Short Report

Exome sequencing detection of two untranslated GFPT1 mutations in a family with limb-girdle myasthenia


The term ‘limb-girdle myasthenia’ (LGM) was first used to describe three siblings with proximal limb weakness without oculobulbar involvement, but with EMG decrement and responsiveness to anticholinesterase medication. We report here that exome sequencing in the proband of this family revealed several sequence variations in genes linked to proximal limb weakness. However, the only mutations that cosegregated with disease were an intronic IVS7-8A>G mutation and the previously reported 3′-UTR c.*22C>A mutation in GFPT1, a gene linked to LGM. A minigene assay showed that IVS7-8A>G activates an alternative splice acceptor that results in retention of the last seven nucleotides of intron 7 and a frameshift leading to a termination codon 13 nucleotides downstream from the new splice site. An anconeus muscle biopsy revealed mild reduction of the axon terminal size and postsynaptic fold simplification. The amplitudes of miniature endplate potentials and quantal release were also diminished. The DNA of the mildly affected father of the proband showed only the intronic mutation along with sequence variations in other genes potentially relevant to LGM. Thus, this study performed in the family originally described with LGM showed two GFPT1 untranslated mutations, which may cause disease by reducing GFPT1 expression and ultimately impairing protein glycosylation.

Conflict of interest

Nothing to declare.

The term ‘limb-girdle myasthenia’ (LGM) was introduced by McQuillen in 1966 to describe a family whose members displayed myopathic proximal weakness without oculobulbar involvement or abnormal muscle biopsy, but with neurophysiologic evidence of failure of neuromuscular transmission and sustained improvement by anti-cholinesterase therapy (1). Since this original report, many patients with similar clinical features as described by McQuillen have been observed, both in sporadic and familial settings (2–7). The first study aimed at the elucidation of the pathogenic basis of familial LGM was conducted by Slater et al. (8), who performed motor-point muscle biopsies in eight patients from seven kindreds affected with LGM. All muscles biopsies were thoroughly investigated with intracellular microelectrode studies.
of neuromuscular transmission as well as with light and electron microscopy of the neuromuscular junction (NMJ). The main electrophysiological and morphological findings in the patients studied by Slater et al., which included combined pre- and postsynaptic failure of neuromuscular transmission, small NMJs and underdeveloped postsynaptic folds, led the authors to conclude that the disorder may arise from defective formation or maintenance of the synaptic structure of the NMJ. Indeed, it was subsequently demonstrated that in most of the patients studied by Slater et al., the defect of neuromuscular transmission was caused by mutations in Dok7, an indispensable protein for the normal development and maintenance of the NMJ (9).

More recently, linkage analysis and whole-exome sequencing (WES) uncovered the association between familial LGM with tubular aggregates and mutations in GFPT1 (MIM 138292) and DPAGT1 (MIM 191350) (10, 11).

Despite these impressive advances recently made in understanding the pathogenesis of familial LGM, the molecular basis for the disorder in the family with LGM originally described by McQuillen has remained elusive.

To elucidate the underlying genetic defect in this first family described with LGM, we conducted WES using DNA extracted from peripheral blood. Mutations in several genes were found to be potentially relevant to the phenotype of these patients, but mutations in GFPT1 best account for the pattern of inheritance in the affected siblings and their descendents.

Materials and methods

Muscle biopsy

A biopsy of the anconeus muscle, intracellular recordings, electron microscopy studies and morphometric analysis of the NMJ was performed as previously described (12).

DNA amplification and sequencing

DNA extracted from peripheral blood was amplified using polymerase chain reaction (PCR) and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). This study was approved by the institutional review board of the University of California, Davis. All patients signed an informed consent form.

Whole-exome capture and massively parallel sequencing

A shotgun sequencing library was constructed with 3 μg genomic DNA and the library was hybridized to NimbleGen microarrays (NimbleGen Systems, Madison, WI) for exome capture. Exome sequencing was performed on the Illumina Hiseq platform with paired-end 76 base reads (Illumina, Inc. San Diego, CA). A full description of the whole-exome methods is included in Appendix S1.

Expression studies

Construction of minigene

To assess the effects of the intron 7 mutation on nascent pre-mRNA splicing, we constructed a GFPT1 minigene covering exons 7 and 8, and the intron 7. Human genomic DNA from the patient and a control were amplified with a forward primer (FP) located in exon 7 and a reverse primer (RP) in exon 8. The PCR product was analyzed on a 1% agarose gel and purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were sequenced and cloned into a pcDNA4/HisMax TOPO expression vector (Invitrogen, Carlsbad, CA).

Transfection of the GFPT1 minigene

Human embryonic kidney (HEK) 293 cells were transfected with the wild-type (WT) and mutant (MT) minigene constructs as previously described (13).

RT-PCR assay

Total RNA was extracted from HEK 293 transfected with the minigene constructs, using RNeasy Mini Kit (Qiagen) as previously described (13). The reverse transcriptase (RT)-PCR in muscle mRNA was performed using SYBR Green PCR Master Mix (Applied Biosystems) to increase the DNA intensity of the amplicon.

Statistical data

Unless otherwise stated, values are expressed as mean ± SEM with the sample number in parentheses and the significance determined using a two-tailed Student’s t-test.

Results

Clinical report

The patient, 68-year old female, started experiencing weakness at age 13. The initial manifestations of her disease involved difficulties with running, climbing stairs and keeping her arms up, more so at the end of the day. The worsening of her symptoms at the end of the day prompted a neostigmine test, which resulted in marked improvement of her strength. A neurologic examination at age 19 revealed a waddling gait, mild facial weakness but no ptosis and intact extraocular movements. Her muscles of the neck, proximal limbs, wrist extension and foot dorsiflexion were all weak and her deep tendon reflexes were diminished. Repetitive stimulation of the left ulnar nerve at 10 Hz revealed no decrement, but the needle electromyography (EMG) examination showed signs of myopathy. Her creatine phosphokinase in serum was mildly increased; however, a biopsy of the deltoid muscle was interpreted to be normal. The patient was subsequently studied with additional muscle biopsies, including a biopsy of the left anconeus described below. Throughout her life she responded well to anticholinesterase medication.
and 3,4-diaminopyridine. Her family history is relevant for the presence of similar symptoms and response to medication in her two younger brothers. The age of onset was similar to hers in her middle brother and late – about 30 – in her youngest brother. In addition, her asymptomatic father was found to have proximal and distal limb weakness on manual examination by McQuillen (1) and one of us (J. M. S), and myopathic features by EMG testing (1).

Muscle biopsy

To investigate the nature of the underlying failure of neuromuscular transmission, a biopsy of the left anconeus muscle was performed at age 45. The light microscopy of the anconeus muscle showed mild type I fiber predominance and type II fiber atrophy. The findings of the electron microscopy of the NMJ were highly variable. Some NMJ showed simplification of postsynaptic folds (Fig. 1b), while other junctions showed marked widening of the primary synaptic cleft and small nerve terminals partially encased by the Schwann cell (Fig. 1b,d). Yet, many NMJ, except for mild reduction of the size of the nerve terminal, were essentially normal (Fig. 1c). Minimally, non-specific degenerative changes such as myelin figures were rarely seen in subsarcolemmal regions (Fig. 1c), but no tubular aggregates were observed. The morphometric analysis revealed that in comparison with controls, the average width of the primary synaptic clefts was increased. The endplate index (EI = length of the postsynaptic membrane to the length of the presynaptic membrane) was also diminished if only secondary clefts in continuity with the primary cleft were considered. However, if secondary clefts not in continuity with the primary cleft were also included there was no difference between the patient and controls. Similarly the mean number of secondary clefts per micron of primary cleft was not reduced. The mean nerve terminal area was mildly diminished, although this was not statistically significant in comparison with controls (Table 1). The muscle specimen was also studied with in

![Fig. 1. Ultrastuctural analysis. (a) A normal neuromuscular junction (NMJ) from a control individual showing normal nerve terminal (asterisk), primary synaptic cleft (horizontal arrow) and secondary synaptic clefts (vertical arrow). (b) Abnormal NMJ from the patient showing marked simplification of postsynaptic folds (white arrow heads), a few secondary folds unconnected with the primary synaptic cleft (vertical arrow), marked increased width of the primary synaptic cleft (horizontal arrow) and a nerve terminal retracted from the endplate and partially surrounded by the Schwann cell (vertical arrow heads). (c) A relatively normal NMJ from the patient showing only mildly decreased size of the nerve terminal (asterisk) and normal postsynaptic folds (horizontal and vertical arrows). Note the presence of subsarcolemmal myelinoid bodies (arrow heads), which represents a non-specific finding. (d) An abnormal NMJ from the patient showing a small size nerve terminal (asterisk) encased by the Schwann cell (arrow heads), and increased diameter of the primary cleft (horizontal arrow) with normal postsynaptic folding and secondary synaptic clefts (vertical arrow). The calibration mark represents 1 μm in (a) and (d), 1.5 μm in (c), and 2 μm in (b).](image-url)
Table 1. Morphometric data

<table>
<thead>
<tr>
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<th>Patient</th>
<th>Controls</th>
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<tr>
<td>EI</td>
<td>5.32 ± 0.88a (n = 8)</td>
<td>11.71 ± 2.36 (n = 12)</td>
</tr>
<tr>
<td>Secondary clefts per primary cleft length</td>
<td>2.34 ± 0.28 (n = 8)</td>
<td>1.79 ± 0.14 (n = 12)</td>
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<tr>
<td>Nerve terminal area (μm²)</td>
<td>5.79 ± 1.66 (n = 8)</td>
<td>7.34 ± 0.93 (n = 12)</td>
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<tr>
<td>Cleft width (μm)</td>
<td>0.095 ± 0.006a (n = 8)</td>
<td>0.07 ± 0.004 (n = 12)</td>
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EI, endplate index (postsynaptic membrane length/presynaptic membrane length). 11.33 ± 1.58 (n = 8) considering secondary clefts not in continuity with the primary synaptic cleft.

*p < 0.05.

**vitro** microelectrode recordings which showed that the mean miniature endplate potential (MEPP) amplitude of 0.49 ± 0.04 mV (n = 25) in the patient was reduced in comparison with the mean MEPP amplitude of 1.02 ± 0.05 mV (n = 56) in controls (p < 0.001). MEPP frequencies in the patient [2.83 ± 0.5/min (n = 23)] were also reduced, but this was not significant in comparison with controls [3.97 ± 0.5/min (n = 50)]. The mean quantal content (QC) of endplate potentials (EPPs) recorded in the presence of 8 mM Mg²⁺ in our patient was 2.33 ± 0.76 (n = 9), which was decreased in comparison with the mean EPP QC of 5.07 ± 1.1 (n = 9) in controls (p < 0.05). However, due to the large variability of values of QC in the patient and controls, the difference was statistically significant only using a one-tail Student’s t-test.

Mutational analysis

We performed mutational analysis using conventional chain-terminator sequencing in: CHRNA1 (100690), CHRNB (100710), CHRND (100720), CHREN (100725), RAPSN (601592), MUSK (601296), DOK7 (610285), AGRN (103320) and GFPT1 (138292), and found no abnormalities in translated areas of these genes that could account for the syndrome. Next, we conducted exome sequencing as described above. Using the filtering procedure detailed in the Materials and methods section, we generated a list of 63 candidate variants. The list was then further restricted by only including single nucleotide variants (SNVs) and indels that met at least one of the following characteristics: missense, nonsense, stop-gain, stop-lost, frameshift, within 10bp of a splice site, or coding indels. The application of these filters resulted in a list of 26 candidate variants, which were individually examined as described in Table S1. Additional variants were removed if they were tagged by the GATK filter flag or were seen in ≥1% of 1200 exomes sequenced at the University of Washington. Inclusion in the Single Nucleotide Polymorphism Database (dbSNP) was not used as a filter for potential pathogenic variants.

Sequence variations reported by WES in GFPT1, DYSF (603099), BIN1 (601248), NEB (161650), COL6A3 (120250), SYNE1 (608441) and GNE (603824) were all confirmed by Sanger sequencing in the DNA of the proband. However, the reported variants in BIN1, NEB, COL6A3 and GNE were present in only one of the affected siblings and hence did not cosegregate with disease. In contrast, the SNV in DYSF [NM_003494.3:c.(4024 C>T, p. R1342W)], was present in both affected brothers, her father and two of her asymptomatic nephews. Since WES did not reveal additional DYSF variations of potential pathogenic nature and DYSF mutations are typically recessive, the single heterozygous DYSF variation found by WES was considered unlikely to be associated with disease in this family.

Finally, the combination of the two mutations in GFPT1 (IVS7-8A>G and c.*22C>A) reported by WES in the patient was also seen in the two siblings affected with myasthenic symptoms, but not in other members of the family, hence cosegregating with disease (Fig. 2). The c.*22C>A mutation was previously reported in patients with LGM, but IVS7-8A>G was novel and not listed in the EVS database from NHLBI http://evs.gs.washington.edu/EVS/. On the other hand, the 3'-UTR variant was seen in a heterozygous state in 21 of 4279 individuals and it was never found in a homozygous state in the European ancestry dataset from NHLBI.

Expression studies

To investigate the effects of IVS7-8A>G on gene splicing, we transfected HEK cells with a GFPT1 minigene spanning the whole exons 7 and 8, and intron 7 carrying the IVS7-8A>G mutation (Fig. 3a). RT-PCR of RNA isolated from HEK cells transfected with the WT construct using a FP starting at the first nucleotide of exon 7 and a RP starting at the last nucleotide of exon.

**Fig. 2.** Family tree and mutational analysis. The patient (arrow) and her brothers with myasthenic symptoms are depicted in black and the father with proximal and distal limb weakness and myopathic EMG with diagonal shading. The patient and her brothers, but not her asymptomatic daughters, are heterozygous for IVS7-8A>G. Her father and two of her asymptomatic nephews are also heterozygous for IVS7-8A>G. The patient, her brothers, her two daughters and one nephew, but not her father, are heterozygous for c.*22C>A. The graph shows that IVS7-8A>G and c.*22C>A are clearly located in different alleles and that only individuals possessing both mutations are affected with myasthenic symptoms.
Maselli et al.

Fig. 3. Results of a GFPT1 minigene transfection into HEK cells. (a) The GFPT1 minigene construct was obtained by polymerase chain reaction (PCR) amplification of genomic DNA from the patient and a control using a forward primer (FP) starting at the first nucleotide of exon 7 and a reverse primer (RP) starting at the last nucleotide of exon 8. The PCR product (not shown in the figure) comprised the 62 bp of exon 7, the 80 bp of exon 8 and the 1927 bp of the interspaced intron 7. The mutant allele of the patient differs from the wild-type allele by the presence of the IVIS-8A>G mutation (highlighted in purple). The canonical and the novel splice sites created by the mutation are bolded. (b) Complementary DNA (cDNA) from cells transfected with the constructs made with control DNA showed a single band of 142 bp. By contrast, cDNA from cells transfected with the minigene made with the mutant DNA from the patient showed an additional band. (c) Sequencing of the wild-type band revealed sequences of correctly spliced exons 7 and 8 without interspaced intronic sequences. (d) Sequencing of both mutant bands revealed a normally spliced allele along with an allele that retains the last seven nucleotides of intron 7.

8 showed a single 142 bp band (Fig. 3b). Sequencing of this band showed correctly spliced exons 7 and 8 (Fig. 3c). In contrast, RT-PCR of RNA from cells transfected with the MT minigene showed an extra band. Sequencing of both MT bands showed a correctly spliced sequence and a superimposed frameshift mutation resulting from the retention of the last seven nucleotides of intron 7 (Fig. 3d). The frameshift results in a stop codon 13 nucleotides downstream from the insertion. Additionally, the last 15 nucleotides of the alternative sequence were novel and did not match any sequences of exons or introns of GFPT1.

Discussion

In this study, the use of WES in the proband of the family first described with LGM showed two heteroallelic untranslated mutations in GFPT1, a gene recently linked to LGM associated with tubular aggregates (MIM 610542) (10).

From a clinical perspective, the proximal limb weakness without ocular or bulbar involvement, the EMG decrement and the good response to anticholinesterase medication in the three affected patients of this family are all consistent with LGM phenotype due to mutations in GFPT1. This phenotype is clearly different from that of DOK7 mutations, which often involves facial, bulbar and respiratory involvement; lack of response to anticholinesterase medication; and frequent improvement with ephedrine treatment (14). Although the muscle biopsy performed in the proband in our institution did not show tubular aggregates, it should be noted that not all patients with congenital myasthenic syndrome (CMS) due to GFPT1 mutations show tubular aggregates in the muscle biopsy (10, 14). Conversely, LGM with tubular aggregates is not always due to mutations GFPT1, because in some patients the condition results from mutations in DPAGT1 (11).

The use of WES in the proband of this family not only resulted in the identification of GFPT1 mutations previously overlooked by conventional gene sequencing but also allowed us to verify that no other mutations that could explain the disease were present in other genes with proven association with CMS and muscle weakness.

One of the mutations present in this family, the 3′-UTR mutation c.*22C>A has been identified in several patients with LGM and tubular aggregates and has also been found to associate with reduced amounts of GFPT1 levels in muscle biopsies of affected patients carrying this mutation (10, 14). Nevertheless, the precise mechanism by which c.*22C>A results in disease is uncertain. Conceivably c.*22C>A, as many other mutations located in the 3′-untranslated (UTR) region causing human disease, may affect regulatory motifs participating on gene translation (15, 16), but this mechanism has yet to be proven.

On the other hand, the expression studies presented here showed that by activating an alternative novel splicing site IVS7-8 A>G results in an anomalous splicing leading to retention of part of intron 7, a frameshift and an early stop codon that predicts truncation of the protein.

Thus, both mutations, c.*22C>A and IVS7-8 A>G appear to result in reduced levels of GFPT1 expression,
Exome sequencing detection of two untranslated GFPT1 mutations

albeit by seemingly different and independent mechanisms. The analysis of the patient's pedigree revealed that unaffected members were heterozygous for either one mutation and that the patient and her affected brothers were compound heterozygous for both mutations. There are several explanations for this observed segregation pattern. One possible explanation is that the disease is inherited in an autosomal recessive manner, the proband's father is a phenocopy, and his symptoms arise from other causes, perhaps exacerbated by being heterozygous for the splice site mutation. An alternative explanation is that the disease is inherited in an autosomal dominant manner, with partial penetrance. In this model, the proband's nephews carrying the IVS7-8 A→G mutation are presymptomatic.

It was recently proposed that, by disrupting acetylcholine receptor (AChR)-subunit glycosylation, mutations in GFPT1 and DAPtg1 result in inefficient export of AChR to the cell surface membrane causing reduced expression of endplate AChRs (11). This proposed pathogenetic mechanism provides a plausible explanation for the structural and physiologic signs of postsynaptic deficit that we found in the muscle biopsy of the proband.

Furthermore, since practically all proteins of the extracellular matrix are glycosylated (17) and all synaptic basal-lamina associated CMS so far described have severe structural and physiologic presynaptic abnormalities (18–20), altered protein glycosylation could also explain the presynaptic deficit that we found in our patient.

Finally, the molecular findings of this report emphasize the importance in genetic disorders of a careful analysis of untranslated areas of genes as these regions may possess, as in this family, important determinants of disease.

Supporting Information

The following Supporting information is available for this article:
Appendix S1. Supplementary exome methods.
Table S1. Single nucleotide variants.

Additional Supporting information may be found in the online version of this article.

Acknowledgements

We thank the patient and her relatives for their enthusiastic participation in this study and their encouragement of this research. This work was partially supported by grants from NIH (SR01NS049117-03), the Muscular Dystrophy Association and The Myasthenia Gravis Foundation of California to R.A.M. and the Muscular Dystrophy Association (MDA 4090 and MDA 186447) to M.A.H.

References