to reduced AChR clustering.[9] Additionally, the predominant

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subclass of LRP4-Ab in patients with dSN-MG is immunoglobulin G1 (IgG1)[8] and LRP4-Ab might be associated with complement activation.[10] At the NMJ of experimental autoimmune MG mice, LRP4-Ab induced complement activation and IgG deposits were observed.[11] LRP4-Ab is often detected using cell-based assays (CBAs) that use cells that express the LRP4 recombinant protein on their membrane. Because CBAs are costly and time-consuming,[12] CBA is used to detect LRP4-Ab in very few laboratories worldwide. LRP4-Ab measurements are therefore very limited, which explains why the characteristics of LRP4-Ab-positive MG are not well known.

The aim of this study was to develop an in-house CBA to detect LRP4-Ab and to apply it to detecting LRP4-Ab in samples from patients with MG.

METHODS

Patients

This study collected 252 serum samples from October 2016 to March 2022. From patients with MG, 202 serum samples were collected: 134 AChR-Ab-positive, 15 MuSK-Ab-positive, and 53 double-seronegative sera. We collected 38 serum samples from patients with various neuromuscular diseases: 18 with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), 8 with Lambert-Eaton myasthenic syndrome (LEMS), 3 with amyotrophic lateral sclerosis (ALS), 7 with polyneuropathy, 1 with Isaacs' syndrome, and 1 with sensory neuronopathy. In addition, 12 sera were collected from healthy controls (Table 1).

All participants provided written informed consent, and the Institutional Review Board of Yonsei University Severance Hospital approved this study (IRB No. 4-2021-1328).

Development of HEK293 cells expressing LRP4

LRP4 cDNA fused into the Sgf I and Mlu I sites of the pCMV6-AC-GFP vector (Origene, Rockville, MD, USA) was used to develop a CBA for detecting LRP4-Ab. The vector included ampicillin and neomycin antibiotic resistance genes for *E. coli* and cell selection, respectively. LRP4 plasmids were expressed in mammalian cells as a tagged protein using a C-terminal tGFP tag. After transformation using MacCell™ DH5α 107 (iNtRON Biotechnology, Seongnam, Republic of Korea) as competent cells, bacteria were selected on antibiotic plates. Bacteria with the LRP4 plasmid were antibiotic-resistant, and the LRP4 plasmids were isolated. Human embryonic kidney 293 (HEK293) cells (Korean Cell Line Bank, Seoul, Republic of Korea) were incubated in a 24-well cell culture dish (SPL Life Sciences, Pocheon, Republic of Korea) at 37°C. The cloned pCMV6-LRP4-tGFP or pCMV6-AC-GFP (empty vector) were then transfected into HEK293 cells using TransIT-2020 (Mirus Bio Corporation, Madison, WI, USA). The transfected cells were cultured in a cell culture incubator at 37°C and 5% CO_2.

Table 1. Serum samples applied to the cell-based assay for LRP4-Ab

	n
Total	252
MG	202
AChR-Ab positive	134
MuSK-Ab positive	15
Double seronegative	53
Other neuromuscular diseases	38
CIDP	18
LEMS	8
Polyneuropathy	7
ALS	3
Isaacs' syndrome	1
Sensory neuronopathy	1
Healthy control	12

AChR-Ab, autoantibodies against the acetylcholine receptor; ALS, amyotrophic lateral sclerosis; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; LEMS, Lambert-Eaton myasthenic syndrome; LRP4-Ab, autoantibodies against low-density lipoprotein receptor-related protein 4; MG, myasthenia gravis; MuSK-Ab, antibodies against muscle-specific tyrosine kinase.

The expressions of the mRNA and protein of LRP4 were confirmed using the reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting, respectively. The following primer pairs were used for the RT-PCR: LRP4, 5'-ACC TACCTGTTCCCCTCTTGA-3' (forward) and 5'-GTCCT GCTCATCCGAGTCATC-3' (reverse); GAPDH, 5'-TGTGGGCATCAATGGATTTGG-3' (forward) and 5'-ACACCATGTATTCCGGGTCAAT-3' (reverse). Untransfected HEK293 cells and HEK293 cells transfected using empty vectors were used as negative controls. Western blotting was performed using rabbit polyclonal anti-LRP4 (Abcam, Cambridge, UK) and β-actin (Invitrogen, Waltham, MA, USA) antibodies. A secondary horseradish peroxidase-conjugated antibody was added and immunocomplex detection was achieved using an enhanced chemiluminescence method.

Immunocytochemistry and CBA

HEK293 cells were plated on a coverslip (SPL Life Sciences) coated with poly-L-lysine (Sigma Aldrich) and placed in wells in cell culture plates. The cells were grown in DMEM (GE Healthcare, Chicago, IL, USA), which contained 10% FBS (GE Healthcare) and 5% penicillin (Invitrogen) at a 70%–80% confluence. The cells were transfected using pCMV6-LRP4-tGFP or empty vectors using TransIT-2020. After 48 hours, immunofluorescence staining was performed. To develop the CBA, immunocytochemistry was performed using

untransfected HEK293 cells, the cells transfected with the empty vectors, and the LRP4-transfected cells. For the CBA to detect LRP4-Ab in the sera of the patients, the assay was performed using the LRP4-transfected cells. The cells were fixed with 4% paraformaldehyde (Tech & Innovation, Chuncheon, Republic of Korea) in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Carlsbad, CA, USA) for 15 minutes at room temperature. After washing, the cells were incubated with rabbit polyclonal LRP4 antibody (Abcam; final concentration of 50 μg/mL) or serum samples of the patients and healthy controls (diluted at 1:20) for 1 hour at room temperature. After washing with PBS, the cells were incubated with goat antirabbit IgG conjugated with Alexa Fluor 594 (AF594, Invitrogen; dilution of 1:750) to detect rabbit polyclonal LRP4 antibodies, or with goat antihuman IgG (H+L) conjugated with AF594 (Invitrogen; dilution of 1:750) to detect LRP4-Ab in the sera of the patients for 45 minutes at room temperature in the dark. After washing with DPBS, the coverslips were gently removed from the cell culture plates and placed on microscope slides with VECTASHIELD Antifade Mounting Medium, which contained 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). The slides were stored in the dark at 4°C before being observed using fluorescence microscopy (Zeiss, Oberkochen, Germany).

The CBA results were evaluated using a fluorescence microscope. The fluorescence intensity on the membranes of LRP4-transfected HEK293 cells and the overlapping of GFP-tagged LRP4 (green fluorescence) and AF594 label (red fluorescence) antibodies were evaluated using the following visual scoring system: 0 for no labeling of the transfected cells, 0.5 for very weak labeling of a few cells with no definite colocalization, 1 for weak labeling of some cells with colocalization, 2 for labeling of 20%–50% of cells with accurate colocalization, 3 for labeling of 50%–80% of cells with perfect colocalization, and 4 for labeling of all transfected cells with perfect colocalization.[5,13,14] A score of 1 or more was considered a positive result. The results were interpreted via the consensus of two independent investigators (M.J.K. and S.W.K.) who were blinded to the clinical data.

RESULTS

LRP4 cDNA transfection into HEK293 cells

LRP4 cDNA was transfected into HEK293 cells to overexpress LRP4 on the cell membranes. As LRP4 was tagged using GFP, the protein was expressed as green fluorescence. Untransfected HEK293 showed only DAPI (blue) at the nuclear location and no green fluorescence. In HEK293 cells transfected with the empty vector, GFP expression was confirmed using green fluorescence in the cytosol of the cells. When the GFP-tagged LRP4 plasmid was transfected, green fluorescence was observed along the cell membrane (Fig. 1A).

To evaluate the binding between LRP4 and anti-LRP4 antibody on the LRP4-transfected cells, the cells were stained using anti-LRP4 and secondary anti-IgG antibodies conjugated with AF594. Red fluorescence was not detected on the transfected cells not treated with anti-LRP4 antibody. On the other hand, red fluorescence and green fluorescence were observed along the membranes of the transfected cells incubated with anti-LRP4 antibody. This indicated that anti-LRP4 antibody was bound to LRP4 on the membranes of LRP4-transfected HEK293 cells (Fig. 1B).

Confirmation of the expressions of mRNA and protein of LRP4 in LRP4-HEK293 cells

To validate the success of LRP4 transfection, the expressions of mRNA and protein of LRP4 were evaluated using RT-PCR and Western blotting, respectively, in three groups of cells: untransfected cells, cells transfected with the empty vector, and LRP4-transfected cells (Fig. 2). LRP4 mRNA expression with a band size of 109 base pairs in LRP4-transfected cells was detected using RT-PCR. The expression of the GAPDH housekeeping gene was confirmed in all three cell groups (Fig. 2A). LRP4 protein expression was confirmed using Western blotting. The sizes of the LRP4 protein and GFP were 212 and 26 kDa, respectively. The size of the LRP4-GFP fusion protein was therefore 238 kDa. Western blotting showed bands with a size of 238 kDa on the lanes of LRP4-transfected cells. β-actin expression was confirmed in all three cell groups (Fig. 2B).

Application of CBA for LRP4 antibody associated with MG

To evaluate the status of autoantibody against LRP4 in the sera of the patients, immunocytochemistry was performed using a CBA. Nuclei were stained using DAPI, and LRP4 protein overexpression was confirmed using GFP (green fluorescence). The serum of each patient was stained using antihuman IgG antibody conjugated with AF594. For the CBA scoring system, percentages of green fluorescence and red fluorescence in colocalized cells along the cell membrane were determined (Fig. 3). The CBA for LRP4-Ab and the visual scoring system were used among 252 sera with unknown antibody status. Among the serum samples, the sera of two patients were scored as 1 (Patient 1 and Patient 2), and that of one patient was scored as 2 (Patient 3) (Fig. 3B).

A visual score of 0.5 was given to 78 serum samples. Among them, 32 patients had AChR-Ab, 7 had MuSK-Ab, 16 had dSN-MG, 6 had CIDP, 3 had LEMS, 5 had polyneuropathy, 2 had ALS, and 7 were healthy controls. A visual score of 0

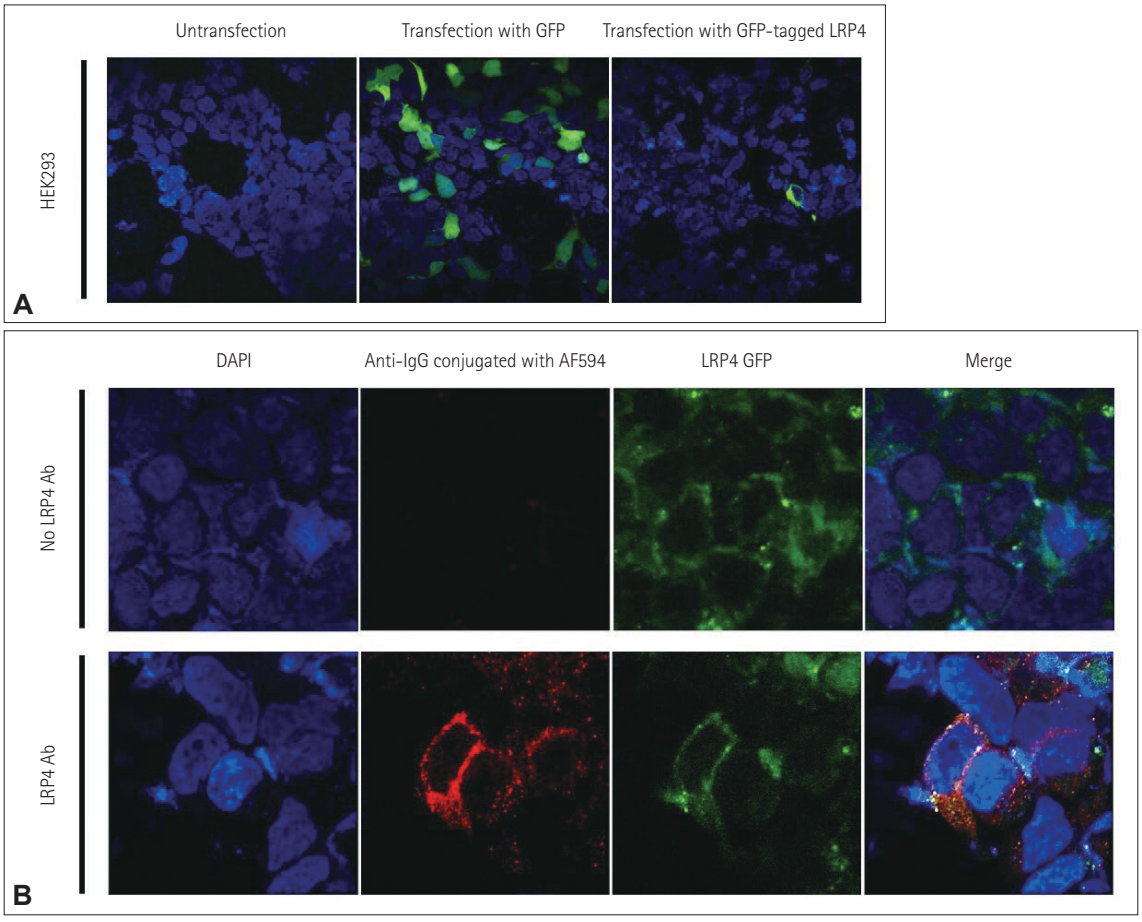

Fig. 1. Low-density LRP4 expression on the membrane of a LRP4-transfected HEK293 cell. A: Untransfected HEK293 cells shows only DAPI at the nuclear location and no green fluorescence. In HEK293 cells transfected with a plasmid containing only GFP, GFP expression is confirmed by green fluorescence in the cytosol of the cells. When the GFP-tagged LRP4 plasmid was transfected, green fluorescence can be observed along the cell membrane. B: LRP4-transfected HEK293 cells were incubated with commercially available polyclonal anti-LRP4 antibody and anti-IgG antibody conjugated with AF594. Red fluorescence is observed along the membranes of LRP4-transfected HEK293 cells, which had green fluorescence on their surfaces. This indicates the binding of anti-LRP4 antibody to LRP4 on the cell surface of LRP4-transfected HEK293 cells. AF594, Alexa Fluor 594; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; IgG, immunoglobulin G; LRP4, lipoprotein receptor-related protein 4; LRP4-Ab, autoantibodies against low-density lipoprotein receptor-related protein 4.

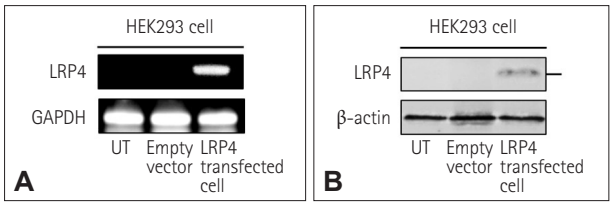

Fig. 2. Confirmation of mRNA and protein of LRP4 in LRP4-transfected HEK293. A: The reverse-transcription polymerase chain reaction reveals the expression (band at 109 bp) of LRP4 mRNA in the LRP4-transfected HEK293 cells, whereas no bands are detected in either untransfected HEK293 cells or HEK293 cells transfected with empty vectors. B: Western blotting also confirms LRP4 expression (band at 238 kDa) in the LRP4-transfected HEK293 cells. No bands are evident in the lanes of untransfected HEK293 cells or HEK293 cells transfected with empty vectors. bp, base pairs; empty vector, plasmids that do not contain the LRP4 gene; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK293, human embryonic kidney 293; LRP4, lipoprotein receptor-related protein 4; UT, untransfected cells.

was given to 171 serum samples. Of them, 102 patients had AChR-Ab, 8 had MuSK-Ab, 34 had dSN-MG, 12 had CIDP, 1 had Isaacs' syndrome, 5 had LEMS, 2 had polyneuropathy, 1 had ALS, 1 had sensory neuronopathy, and 5 were healthy controls.

Clinical features of the patients

Patient 1

A 24-year-old female presented with eyelid ptosis and diplopia for 1 year. Her ocular symptoms had diurnal variation and fatigability. A neurological examination demonstrated the weakness of eye closure and fatigable eyelid ptosis. Other than in the extraocular muscles and orbicularis oculi, motor power was normal. Repetitive nerve stimulation of the right abductor digiti minimi, flexor carpi ulnaris, orbicularis oculi,

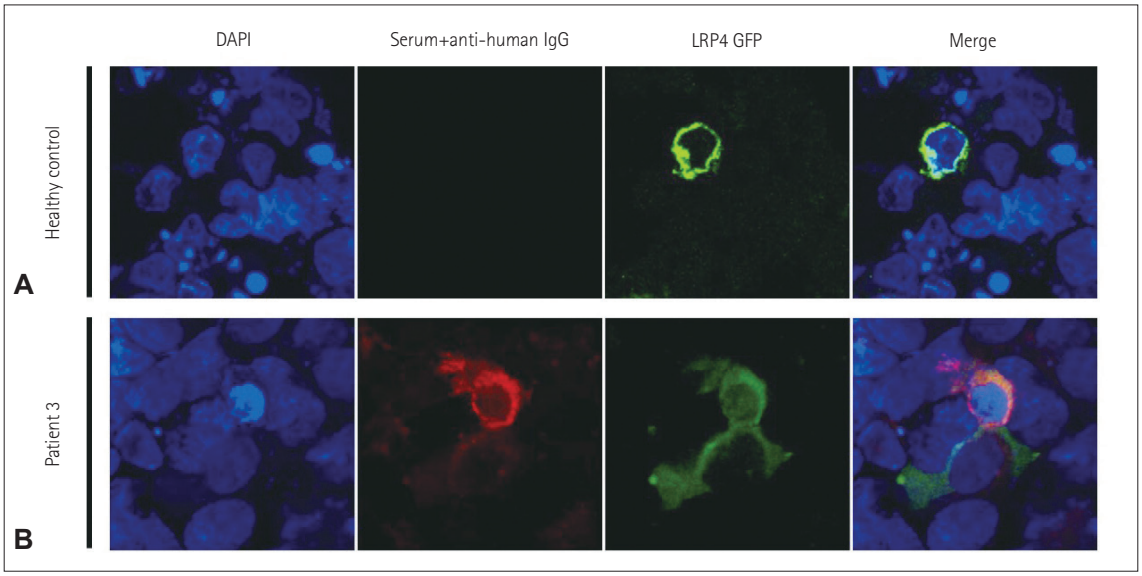

Fig. 3. Cell-based assay to detect autoantibodies against LRP4 (LRP4-Ab). A: Red fluorescence is not observed in a serum sample of a healthy control. B: Red fluorescence colocalized with green fluorescence is observed in a serum sample of Patient 3, indicating that the serum sample of this patient contains LRP4-Ab. DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; IgG, immunoglobulin G; LRP4, lipoprotein receptor-related protein 4; LRP4-Ab, autoantibodies against low-density lipoprotein receptor-related protein 4.

nasalis, and trapezius muscles showed normal responses. Chest computed tomography (CT) revealed suspected soft-tissue attenuation in the anterior mediastinum, which suggested thymic hyperplasia. The ocular symptoms improved after pyridostigmine bromide treatment. AChR-Ab and MuSK-Ab were negative. The symptoms responded to the pyridostigmine bromide and prednisolone treatments.

Patient 2

A 47-year-old male presented with eyelid ptosis and diplopia for 7 months. Their ocular symptoms had diurnal variation and fatigability. A neurological examination demonstrated eye closure weakness and fatigable eyelid ptosis. Other than in the extraocular muscles and orbicularis oculi, motor power was normal. Repetitive nerve stimulation of the left abductor digiti minimi, flexor carpi ulnaris, orbicularis oculi, nasalis, and trapezius muscles showed normal responses. The ocular symptoms improved significantly after neostigmine injection. Chest CT revealed no anterior mediastinal mass. AChR-Ab and MuSK-Ab were negative. The symptoms were resolved after pyridostigmine bromide and prednisolone treatments.

Patient 3

A 48-year-old female presented with leg weakness, which had diurnal variation and fatigability. Symptoms of arm weakness, difficulty in mastication, and a nasal voice appeared 2 months later. A neurological examination demonstrated moderate weakness in the proximal limb muscles. Extraocular and orbicularis oculi muscle functions were normal. Repetitive nerve stimulation showed decremental responses on the right abductor digiti minimi, flexor carpi ulnaris, orbicularis oculi, nasalis, and trapezius muscles. Chest CT revealed no abnormalities in the anterior mediastinum. AChR-Ab and MuSK-Ab were negative. The symptoms temporarily responded to pyridostigmine bromide treatment. There was an insufficient response to prednisolone treatment. The symptoms resolved after adding tacrolimus to the treatment.

DISCUSSION

The purpose of this study was to develop a CBA to detect LRP4 antibodies. LRP4 plasmid vector was transfected to HEK293 cells. The expressions of mRNA and protein of LRP4 in the transfected HEK293 cells were confirmed using RT-PCR and Western blotting, respectively. Immunocytochemical staining with anti-LRP4 antibody demonstrated LRP4 expression on the cell membrane and the binding of LRP4 and anti-LRP4 antibody. CBA for detecting LRP4-Ab using these LRP4-transfected HEK293 cells found 3 MG patients with LRP4-Ab from 202 patients with MG including 53 with dSN-MG.

Diagnostic CBAs involve transfection of cells with the cDNA that encodes the relevant protein, which is usually a membrane protein. The transfection leads to the expression of the protein on the cell membranes. The protein and antibody binding can then be evaluated using immunocytochemistry and scored using a visual scoring system. CBA has some advantages. Compared with other antibody assays such as radioimmunoprecipitation assay (RIA) and enzyme-linked immunosorbent as-

say (ELISA), CBAs provide a more physiological environment that permits the expression of proteins in native conformation with posttranslational processing. In addition, because the protein is expressed on intact live cell membranes, antibodies against intracellular epitopes, which are usually nonpathogenic, cannot bind to the protein and are not detected. For these reasons, CBAs are more sensitive and specific in detecting pathogenic antibodies compared with RIA and ELISA, which use recombinant proteins in solutions or on solid surfaces.[12]

The detection of novel antibodies for the proteins for NMJ is needed to identify new antigens associated with MG. A CBA for LRP4-Ab was developed recently and involves LRP4 cDNA transfection to express the LRP4 protein on the cell membrane.[4,8,15] Previous studies of CBAs for LRP4-Ab found that 50%,[4] 19%,[8] 15%,[16] 8%,[17] and 4%[6] of patients with dSN-MG were LRP4-Ab positive. Two studies that used CBA failed to identify LRP4-Ab-positive patients with MG.[18,19] Some studies used ELISA or luciferase immunoprecipitation. Two studies that used ELISA found prevalence rates of 9% and 15%.[20,21] One study that used luciferase immunoprecipitation found that 9 of 300 (3%) patients with AChR-Ab-negative MG were LRP4-Ab positive.[15] According to the previous studies, frequencies of LRP4-Ab in dSN-MG varied and CBAs seemed more sensitive in detecting LRP4-Ab than ELISA or luciferase immunoprecipitation. In the present study, the proportion of LRP4-Ab in dSN-MG was 5.7%, which was lower than in previous CBA studies. The subjects in most studies of LRP4-Ab in MG were from Europe or North America; however, the frequencies in two studies from Japan and China were 3% and 4%, respectively, which were similar to that in the present study.[6,15] The low prevalence of LRP4-Ab positivity in MG found in the present study might be due to the racial characteristics of patients with MG. In addition, the proportion of ocular and generalized patients among the patients with dSN-MG may have affected the frequency of LRP4-Ab positivity. In the present study, 21 of the 53 patients with dSN-MG only had ocular symptoms, but this proportion was not clearly reported for most previous studies. It was therefore difficult to compare the proportions of patients with ocular MG among the studies and to analyze the association between the proportion of patients with ocular MG and the frequency of positive LRP4-Ab patients. Future studies should clearly describe the clinical characteristics of the subjects.

Based on the previous studies of LRP4-Ab in MG, the age at onset was lower and there was a female predominance in LRP4-Ab positive compared with LRP4-Ab negative MG patients.[22] LRP4-Ab-positive patients with MG tended to have mild symptoms and often isolated ocular weakness (i.e., ocular myasthenia).[22] LRP4-Ab-positive patients with MG also responded well to the pyridostigmine bromide and prednisolone treatments. In the present study, two patients with LRP4-Ab also only had mild ocular symptoms and responded to the pyridostigmine bromide and prednisolone treatments. However, there have been a few studies that found different clinical features. Two previous studies that used ELISA to detect LRP4-Ab found that moderate to severe weakness and generalized disease pattern were more common than mild weakness and isolated ocular weakness.[15,20] In the present study, one LRP-Ab-positive patient with MG had moderate generalized symptoms, which was temporarily responsive to pyridostigmine bromide treatment. Treatment with prednisolone was insufficient and tacrolimus was also added. The clinical characteristics of LRP4-Ab-positive patients with MG have varied between previous studies, and so further studies are needed. The reason for this discrepancy is not yet known, but several reasons may be considered. LRP4-Ab-positive MG may be a disease with a broad clinical spectrum, hence requiring evaluations of more patients in order to better describe it. Another possibly influencing factor is the titer of LRP4-Ab. Higher LRP4-Ab titers could be associated with more severe and generalized clinical features. In the present study, Patient 3 presented with a worse disease condition and higher LRP4-Ab visual score than Patient 1 and Patient 2. However, CBA is only a semiquantitative assay. Further studies using more quantitative methods are necessary to verify this hypothesis. CBA and ELISA are the most commonly used methods for detecting LRP4-Ab in patients with MG, but no studies have compared these two methods. It is also necessary to evaluate whether the different LRP4-Ab assay methods are associated with differences in clinical features.

This study had several limitations. First, there is no gold-standard test for LRP4-Ab that can be used to compare the performance of the present CBA. The possibility of false-positive results cannot be ruled out. Further studies using different methods for detecting LRP4-Ab are necessary. To reduce the possibility of false-positive results, we are also planning to modify the CBA technique. In the present study, secondary antibodies for the whole IgG (H+L) were used in the CBA. Because all immunoglobulin classes share light chains and whole IgG secondary antibodies recognize both heavy and light chains, the use of whole IgG (H+L) secondary antibodies might result in cross-reactivity to other immunoglobulins. On the other hand, Fc-specific secondary antibodies do not result in cross-reactivity with other immunoglobulin classes due to the absence of light chains in the Fc fragment. Replacement of whole IgG (H+L) secondary antibodies with Fc-specific secondary antibodies may therefore increase the signal-to-noise ratio of the CBA. Second, the efficiency of LRP4 transfection for HEK293 cells was not high and only a few cells

were transfected. For more stable and accurate testing, additional research is needed to identify techniques that increase transfection efficiency. We are planning studies using cotransfection of LRP4 and its chaperone protein, low-density lipoprotein receptor-related protein-related protein 1, in order to enhance cell membrane expression,[18] or LRP4 transfection without GFP instead of GFP-tagged LRP4 in order to reduce the plasmid size.

In conclusion, a CBA for detection of LRP4-Ab associated with MG has been developed. LRP4-Ab could be found by applying the sera of patients with MG to the CBA. The clinical features of the patients with LRP4-Ab detected by the present CBA were consistent with the known clinical characteristics of LRP4-Ab-positive MG. The present CBA can be used to determine the presence of LRP4-Ab.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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