Impaired Synaptic Development, Maintenance, and Neuromuscular Transmission in LRP4-Related Myasthenia

Duygu Selcen, MD; Bisei Ohkawara, PhD; Xin-Ming Shen, PhD; Kathleen McEvoy, MD; Kinji Ohno, MD, PhD; Andrew G. Engel, MD

IMPORTANCE Congenital myasthenic syndromes (CMS) are heterogeneous disorders. Defining the phenotypic features, genetic basis, and pathomechanisms of a CMS is relevant to prognosis, genetic counseling, and therapy.

OBJECTIVES To characterize clinical, structural, electrophysiologic, and genetic features of a CMS and to search for optimal therapy.

DESIGN, SETTINGS, AND PARTICIPANTS Two sisters with CMS affecting the limb-girdle muscles were investigated between 2012 and 2014 at an academic medical center by clinical observation, in vitro analysis of neuromuscular transmission, cytochemical and electron microscopy studies of the neuromuscular junction, exome sequencing, expression studies in HEK293 and COS7 cells, and for response to therapy, and they were compared with 15 historical control participants.

MAIN OUTCOMES AND MEASURES We identified the disease gene and mutation, confirmed pathogenicity of the mutation by expression studies, and instituted optimal pharmacotherapy.

RESULTS Quantitative analysis of single EP regions was done for all 15 control participants and microelectrode studies of neuromuscular transmission and α-bgt binding sites per EP was conducted for 13 control participants. Examination of the older sister’s intercostal muscle end plates (EPs) showed them to be abnormally small, with attenuated reactivities for the acetylcholine receptor and acetylcholinesterase. Most EPs had poorly differentiated or degenerate junctional folds, and some appeared denuded of nerve terminals. The amplitude of the EP potential (EPP), the miniature EPP, and the quanta l content of the EPP were all markedly reduced. Exome sequencing identified a novel homozygous p.Glu1233Ala mutation in low-density lipoprotein receptor-related protein 4 (LRP4), a coreceptor for agrin to activate muscle-specific tyrosine kinase (MuSK), which is required for EP development and maintenance. Expression studies indicate that the mutation compromises the ability of LRP4 to bind to, phosphorylate, and activate MuSK. Treatment with albuterol sulfate improved the patient’s symptoms. A previously identified patient harboring 2 heterozygous mutations in LRP4 had structurally abnormal intercostal EPs but no identifiable defect of neuromuscular transmission at these EPs.

CONCLUSIONS AND RELEVANCE We identified a second CMS kinship harboring mutations in LRP4, identified the mechanisms that impair neuromuscular transmission, and mitigated the disease by appropriate therapy.

Published online June 8, 2015.

Copyright 2015 American Medical Association. All rights reserved.
The development and maintenance of the neuromuscular junction depends crucially on the agrin-MuSK-LRP4 signaling system. Low-density lipoprotein receptor-related protein 4 (LRP4) and muscle-specific tyrosine kinase (MuSK) are anchored in the postsynaptic membrane. Agrin, secreted into the synaptic space by the nerve terminal, binds to multiple sites on the extracellular domain of LRP4, which then binds to the extracellular domain of MuSK on the postsynaptic membrane. This results in phosphorylation and activation of MuSK and clustering of MuSK and LRP4.1-2 Activated MuSK, in concert with DOK7 and other postsynaptic proteins, acts on rapsyn to concentrate the acetylcholine receptor (AChR) in the postsynaptic membrane and promotes postsynaptic gene expression and differentiation. Clustered LRP4 also enhances presynaptic differentiation.3

Several reports have described congenital myasthenic syndromes (CMSs) caused by mutations in agrin4-6 and MuSK,7-11 but only 1 report has described mutations in LRP4.12 This was the case of a 14-year-old girl with moderately severe fatigable limb-girdle weakness, dysplastic synaptic contacts, borderline end-plate (EP) AChR deficiency, smaller-than-normal individual EP regions, but no demonstrable defect of neuromuscular transmission at intercostal muscle EPs. The patient’s weakness continued to progress, and by 24 years of age, she was barely able to walk. Herein we describe 2 young adult sisters with LRP4-related myasthenia caused by a novel homozygous LRP4 mutation. Intercostal muscle studies of the older sister reveal structurally and functionally abnormal EPs and EP AChR deficiency. Expression studies indicate that the mutant protein hinders LRP4 from binding to, activating, and phosphorylating MuSK.

To determine whether an identified nucleotide variant caused abnormal splicing, we isolated cDNA from the muscle specimens obtained from control participants and the 2 patients. To detect any alternative transcript, we amplified the cDNA from exons 25 to 29 and exons 26 to 28 with primers designed for the cDNA of the 2 patients and 2 control participants.

**Structural Studies**

Intercostal and serratus anterior muscle specimens were obtained from the older sister and from control participants without muscle disease undergoing thoracic surgery. Cryosections were used to colocalize the AChR and acetylcholinesterase as previously described.13 End plates were localized for electron microscopy14 and quantitatively analyzed15 by established methods. Peroxidase-labeled α-bungarotoxin was used for the ultrastructural localization of AChR.16 The number of AChRs per EP was measured with iodine 125-labeled α-bungarotoxin.17

**In Vitro Electrophysiology Studies**

Quantitative analysis of single EP regions was performed for all 15 control participants and microelectrode studies of neuromuscular transmission and α-bgt binding sites per EP was conducted for 13 control participants. Intracellular microelectrode studies were performed on an intercostal muscle specimen obtained from the 34-year-old sister (patient 1). The amplitude of the miniature EP potential (MEPP) and the quantal content of the EP (m) were determined as previously described.18-20

**Plasmids**

We used the following previously constructed plasmids: (1) full-length human LRP4 cDNA for the luciferase assay and cell surface binding assays; (2) mouse Musk cDNA for luciferase assay; (3) the extracellular domain of mouse Musk cDNA and a fraction (amino acids 1141-1937) of rat Agrn cDNA, both of which were fused to an myc-tag and alkaline phosphatase (MuSKect-mycAP and agrin-mycAP) for cell surface binding assay; and (4) human MusK cDNA with a flag-tag at the N-terminal end for coimmunoprecipitation assay. Mutant LRP4 plasmid carrying p.Glu1233Ala was generated by the QuikChange Site-Directed Mutagenesis kit (Stratagene).12 The ATF2-luciferase (ATF2-luc) reporter12 and the phRL-TK Renilla luciferase vector (Promega) were used for the luciferase reporter assay.

**Cell Cultures**

HEK293 and COS7 cells were cultured in the Dulbecco Modified Eagle Medium supplemented with 10% fetal calf serum, and transfected with FuGENE 6 transfection reagent (Roche). The agrin-mycAP and MuSKect-mycAP proteins were produced as previously described.12 Recombinant rat C-terminal agrin (10 ng/mL; R&D Systems) was used for agrin treatment, except for the cell-binding assays.

** Luciferase Assays**

We used an ATF2-luc reporter to monitor MuSK activation. The basis for this approach is that agrin induces JNK activation in myotubes21 and that a previous report22 has demonstrated in-
teraction between JNK and ATF2. This suggested that report-ers regulated by JNK might reflect MuSK activation. We there-fore tested several JNK reporters and found that ATF2-luc reporter responded to MuSK, LRP4, and agrin in a dose-dep-endent manner. The HEK293 cells were transfected with ATF2-luc and pRL-TK along with the MUSK cDNA and the LRP4 cDNA. Cells were cultured for 24 hours in a 96-well plate with or without 10 ng/mL of agrin in the medium. Cells were lysed with the Passive Lysis Buffer (Promega) and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicate.

Western Blotting
HEK293 cells transfected with MuSK and LRP4 plasmids were cultured for 24 hours in the presence of 10 ng/mL of agrin, as previously described. The primary antibodies were mouse monoclonal anti-FLAG M2 (Sigma-Aldrich; F3165, dilution 1:4000), anti-β-actin (Santa Cruz Biotechnology; sc-47778, dilution 1:200), and the goat polyclonal anti-LRP4 (Abcam; ab85697, dilution 1:1000). The secondary antibodies were goat antimouse IgG (GE Healthcare; NA931V, dilution 1:6000) and mouse antigoat IgG (Santa Cruz Biotechnology; sc-2345, dilution 1:6000) conjugated to horseradish peroxidase.

Biotinylation Assay
HEK293 cells transfected with plasmids harboring wild-type LRP4 or its Glu233Ala mutant were cultured for 48 hours. Cell surface proteins were isolated by biotinylating the cell surface proteins and precipitating the bound proteins with streptavidin beads, as previously described.

Results

Clinical Data
Two young adult sisters with CMS (patient 1 in her mid-30s and patient 2 in her early 20s) who were born to nonconsanguin-eous parents, were investigated. Their parents and other sib-lings are unaffected.

Patient 1 was born after normal gestation and delivery. months. Developmental milestones (sitting and walking) were slightly delayed; after beginning to walk, she fell frequently. As a young child, she had mild difficulty chewing and swallowing. She never climbed steps or kept up with her peers in physical activities. Her weakness worsened around her menses. In her teens, she could only walk a short distance with support and became dependent on a wheelchair. On initial examination, she could barely rise from the sitting position without support, and her gait was waddling, hyperlordotic, and intoeing. Her weakness was confined to the axial and limb muscles with selective severe involvement of the dorsal forearm muscles. Her tendon reflexes were hypoactive. Her vital capacity was reduced to 49%, and the maximal inspiratory and expiratory pressures were reduced to 43% and 22% of normal, respectively. Repetitive nerve stimulation at 2 Hz revealed a decremental response of 37% in the trapezius and of 14% in facial muscles of the fourth compared with the first evoked compound muscle action potential (CMAP) (normal, <10%). Repetitive CMAPs, typical of the slow-channel myasthenic syndrome or EP acetylcholinesterase deficiency, were absent. Brief strenuous exercise did not appreciably improve the decremental response or potentiate the first evoked CMAP as in the CMS-caused synaptotagmin-2 deficiency, in some patients harboring mutations in agrin, or in the Lambert-Eaton syndrome. Needle electromyographic studies of multiple muscles revealed an increased proportion of short-duration, polyphasic motor unit potentials without spontaneous electrical activity, a common finding in myas-thenic disorders owing to a variable proportion of muscle fi-bers in motor units failing to generate an action potential.

After treatment with 4 mg of albuterol sulfate twice daily for 1 week, patient 1 rose up from the sitting position 10 times without support, and the electromyographic decrement in her trapezius muscle decreased to 25%. One week later, she climbed 10 steps, walked 450 m (500 yd), and was able to groom herself independently. Additional treatment with pyridostig-mine bromide or 3,4-diaminopyridine made her weaker and had to be discontinued.

Patient 2 appeared normal at birth and during infancy. She walked at a developmentally appropriate age but never learned to run or jump, found it difficult to climb steps, and could not keep up with her peers in physical activities. By late adoles-cence, her arm-elevation time was 22 seconds, and her gait was lordotic and waddling; she required assistance to rise from a sitting position and could climb only 20 steps before having to rest. There was mild weakness of the cervical and proximal arm muscles and mild to moderately severe weakness of the hip girdle muscles. Her tendon reflexes were hypoactive. Respiratory function tests showed that her vital capacity was reduced to 68% and that her maximal inspiratory and expiratory pressures were reduced to 39% and 47% of normal, respectively. After treatment with 4 mg of albuterol twice daily for 2 weeks, her arm and cervical muscles were of normal strength, her arm-elevation time was 60 seconds, and she could climb 44 steps before having to rest. Additional treatment with 60 mg of pyridostigmine twice daily over the next 2 years neither improved nor worsened her condition. A needle electromyographic examination suggested a mild proximal myopa-thy. After receiving albuterol for 2 years, repetitive stimulation of multiple muscles revealed no significant decrement of the evoked CMAP.

Structural Studies
Structural studies were performed in patient 1. Light micro-copy revealed markedly diminished EP size. In transverse frozen sections reacted for acetylcholinesterase, the median length of 34 EPs was 7.2 μm (Figure 1A, B, E, and F); the median length of 69 EPs of 3 control participants was 21.3 μm (P < .001, determined by the use of the rank sum test) (Figure 1C, D, G, and H). Paired fluorescence localization studies revealed reduced synaptic expression of acetylcholinesterase and of the AChR in the EPs of patient 1 (Figure 1E and F) compared with the EPs in the 3 control participants (Figure 1G and H).

Qualitative inspection of 15 EP regions revealed 7 postsyn-aptic regions unoccupied or partially occupied by the nerve

jama-neurology.com  JAMA Neurology August 2015 Volume 72, Number 8 891

Copyright 2015 American Medical Association. All rights reserved.
In transverse sections, the length of acetylcholinesterase (AChE)–reacted end-plate (EP) regions is markedly reduced in the EPs of patient 1 (A and B) compared with the EPs of control participants (C and D). Paired fluorescence studies reveal reduced expression of AChE (green) and acetylcholine receptors (AChRs) (red) in the EPs of patient 1 (E and F) compared with the EPs of control participants (G and H). Electron microscopy reveals simplified postsynaptic regions (I and J) and patchy expression of AChRs (black reaction product) at the EP (J). K, Electron microscopy also reveals a degenerating nerve terminal (NT) separated from a shallow postsynaptic region by a synaptic space containing coarse collagen fibrils; the region on the left shows a degenerate postsynaptic region displaying basal lamina remnants of preexisting folds (asterisk) and no NT. Electron microscopy also reveals a noninnervated postsynaptic region (L) and focal myofibrillar degeneration (M). Scale bars: A-D, 50 μm; E-H, 20 μm; I-L, 1 μm; M, 2 μm.
terminal (Figure 1K and L) and 2 regions displaying degenerating junctional folds (Figure 1K). In contrast, at 157 EP regions of control participants, only 15 postsynaptic regions were unoccupied by the nerve terminal (P < .001, determined by use of the rank sum test), and only 6 postsynaptic regions displayed degenerating folds (P < .03, determined by use of the z score). Most postsynaptic regions had poorly developed junctional folds (Figure 11 and J), and a single nerve terminal was degenerating (Figure 1K). The postsynaptic reaction for AChR, revealed by peroxidase-labeled α-bungarotoxin, was attenuated (Figure 1J). At the light microscopic level, the intercostal muscle specimen showed a single-target formation and small focal decreases of enzyme activity. Consistent with this, at the electron microscopic level, occasional muscle fibers displayed focal myofibrillar degeneration (Figure 1M) that is likely secondary to functional denervation. Morphometric analysis revealed that the size of the nerve terminal was not significantly different from normal but that the postsynaptic area was reduced by 50% (ie, 67% of normal) and that the postsynaptic areawas significantly different from normal but that the postsynaptic area was reduced by 50% (ie, 50% of normal).

Table 1. Quantitative Analysis of Single EP Regionsa

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control Participants (n = 15)</th>
<th>Older Sister</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postsynaptic membrane length, μm</td>
<td>54.9 (5.3)</td>
<td>18.2 (2.00)</td>
</tr>
<tr>
<td>Postsynaptic area, μm2</td>
<td>10.6 (0.79)</td>
<td>7.07 (1.02)</td>
</tr>
<tr>
<td>Postsynaptic membrane density, μm/μm2</td>
<td>5.83 (0.25)</td>
<td>2.88 (0.23)</td>
</tr>
</tbody>
</table>

* More than 1 region can occur at a single end plate (EP).

**Mutation Analysis**

Whole-exome sequencing in both patients (confirmed by Sanger sequencing) revealed a novel homozygous p.Glu1233Ala (c.3698A>C) mutation in exon 26 (Figure 2). The mutated residue is positioned at the edge of the third β-propeller domain of LRP4 and close to the conserved YWTD motif important for β-sheet formation. The unaffected brother and the mother are heterozygous for the mutation. Glu1233 is highly conserved across vertebrates, but not in insects, and is not reported in the Exome Variant Database (Exome Variant Server, National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project, Seattle, Washington [http://evs.gs.washington.edu/EVS/]; January 2015), and it is predicted to be disease causing by MutationTaster but benign by Polymorphism Phenotyping v2. Interestingly, mutations in the central cavity of the third β-propeller domain of LRP4 were previously reported to impair Wnt signaling and cause bone disease, including Cenani-Lenz syndactyly syndrome and sclerosteosis.2,26

Because the A>C variant is the penultimate nucleotide of exon 26 and because MutationTaster predicts it to alter splicing, we isolated cDNA from the patient’s intercostal and serratus anterior muscles and amplified the segment, including exon 26 by 2 different sets of primers. In both specimens, the mutant residue was homozygous, and there was no evidence for abnormal splicing.

Table 2. Microelectrode Studies of Neuromuscular Transmission and α-bgt Binding Sites per EP

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control Participants (n = 13)</th>
<th>Participants or EPs, No.</th>
<th>Older Sister</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP amplitude, mV</td>
<td>28.76 (1.98)</td>
<td>10°</td>
<td>4.66 (0.74)</td>
</tr>
<tr>
<td>MEPP amplitude, mV</td>
<td>1.00 (0.03)</td>
<td>165°</td>
<td>0.43 (0.078)</td>
</tr>
<tr>
<td>Quantal content of EPP at 1 Hz (m)</td>
<td>26.9 (1.0)</td>
<td>91</td>
<td>10.8 (2.36)</td>
</tr>
<tr>
<td>[125I]α-bgt binding sites per EP, No.</td>
<td>12.8 × 10^6 (0.8 × 10^6)</td>
<td>13</td>
<td>3.03 × 10^6</td>
</tr>
</tbody>
</table>

* More than 1 region can occur at a single end plate (EP).
Expression Studies

Expression studies show that Glu1233Ala inhibits the MuSK signaling pathway. During the formation of the neuromuscular junction, binding of agrin to LRP4 induces phosphorylation and activation of MuSK. Activated MuSK activates ATF2 downstream of JNK to induce clustering of AChRs. To investigate the effect of the Glu1233Ala mutation on this signaling pathway, we used a JNK-responsive ATF2-luc reporter that specifically monitors MuSK-dependent stimulation in transfected HEK293 cells. When LRP4 and MuSK are overexpressed, limited ATF2 activation occurs even in the absence of agrin. The addition of agrin to this system further enhances ATF2 activation by wild-type LRP4 but not to mutant LRP4 (Figure 3E). Thus, all 3 experiments indicate that Glu1233Ala in LRP4 inhibits agrin-mediated upregulation of MuSK signaling.

Discussion

The 2 sisters described in this report harbor a homozygous p.Glu1233Ala mutation in the third propeller domain of LRP4 that binds to and activates MuSK. An intercostal muscle biopsy of the older sister revealed very small EPs with poorly developed postsynaptic regions, and postsynaptic regions that were degenerating or denuded of their nerve terminal. The amplitude of the EPP was reduced to 16% of normal owing to the combined decrease of the quantal content of the EPP and of the MEPP amplitude (Table 2). The decreased amplitude of the MEPP can be attributed to decreased input resistance of the EP owing to simplification of the postsynaptic membrane, as well as the attenuated expression of AChRs on the remaining junctional folds. The decreased quantal content of the EPP is adequately explained by the small size of individual EPs, and hence of the total number of synaptic vesicles available for release by nerve impulse. Thus, the observed structural and electrophysiologic abnormalities are consistent with an abrogated role played by LRP4 in the development and maintenance of the neuromuscular junction.
Conclusions

The first reported patient with LRP4-related myasthenia harbored 2 heterozygous mutations in LRP4, p.Glu1233Ala and p.Arg1277His. At 14 years of age, her synaptic contacts were dysplastic, the individual EP regions were smaller than normal, and the EP AChR content slightly reduced, but, paradoxically, in vitro electrophysiology studies of intercostal muscle EPs revealed no abnormality. Interestingly, expression studies of both mutant proteins in the first patient and of the single mutant protein in the 2 sisters in this study revealed impaired LRP4 binding to, activating, and phosphorylating MuSK.

The reason for the much milder clinical, structural, and electrophysiological abnormalities observed in the first identified patient with LRP4-related myasthenia compared with the patients described in this report remains unknown. We initially attributed the milder findings in the first reported patient to...
relative sparing of the intercostal muscles. Alternatively, the structural and physiologic abnormalities at the EPs in LRP4-related myasthenia could worsen with age, consistent with the progressive clinical course in all 3 LRP4-deficient patients observed to date. The greater suppression of ATF2 activation by the Glu1233Ala mutation in LRP4 than by the previously reported Glu1233Lys mutation in LRP4 likely also contributes to the phenotypic differences between the first reported patient and the 2 patients described herein. Finally, single-nucleotide polymorphisms in modifier genes may also contribute to phenotypic differences. That both kinship harbor mutations at the edge of the third β-propeller domain and that 2 of the 3 identified mutations occur at codon 1233 suggest a hot spot for causing CMS.

REFERENCES