Development and Application of Cell-based Assay for LRP4 Antibody Associated with Myasthenia Gravis

Min Ju Kim

Program of Biomedical Engineering

The Graduate School, Yonsei University

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Directed by Professor Ha Young Shin

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Thesis Supervisor: Sung-Rae Cho

Thesis Committee Member #1: Yoon-Ho Hong

Thesis Committee Member #2: Ha Young Shin

The Graduate School
Yonsei University

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ABSTRACT

Development and application of cell-based assay for LRP4 antibody associated with myasthenia gravis

Min Ju Kim

Department of Nanoscience and Technology
The Graduate School, Yonsei University

(Directed by Professor Ha Young Shin)

Background: Myasthenia gravis (MG) is an autoantibody mediated autoimmune disorder of the neuromuscular junction characterized by fatigable muscle weakness. Approximately 90% of patients with MG have autoantibodies against the acetylcholine receptor (AChR) or muscle-specific kinase (MuSK). Among about 10% of MG patients who do not have detectable AChR-Ab or MuSK-Ab (double seronegative), autoantibodies against the low-density lipoprotein receptor-related protein 4 (LRP4-Ab) have been detected recently. The purpose of this study is to develop an in-house cell-based assay (CBA) to detect LRP4-Ab and to apply the CBA for detecting LRP4-Ab in the samples from MG patients.

Method: For the development of CBA, LRP4 complementary DNA (cDNA) was amplified and cloned into the pCMV6-AC-GFP vector. The cloned LRP4 plasmids were transfected into human embryonic kidney 293

(HEK293) cells. The reverse-transcription polymerase chain reaction (RT-PCR) and western blotting were performed to confirm the expression of LRP4 mRNA and protein, respectively. The transfected HEK293 cells were incubated with rabbit anti human LRP4 antibody and goat ant-rabbit IgG conjugated with Alexa Fluor 594. The immunofluorescence staining was evaluated using a fluorescence microscope. After the establishment of CBA, totals of 251 serum samples collected from 201 patients with MG, 38 with other neuromuscular diseases, and 12 healthy controls were applied to the CBA for LRP4-Ab. The presence of LRP4-Abs was determined based on the fluorescence intensity and their localization in fluorescence microscopy.

Results: The expression of LRP4 mRNA and protein in the LRP4 transfected HEK293 cells was confirmed by RT-PCR and western blotting, respectively. In fluorescence microscopy, green fluorescence was observed along the cell surface of the LRP4 transfected cells, indicating the expression of LRP4 on the cell membranes. After adding the LRP4-Ab, the cell membranes were stained with red fluorescence that was colocalized with the green fluorescence. Two of 201 MG patients including 52 double seronegative MG patients were positive for LRP4-Ab. The two MG patients had only ocular symptoms and did not have AChR-Ab and MuSK-Ab. They were responsive to the treatment with acetylcholinesterase inhibitor and prednisolone.

Conclusion: CBA for detection of LRP4-Ab associated with myasthenia gravis was developed. Using CBA, LRP4-Abs were found in the serum from MG patients.

Key words: Autoimmune disorders, Cell-based assay, Low-density lipoprotein receptor-related protein 4, Myasthenia gravis

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I. INTRODUCTION

1. Neuromuscular junction

Neuromuscular junction (NMJ) is a synapse between the end of the motor neuron nerve and the muscle fiber, and its structure and function are highly specialized and complex. NMJ plays a role in converting electrical impulse generated by motor neurons to chemical signal and finally into electrical activity of muscle fibers. When the motor nerve action potential is reached at the motor nerve terminal, calcium influx occurs at the presynaptic terminal and the acetylcholine (ACh) is released. And then, it crosses the synaptic cleft and binds to the ACh receptor (AChR) on the muscle membrane (Cruz et al. 2020). ACh is a neurotransmitter released from motor neurons that bind to receptors in motor end plates and plays an important role in neuromuscular transmission (Conti-Fine, Milani, Kaminski 2006).

High density clustering of AChRs at the post-synaptic membrane is critical for efficient neuromuscular transmission. Impairment of

neuromuscular transmission can be caused by genetic disorders that affect the clustering of AChR (Muller et al. 2006, Nicole et al. 2014, Ohkawara et al. 2014). Signaling by Agrin-LRP4-MuSK complex in NMJ induces AChR clustering (Shen et al. 2013, Zhang et al. 2008). Agrin released from motor nerve terminals binds to LRP4 and activates the phosphorylation of MuSK, which signals through Dok-7 and rapsyn and stabilizes AChR clustering. Defects in LRP4 can affect neuromuscular function and cause congenital myasthenic syndrome (Ohkawara et al. 2014, Selcen et al. 2015).

2. Low-density lipoprotein receptor-related protein 4

Low-density lipoprotein receptor-related protein 4 (LRP4), one of the members of the low-density lipoprotein receptor family, has a large extracellular N-terminal region, a transverse domain, and a short C-terminal region (Zong et al. 2012). LRP4 has multiple domains in its extracellular domain, an LDLa domain and four ß-propeller domains, and an EGF-like motif. The first ß-propeller domain interacts with the c-terminus of agrin to form a dimer. These dimers form a tetramer important for agrin signaling, which prompts the phosphorylation of MuSK. MuSK attaches to and interacts with the ß3 propeller domain of LRP4. (Pevzner et al. 2012, Yu et al. 2021).

3. Myasthenia Gravis

Myasthenia gravis (MG) is an autoantibody mediated autoimmune disorder of the neuromuscular junction characterized by fatigable muscle weakness. Approximately 80% of patients with MG have autoantibodies against the acetylcholine receptor (AChR) and 40–70% of generalized MG patients without AChR antibodies (AChR-Abs) have antibodies against MuSK (MuSK-Abs) (Gilhus et al. 2019, Gilhus, Verschuuren 2015). The

relationship between the path mechanism of MG and these antibodies have been well established. In about 10% of MG patients, AChR-Ab or MuSK-Ab are not detected. These patients are defined as double seronegative MG (dSN-MG) (Poulas et al. 2012). Recently, autoantibodies against the low-density lipoprotein receptor-related protein 4 (LRP4-Ab) have been detected in dSN-MG patients with various frequencies, ranging from 2% to 50% (Hong et al. 2017, Li et al. 2017, Pevzner et al. 2012, Yan et al. 2018, Zisimopoulou et al. 2014). The predominant subclass of LRP4-Ab is IgG1 in dSN-MG patients (Zisimopoulou et al. 2014).

Although the pathomechanism of LRP4-Ab in MG has not been fully defined, LRP4-Ab disrupts the interaction between LRP4 and agrin, leading to reduced AChR clustering (Shen et al. 2013). Additionally, LRP4-Ab might activate complement. At NMJ of experimental autoimmune myasthenia gravis mice induced by LRP4, complement and IgG deposits were demonstrated (Ulusoy et al. 2017). Usually, LRP4-Ab is detected by cell-based assays (CBA) which use cells expressing the LRP4 recombinant protein on their membrane. Because CBAs are costly and time consuming (Rodriguez Cruz et al. 2015b), CBA for detection of LRP4-Ab is not widely used but is performed only in very few laboratories worldwide. Therefore, LRP4-Ab measurement is very limited, and for this reason, the characteristics of LRP4-Ab positive MG are not well known.

4. Aim of study

The aim of this study is to develop an in-house CBA to detect LRP4-Ab and to apply the CBA for detecting LRP4-Ab in the samples from MG patients.

II. MATERIALS AND METHODS

1. Subjects

Total 251 serum samples collected from October 2016 to September 2021. From MG patients, 201 serum samples were collected: 134 AChR ab positive, 15 MuSK-Ab positive, and 52 double seronegative sera. We collected 38 serum samples from various neuromuscular diseases: 18 from chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), eight from Lambert-Eaton myasthenic syndrome (LEMS), three from amyotrophic lateral sclerosis (ALS), seven from polyneuropathy, one from Isaac syndrome, and one from 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 (No. 4-2021-1328).

2. Transformation

LRP4 was fused into the Sgf I and Mlu I sites of the pCMV6-AC-GFP vector (Origene, Rockville, MD, USA). The LRP4 plasmid were expressed in mammalian cells as a tagged protein with a C-terminal tGFP tag. The vector included ampicillin and neomycin antibiotic resistance genes for E.coli and cell selection, respectively. For perform transformation, used MacCell™ DH5α 10^7 (Intron biotechnology, Seongnam-si, Gyeonggi-do, Korea) as competent cells. The plasmid DNA were added 1μl directly to the competent cells and gently to mix on the ice. While incubating the product, heat block controlled the temperature to heat shock for cell mixture. After 30 minutes, the product was heated exactly 90 second in a 42 ℃ and immediately placed on the ice for 5 minutes. Pre-heated super optimal broth

(SOC) medium was added 250 μl to each tube. To cloning the plasmid DNA, each tube was incubated for 1 hour at the 37 ℃ on a shaking incubator. During the incubation, an antibiotic-free luria bertani (LB) agar plate and an antibiotic-containing LB agar plate were dried at the 37 ℃. After incubation, 200 μl of cell mixture was spread widely on all dried LB agar plates. The plates were placed in an incubator overnight at 37 ℃ and 5% CO_2. This experiment was performed in according to the manufacturer's recommendations of MacCell™ DH5α 10^7.

3. Plasmid purification

After transformation, bacteria were selected on antibiotic plates. Bacteria with a plasmid were antibiotic-resistant, and each one was formed a colony. The single colony formed on the plate was selected and incubated in a LB growth medium. After 1 day, the LRP4 plasmids were isolated from the incubated LB medium by plasmid midi kit (Qiagen, Hilden, Germany).

4. Cell culture

Human embryonic kidney 293 (HEK293) cells (Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco's modified Eagle's medium (DMEM, GE Healthcare, Chicago, IL, USA) containing 10% fetal bovine serum (FBS, R&D system, Minneapolis, MN, USA), 100 μg/ml penicillin (Invitrogen, Carlsbad, CA, USA) at 37 ℃ in a humidity-controlled incubator with 5% CO_2.

5. Transfection

A day before transfection, HEK293 (Korean Cell Line Bank) cells were incubated in a 24-well cell culture dish (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) in $0.25x10^6$ cells/well. A day after, the DMEM of HEK293 cell were changed to new DMEM containing 10% FBS without antibiotic. HEK293 cell were incubated for at least 2 hours at 37 °C incubator. The cloned pCMV6-LRP4-tGFP or pCMV6-AC-GFP were transfected into HEK293 (Korean Cell Line Bank) cells using TransIT-2020 (Mirus Bio Corporation, Madison, WI, USA) on 24-well cell culture plate (SPL Life Science). First, 50 μl of Opti-MEM (Gibco, Carlsbad, CA, USA) were placed in each sterile tube. Plasmid DNA were added 0.7 μg/ml in each sterile tube and mixed gently by pipetting. The reagent buffer, 2.1 μl of TransIT-2020, were added in the plasmid contained tube and incubated at the room temperature for 20 minutes. Final step, the DNA/reagent complex mixture was evenly dropped into the wells and the plate was gently shaken to ensure even distribution of the complexes. The transfected cells were cultured in a cell culture incubator at 37 °C and 5% CO_2, with the culture medium changed after 24 hours of incubation.

6. Reverse transcription-polymerase chain reaction

Cells were homogenized on ice in 1 ml Trizol (Thermo Fisher, Rockford, IL, USA) using a pipette, and were incubated for 10 minutes. Total RNA was isolated according to the manufacturer's instructions. The cell was centrifuged for 10 minutes at 12,000 g at 4 °C. Total RNA were precipitated and formed a white gel-like pellet at the bottom of the tube. The supernatant was gently discarded with micropipette and resuspended the pallet in 1 ml of 75% ethanol per 1 ml of Trizol reagent used for lysis. The pellet was briefly

mixed with 75% ethanol using vortex and centrifuged for 5 minutes at 7,500 g at 4 °C. The supernatant was discarded with micropipette and dried the RNA pellet for 8 minutes on air. The RNA was resuspended in 20 μl of RNase-free water (Tech&Innovation, Chuncheon-si, Gangwon-do, Korea) by pipetting up and down. The RNA was measured the concentration using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher).

The expression of LRP4 or GAPDH mRNA was confirmed using the reverse transcription polymerase chain reaction (RT-PCR) with primer pairs. The following primer pairs were used for the RT-PCR: LRP4, forward 5'-ACCTACCTGTTCCCCTCTTGA-3' and reverse 5'-GTCCTGCTCATCCGAGTCATC-3'; GAPDH, forward 5'-TGTGGGCATCAATGGATTTGG-3' and reverse 5'-ACACCATGTATTCCGGGTCAAT-3'. Untransfected HEK293 cells and HEK293 cells transfected with empty vectors were used as negative controls. The conserved sequences were selected by sequence comparison and sequence alignment retrieved from the GenBank of the National Center of Biotechnology Information (NCBI).

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Kita-Ku, Osaka, Japan). The PCR amplification was performed in a MiniAmp Thermal Cyclers (Thermo Fisher). In PCR, annealing was performed at 62 °C. After 30 cycles of the reaction, PCR products were electrophoresed on 1.5% agarose gels at 100 mV and visualized by gel doc.

7. Western blot

The expression of LRP4 protein was confirmed using western blotting. LRP4 transfected HEK293 cells were washed two times with DPBS and lysed in radio-immunoprecipitation assay (RIPA, Thermo Fisher) buffer. The mixture was incubated during 15 minutes on the ice and centrifuged for 15 minutes at 13,000 rpm. The supernatant was gently transferred to the new micro tube by micropipette. The concentration of protein was measured by Lowry assay. A total of 50 μg protein was solubilized in Tris-Glycine 2X SDS sample buffer (Invitrogen) by boiling at 97 ℃ and 4-12% Tris-Glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by electroblotting on 0.45um polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were blocked for 1 hour at room temperature with 5% skim milk (BD, Franklin Lakes, NJ, USA) and 0.05% polysorbate 20 (Sigma Aldrich, St. Louis, MO, USA) in TBS (Biosesang , Seongnam-si, Gyeonggi-do, Korea) and then reacted with the primary antibodies: anti-LRP4 antibody (Abcam, 1: 1,000) and β-actin antibody (Invitrogen, 1: 2,000) dissolved in TBS containing 0.05% polysorbate 20 (Sigma Aldrich) and 1% skim milk (BD). After the membranes had been washed three times, incubations with a secondary horseradish peroxidase (HRP)–conjugated antibody dissolved in TBS containing 0.05% polysorbate 20 and 1% skim milk (BD) were performed for 1 hour at room temperature. For detection of immunocomplex, an enhanced chemiluminescence method was used.

8. Immunocytochemistry

HEK293 (Korean Cell Line Bank) were plated with a 12-mm glass cover (Marieenfeldt senior, Lauda-Königspad, and Ambolspad) coated on a 24-well culture plate of poly-L-lysine (Sigma Aldrich). Cells grew in DMEM (GE Healthcare), which contained 10% FBS (GE Healthcare) and 5%

penicillin (Invitrogen). One day later, cells with 70-80% confluence on a coated 24-well culture dish were transfected using TranSIT-2020. After 48 hours, immunofluorescence staining was performed using the LRP4-transfected HEK239 cells. The culture medium was gently removed, and the cells were rinsed three times with DMEM containing 20 mM HEPES (Tech&Innovation, Chuncheon-si, Gangwon-do, Korea). The cells were fixed with 4% paraformaldehyde (PFA, Tech&Innovation) in Dulbecco's phosphate buffered saline (DPBS, Gibco) for 15 minutes at room temperature. The cells were washed three times with DPBS. The cells were incubated with rabbit polyclonal LRP4 antibody (Abcam, Cambridge, UK; 50 μg/mL final concentration) in DMEM containing 1% bovine serum albumin (Biosesang) and 20 mM HEPES (Tech&Innovation), for 1 hour at room temperature. After washing with PBS, the cells were incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Invitrogen; 1:750) to detect rabbit polyclonal LRP4 antibody for 45 minutes at room temperature in the dark. After washing three times with DPBS, the coverslips were gently removed from the 24-well plates and placed on microscope slides (Marienfeld-superior) with VECTASHIELD antifade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). The slides were stored in darkness at 4 °C before being observed using fluorescence microscopy (Zeiss, Oberkochen, Germany)

9. Cell-based assay

The total of 251 serum samples were applied the established cell-based assay for LRP4-Ab. The procedure of this cell-based assay was carried out in the same manner as the above immunocytochemistry protocol. We prepared LRP4-transfected HEK293 cells and the medium in the cell culture

plate was removed. The washing was repeated three times with a DMEM containing 20 mM HEPES (Tech&Innovation). The cell was fixed with 4% PFA (Tech&Innovation) in DPBS for 15 minutes at room temperature. The cells were washed three times with DPBS. The cells were incubated with sera of patients and healthy controls (diluted 1:20) in DMEM containing 1% BSA (biosesang) and 20 mM HEPES (Tech&Innovation) for 1 hour at room temperature. After washing with PBS, the cells were incubated with goat anti-human IgG (H+L) conjugated with Alexa Fluor 594 (Invitrogen; dilution 1:750) for 45 minutes at room temperature in the dark. After washing three times using DPBS, the coverslips were gently removed from the 24-well plates and placed on microscope slides with VECTASHIELD antifade mounting medium containing DAPI. The slides were stored in darkness at 4 ℃ before being observed using fluorescence microscopy (Zeiss)

The results of CBA were evaluated using a fluorescence microscopy. Fluorescence intensity on the cell membrane of LRP4-transfected HEK293 cells and overlap of GFP-tagged LRP4 (green fluorescence) and Alexa Fluor 594 label antibody (red fluorescence) were evaluated using a visual scoring system as previously: 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 showing perfect colocalization (Hong et al. 2017, Kang et al. 2012, Kim et al. 2021). A score of 1 or more was considered as positive result. The results were interpreted using consensus by two independent investigators who were blinded to the clinical data.

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

	n
Total	251
Myasthenia gravis	201
AChR Ab (+)	134
MuSK Ab (+)	15
Double seronegative	52
Other neuromuscular diseases	38
CIDP	18
LEMS	8
Polyneuropathy	7
ALS	3
Issac syndrome	1
Sensory neuronopathy	1
Healthy control	12

Abbreviation: MG, myasthenia gravis; Ab, antibody; AChR, Acetylcholine Receptor; MuSK, Muscle, skeletal receptor tyrosine-protein kinase; CIDP, Chronic inflammatory demyelinating polyradiculoneuropathy; LEMS, Lambert-Eaton Myasthenic Syndrome; ALS, Amyotrophic lateral sclerosis .

III. RESULTS

1. LRP4 gene transfection into HEK293 cell

LRP4 gene was transfected into HEK293 cells to overexpress LRP4 on cell membrane. As LRP4 was tagged with GFP, the LRP4 protein was expressed in green fluorescence. Untransfected HEK293 showed only DAPI (blue) at the nuclear location and no green fluorescence. In HEK293 cells transfected with a plasmid containing only GFP, GFP expression was confirmed by green fluorescence in cytosol of cell. 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 by anti-LRP4 antibody and secondary anti-IgG conjugated with Alexa Fluor 594. On the transfected cells not treated with anti-LRP4 antibody, red fluorescence was not detected. On the other hand, on the transfected cells incubated with anti-LRP4 antibody, red fluorescence was observed along the membrane of the cells, which had green fluorescence on their surface. This indicates the binding of anti-LRP4 antibody to LRP4 on the cell surface of LRP4-transfected HEK293 cells (Fig. 1B).

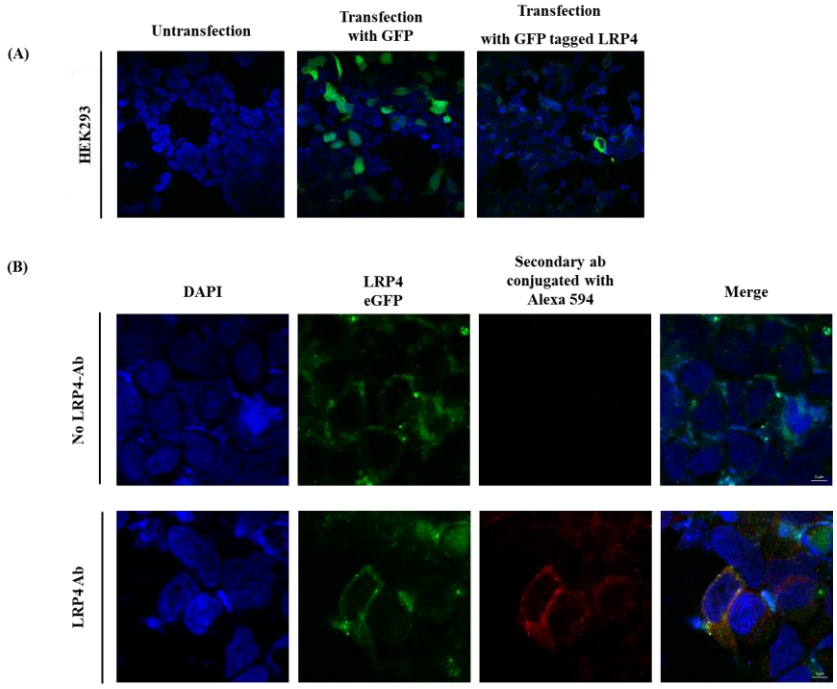

Figure 1. Expression of LRP4 on the membrane of LRP4 transfected HEK293 cell. (A) Untransfected HEK293 showed only DAPI at the nuclear location and no green fluorescence. In HEK293 cells transfected with a plasmid containing only GFP, GFP expression was confirmed by green fluorescence in cytosol of cell. When the GFP-tagged LRP4 plasmid was transfected, green fluorescence was 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 Alexa Fluor 594. Red fluorescence was observed along the membrane of LRP4-transfected HEK293 cells, which had green fluorescence on their surface. This indicates the binding of anti-LRP4 antibody to LRP4 on the cell surface of LRP4-transfected HEK293 cells. HEK293, human embryonic kidney 293; LRP4, low-density lipoprotein receptor-related protein 4; DAPI,

4′,6-diamidino-2-phenylindole; GFP, Green fluorescent protein; IgG, Immunoglobulin G.

2. Confirmation of LRP4 mRNA and protein expression in LRP4-HEK293 cells

To validate the success of LRP4 transfection, the expression of LRP4 mRNA and protein were evaluated using RT-PCR and western blotting, respectively, in three groups of cells, i.e., untransfected cells, empty vector transfected cells, and LRP4 transfected cells (Fig. 2). With RT-PCR, LRP4 mRNA expression with band size on 109 base pair was confirmed in LRP4 transfected cells. The expression of GAPDH house gene was confirmed in all three cell groups (Fig. 2A). LRP4 protein expression was confirmed with western blotting. Size of LRP4 protein is 212 kDa and that of GFP is 26 kDa. Therefore, the size of LRP4-GFP fusion protein is 238kDa. The result of western blot showed the band with size 238 kDa on the lane of LRP4 transfected cells. The expression of ß-actin was confirmed in all three cell groups (Fig. 2B).

Through these two molecular experiments, it was confirmed that the expression of LRP4 in HEK293 cells was successful.

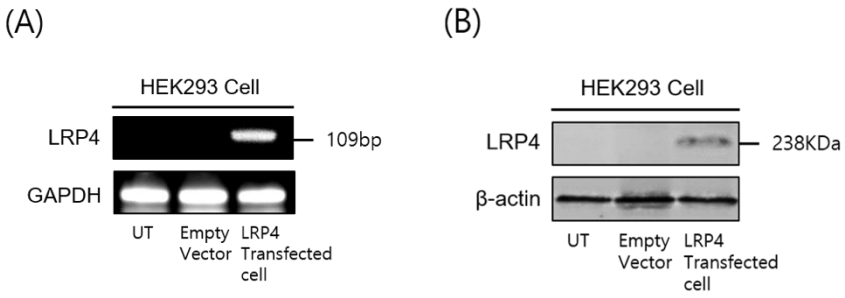

Figure 2. Confirmation of LRP4 mRNA and protein in LRP4-transfected HEK293. (A) Reverse-transcription polymerase chain reaction revealed the expression (band at 109 bp) of LRP4 mRNA in the LRP4-transfected HEK293 cells, whereas no bands were detected in either untransfected HEK293 cells or HEK293 cells transfected with empty vectors. (B) Western blotting also confirmed LRP4 expression (band at 238 kDa) in the LRP4-transfected HEK293 cells. Lanes of untransfected HEK293 cells or HEK293 cells transfected with empty vectors showed no bands. GAPDH, glyceraldehyde 3-phos-phate dehydrogenase; HEK293, human embryonic kidney 293; LRP4, low-density lipoprotein receptor-related protein 4; UT, untransfected cells; Empty Vector, HEK293 cells transfected with plasmids that do not contain the LRP4 gene; bp, base pair; kDa, kilo Dalton.

3. Application of Cell-based Assay for LRP4 Antibody Associated with Myasthenia Gravis

To evaluate the status of autoantibody against LRP4 in patient's sera, immunocytochemistry was proceeded as cell-based assay. Nuclei were stained with DAPI and overexpressed LRP4 protein was confirmed with GFP. Each patient's serum stained with anti-human IgG antibody conjugated with Alexa Fluor 594. For the CBA scoring system, percentage of co-localized cells of green and red fluorescence along the cell membrane were determined (Fig. 3). Sera of which presence or absence of LRP4-ab was confirmed were applied to the CBA scoring system (Fig. 3A and 3B). Stain with LRP4 antibody negative serum showed no red fluorescence labelling on the LRP4 transfected cells (Fig. 3A). Therefore, it was scored as 0. Stain with LRP4 antibody positive serum demonstrated red fluorescence labelling co-localized with green fluorescence which indicated the overexpression of GFP tagged LRP4 on cell membrane (Fig. 3B). Approximately 60% cells with perfect co-localization were shown, so the score was 3. A total of 251 patients' sera with unknown antibody status were applied to the CBA for LRP4-Ab and scored according to the visual scoring system. Among the serum samples, two patients' sera were scored as one (Fig. 3C and 3D). The visual score was 0.5 for 78 serum samples and 0 for 171 serum samples, respectively.

The visual score was 0.5 for 78 serum samples. Among them, 32 from AChR Ab positive, 7 from MuSK Ab positive, 16 from dSN-MG, 6 from CIDP, 3 from LEMS, 5 from polyneuropathy, 2 from ALS patients, and 7 from healthy controls. The visual score was 0 for 171 serum samples. Of the 171 samples, 102 from AChR Ab positive, 8 from MuSK Ab positive, 34 from dSN-MG, 12 from CIDP, 1 from ISSAC syndrome, 5 from LEMS, 2 from polyneuropathy, 1 from ALS, 1 from sensory neuronopathy patient, and 5 from healthy controls.

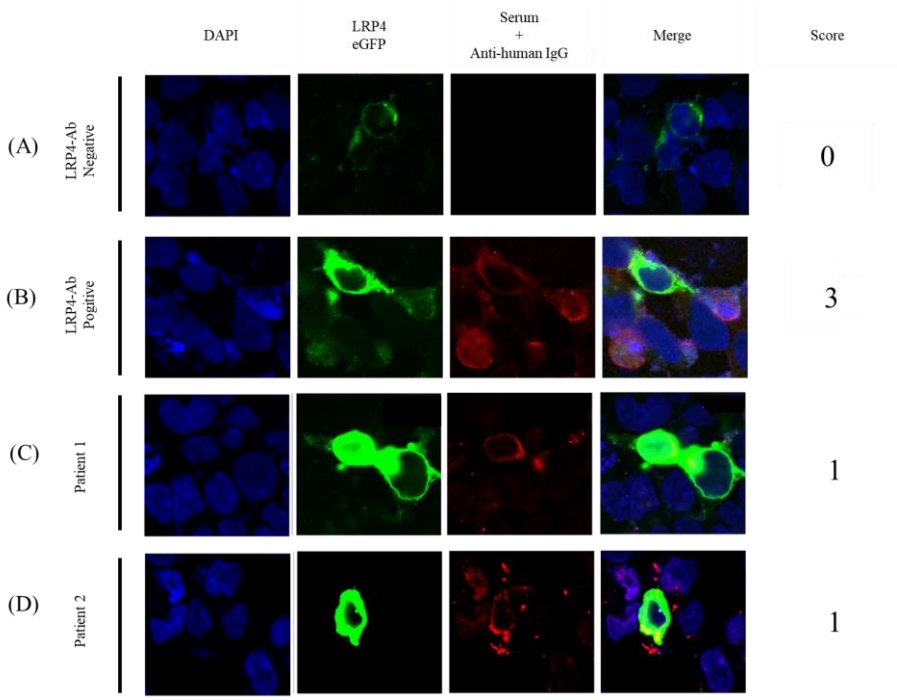

Figure 3. Cell-based assay to detect LRP4-Ab. (A and B) LRP4-transfected HEK293 cells were incubated with serum samples with known LRP4-Ab status and secondary anti-human IgG antibody conjugated with Alexa Fluor 594. (A) When serum sample from patient without LRP4-Ab was applied, red fluorescence was not observed. (B) When serum sample from patient with LRP4-Ab was applied to the LRP4-transfected cells, red fluorescence was observed along the cell membrane, which had green fluorescence. (C and D) Two serum samples reacted with LRP4-transfected HEK293 cells, indicated by red fluorescence co-localized green fluorescence**.** Ab, antibody; HEK293, human embryonic kidney 293; LRP4, low-density lipoprotein receptor-related protein 4; DAPI, 4′,6-diamidino-2-phenylindole; GFP, Green fluorescent protein; IgG, Immunoglobulin G.

A. Patient 1

A 24-year-old woman presented with eyelid ptosis and diplopia for one year. Her ocular symptoms showed diurnal variation and fatiguing nature. Neurologic examination demonstrated the weakness of eye closure and fatigable eyelid ptosis. Without extraocular muscles and orbicularis oculi, motor power was normal. Repetitive nerve stimulation of right abductor digiti minimi, flexor carpi ulnaris, orbicularis oculi, nasalis, trapezius muscles was normal. Chest computed tomography (CT) study revealed suspected soft tissue attenuation in the anterior mediastinum, suggestive of thymic hyperplasia. Her ocular symptoms improved after treatment with pyridostigmine bromide. AChR-Ab and MuSK-Ab were negative. Her symptoms were responsive to the treatment with pyridostigmine bromide and prednisolone.

B. Patient 2

A 47-year-old man presented with eyelid ptosis and diplopia for 7 months. His ocular symptoms showed diurnal variation and fatiguing nature. Neurologic examination demonstrated the weakness of eye closure and fatigable eyelid ptosis. Without extraocular muscles and orbicularis oculi, motor power was normal. Repetitive nerve stimulation of left abductor digiti minimi, flexor carpi ulnaris, orbicularis oculi, nasalis, trapezius muscles was normal. His ocular symptoms improved after neostigmine injection significantly. Chest computed tomography (CT) study revealed no anterior mediastinal mass. AChR-Ab and MuSK-Ab were negative. His symptoms resolved after treatment with pyridostigmine bromide and prednisolone.

IV. DISCUSSION

The purpose of this study was development of CBA to detecting LRP4 antibody. LRP4 plasmid vectors was transfected to HEK293 cells for CBA. The expression of LRP4 mRNA and protein in the transfected HEK293 cells was confirmed by RT-PCR and western blotting, retrospectively. Immunocytochemical staining with anti-human LRP4 antibody showed the expression of LRP4 on the cell membrane and the binding of LRP4 and anti-human LRP4 antibody. Using this LRP4-transfected HEK293 cells, CBA for detection of LRP4-Ab in the sera from 201 MG patients including 52 dSN-MG patients found two MG patients who had LRP4-Ab.

Diagnostic CBAs involve transfection of cells, usually HEK cells, with the cDNA encoding the protein interested which is commonly membrane proteins. The transfection leads the expression of the protein on the membrane of cells. Then the protein and antibody binding can be evaluated with immunocytochemistry and scored using visual scoring system. The CBA has some advantages. Compared to other antibody assays such as radioimmunoprecipitation assay (RIA) and enzyme-linked immunoassay (ELISA), CBAs provide a more physiologic environment that permits expression of proteins in native conformation and post-translational processing. In addition, because the protein is expressed on intact live cell membrane, the antibodies to intracellular epitopes, which are usually non-pathogenic, cannot bind to the protein and is prevented from being detected. For these reasons, CBAs are more sensitive and specific tests for detection of pathogenic antibodies, compared to the RIA and ELISA, which use recombinant proteins in solution or solid surface (Rodriguez Cruz et al. 2015b).

To identify new antigens associated with MG, the development CBAs for the proteins of NMJ was prompted. The CBA for LRP4-Ab were

developed recently and involves the transfection of LRP4 to express LRP4 on the cell membrane (Higuchi et al. 2011, Pevzner et al. 2012, Zisimopoulou et al. 2014). The previous studies of CBAs for LRP4-Ab have reported the frequencies of 50% (Pevzner et al. 2012), 19% (Zisimopoulou et al. 2014), 15% (Marino et al. 2015), 8% (Cossins et al. 2012), and 4% (Li et al. 2017) of LRP4-Ab positive MG patients among the dSN-MG patients. Two studies using CBA failed to identify LRP4-Ab positive MG patients (Gallardo et al. 2014, Rodriguez Cruz et al. 2015a). There were studies using ELISA or luciferase immunoprecipitation. Two studies using ELISA found a prevalence of 9% and 15% (Rivner et al. 2020, Zhang et al. 2012). One study using luciferase immunoprecipitation found a prevalence of 3% LRP4-Ab positive among 300 patients with AChR-Ab negative MG (Higuchi et al. 2011). According to the previous reports, the range of the frequency of LRP4-Ab in double seronegative MG was wide and CBAs seem to have a higher sensitivity for the detection of LRP4-Ab than ELISA or luciferase immunoprecipitation. In the present study, the frequency of LRP4-Ab in double seronegative MG was 3.9%, which was relatively low compared to the previous CBA studies. The subjects were from Europe or North America in the most LRP4-Ab studies in MG, whereas, in the two studies from Japan and China, the frequency was 3% and 4% respectively, which is similar to that of the present study (Higuchi et al. 2011, Li et al. 2017). So, the low LRP4-Ab positive MG of the present study might be the ethnic characteristics of MG patients. In addition, the proportion of ocular and generalized MG patients among the dSN-MG patients may have affected the frequency of LRP4-Ab positive. In the present study, 21 patients had only ocular symptoms among the 53 dSN-MG. However, most previous studies have not clearly described this ratio. So, it was difficult to compare the proportion of ocular MG patients among the studies and to analyze the

association between the proportion of ocular MG patients and the frequency of LRP4-Ab positive. Future studies should clearly describe the clinical characteristics of the subject.

Based on the previous studies for LRP4-Ab in MG, the onset of age is earlier, and female is more common in LRP4-Ab positive than LRP4-Ab negative MG patients (Bacchi, Kramer, Chalk 2018). LRP4-Ab positive MG patients tend to have mild symptoms in severity and often isolated ocular weakness, i.e., ocular myasthenia (Bacchi, Kramer, Chalk 2018). In addition, LRP4-Ab positive MG responds well to the treatment with pyridostigmine bromide and prednisolone. In the present study, the two patients with LRP4-Ab had also only mild ocular symptoms and were responsive to the treatment with pyridostigmine bromide and prednisolone. However, there were a few of studies that reported different clinical features. Higuchi et al and Rivner et al reported that moderate to severe weakness and generalized disease pattern is more common that mild weakness and isolated ocular weakness (Higuchi et al. 2011, Rivner et al. 2020). These two studies used ELISA to detect LRP4-Ab. So far, no studies have been conducted comparing the CBA and ELISA for detection of LRP4-Ab in MG patients. It is necessary to evaluate whether these differences in clinical features are due to the LRP4-Ab assay method.

LRP4-Ab have been also detected in neurological disorders other than MG, including amyotrophic lateral sclerosis (ALS) and neuromyelitis optica. In the previous studies for LRP4-Ab in ALS, the frequency of LRP4-Ab was 23% (Tzartos et al. 2014), 10% (Rivner et al. 2017), and 5% (Lei et al. 2019). Two patients with neuromyelitis optica were positive for LRP4-Ab (Zhang et al. 2012). The present study included CIDP, LEMS, polyneuropathy, ALS, Issac syndrome, and sensory neuronopathy as other neurological disease. LRP4-Ab was not detected in all the other neurological disorder. However,

the number of patients were small. In the case of ALS, this study included only three patients. Studies on many ALS patients is being planned.

This study has several limitations. First, there are no gold standard test for LRP4-Ab to compare the performance of the present CBA. The possibility of false positive results cannot be ruled out. However, the CBA results using the sera with known LRP4-Ab status were consistent with the results of CBA performed in the verified laboratory. Further study using different methods for detection of LRP4-Ab is necessary. In addition, to reduce the possibility of false positive results, we are planning to modify the CBA technique. In the present study, secondary antibody for whole IgG (H + L) was used for the CBA. Because all immunoglobulin classes shared light chains and whole IgG secondary antibody recognizes not only heavy chains but also light chains, the use of whole IgG secondary antibody may result in cross-reactivity to other immunoglobulins. On the contrary, 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. Therefore, replacement of whole IgG secondary antibody with Fc-specific secondary antibody may enhance signal-to-noise ratio in CBA. Second, the efficiency of LRP4 transfection to HEK293 cells was not high and only a few cells were transfected. For more stable and accurate testing, additional research is needed to find techniques to increase the transfection efficiency. We are planning studies using co-transfection of LRP4 and its chaperone protein, low density lipoprotein receptor-related protein-associated protein 1, in order to enhance cell membrane expression (Rodriguez Cruz et al. 2015a) or using transfection of LRP4 without GFP instead of GFP tagged LRP4 in order to reduce the size of plasmid.

V. CONCLUSION

CBA for detection of LRP4-Ab associated with myasthenia gravis was developed and validated. LRP4-Ab could be found by applying sera from MG patients 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.

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ABSTRACT (in Korean)

중증 근무력증 관련 항LRP4 항체에 대한 세포 기반 분석의 개발 및 적용

< 지도교수 신 하 영 >

연세대학교 대학원 생체공학협동과정

김 민 주

배경: 중증근무력증은 골격근의 근력약화를 특징으로 하는 신경근접합부의 자가항체 매개 자가면역질환이다. 중증근무력증 환자의 약 90%는 아세틸콜린수용체(AChR) 또는 근육-특이-키나아제(MuSK)에 대한 자가항체를 지니고 있다. 아세틸콜린수용체항체(AChR-Ab) 또는 MuSK 항체(MuSK-Ab)가 검출되지 않는 MG 환자들에서 저밀도지단백수용체관련-단백질 4(LRP4)에 대한 자가항체(LRP4-Ab)가 최근에 확인되었다. 이 연구의 목적은 중증근무력증과 연관되어 있는 LRP4-Ab 를 검출하기 위한 세포기반분석법(CBA)을 개발하고 이

세포기반분석법에 중증근무력증 환자의 샘플들을 적용하여 LRP4-Ab 를 분석하는 것이다.

방법: CBA 의 개발을 위해 LRP4 cDNA 가 삽입된 pCMV6-AV-GFP 벡터를 이용하였다. 클로닝 된 LRP4 플라스미드를 인간배아신장-293(HEK293)세포에 형질감염(transfection)을 시켰고, LRP4 mRNA 와 단백질의 발현을 확인하고자 역전사중합효소연쇄반응(RT-PCR)과 웨스턴블랏을 이용하였다. 형질감염시킨 HEK293 세포를 Rabbit anti-human LRP4 항체 및 Alexa Fluor 594 와 접합된 Goat anti- rabbit 와 함께 반응을 시켰다. 면역형광염색은 형광현미경을 사용하여 평가하였다. CBA 확립 후, MG 환자 201 명, MG 이외의 다른 신경근질환 환자 38 명, 그리고 건강한 대조군 12 명으로부터 얻은 혈청 샘플을 LRP4-Ab 에 대한 CBA 에 적용하였다. LRP4-Ab 의 존재는 형광 강도 및 형광 현미경에서 이들의 co-localization 을 기반으로 결정하였다.

결과: LRP4 가 형질주입된 HEK293 세포에서 LRP4 mRNA 와 단백질의 발현을 각각 RT-PCR 과 웨스턴블랏으로 확인하였다. 형광현미경에서 LRP4 가 형질감염된 세포의 세포 표면을 따라 녹색 형광이 관찰되었고, 세포막에서 LRP 의 발현을 확인하였다. 항-LRP4 항체를 첨가한 후, LRP4 형질주입된 HEK293 세포막에서 녹색 형광과 적색 형광이 확인되으며, 이 두 형광이 발현된 위치는 서로 일치하였다. 이렇게 개발된 세포기반분석법을 이용하여 총 201 명의 MG 환자 중 2 명(1%)에서 LRP4-Ab 가 검출되었다. 이들 2 명의 MG 환자는 모두 안구 증상만 있었고, AChR-Ab 는 음성이었으며 아세틸콜린에스트라제 억제제와 프레드니솔론 치료에 반응을 보였다.

결론: 이 연구를 통하여 MG 와 관련된 LRP4-Ab 검출을 위한 CBA 가 개발하였고, CBA 를 이용하여 MG 환자의 혈청에서 LRP4-Abs 를 확인하였다. 이 연구를 통하여 개발된 CBA 를 이용하여 MG 환자들에서 LRP4-Ab 를 검출할 수 있고 따라서 희귀질환인 MG 의 진단에 도움이 될 것으로 기대된다.

핵심 되는 말: 중증근무력증, 저밀도지단백수용체관련-단백질4, 세포기반분석법, 자가항체 매개 자가면역질환

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