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Report

myotilin Mutation Found in Second Pedigree with LGMD1A

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Limb-girdle muscular dystrophy 1A (LGMD1A [MIM 159000]) is an autosomal dominant form of muscular dystrophy characterized by adult onset of proximal weakness progressing to distal muscle weakness. We have reported elsewhere a mutation in the *myotilin* gene in a large, North American family of German descent. Here, we report the mutation screening of an additional 86 families with a variety of neuromuscular pathologies. We have identified a new *myotilin* mutation in an Argentinian pedigree with LGMD1 that is predicted to result in the conversion of serine 55 to phenylalanine (S55F). This mutation has not been found in 392 control chromosomes and is located in the unique N-terminal domain of *myotilin*, only two residues from the T57I mutation reported elsewhere. Both T57I and S55F are located outside the α-actinin and γ-filamin binding sites within *myotilin*. The identification of two independent pedigrees with the same disease, each bearing a different mutation in the same gene, has long been the gold standard for establishing a causal relationship between defects in a gene and the resultant disease. As a description of the second known pedigree with LGMD1A, this finding constitutes that gold standard of proof that mutations in the *myotilin* gene cause LGMD1A.

The limb-girdle muscular dystrophies (LGMDs) include a clinically diverse group of dominant and recessive disorders characterized by proximal muscle weakness, elevated serum creatine kinase values, and absent or reduced deep-tendon reflexes. Among the autosomal recessive limb girdles, mutations in calpain 3 cause LGMD2A (Richard et al. 1995), mutations in dysferlin cause LGMD2B (Bashir et al. 1998), and mutations in telethonin cause LGMD2G (Moreira et al. 2000). LGMD2C through LGMD2F are all caused by defects in members of the dystrophin glycoprotein complex: γ-, α-, β-, and δ-sarcoglycan (Roberds et al. 1994; Bonnemann et al. 1995; Lim et al. 1995; Noguchi et al. 1995; Nigro et al. 1996). Mutations in TRIM32, a putative E3-ubiquitin-ligase gene, have been identified as the cause of LGMD2H (Frosk et al. 2002). Among the dominant forms, four have been mapped: LGMD1B to 1q11-21 (van der Kooi et al. 1997), LGMD1D to 6q23 (Messina et al. 1997), LGMD1E to 7q (Speer et al. 1999), and vocal cord and pharyngeal weakness with autosomal dominant distal myopathy to 5q (Feit et al. 1998). Caveolin 3 mutations have been described in LGMD1C (McNally et al. 1998; Minetti et al. 1998).

LGMD1A (MIM 159000) is characterized by onset of proximal muscle weakness at a mean age of 27 years, later progressing to include distal weakness. Approximately half of the affected individuals exhibit a distinctive nasal, dysarthric pattern of speech. Tightened heel cords and reduced knee and elbow deep-tendon reflexes are frequently seen. CK levels are elevated, ranging from 1.6-fold to 9-fold higher than the normal limit of 120 IU/l for males and 80 IU/l for females. Biopsy of affected individuals shows variations in fiber size, fiber splitting, and other hallmarks of degeneration; a large number of rimmed vacuoles; and patches of striking Z-line streaming, similar to that seen in nemaline myopathy (Hauser et al. 2000). Elsewhere, we have reported a mutation in the *myotilin* gene (*TTID*, *MYOT* [NCBI accession number NM_006790; MIM 604103]) in the only known LGMD1A pedigree, Duke family 39 (Hauser et al.

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2000). Myotilin protein is localized to the Z-line and binds to α-actinin (Salmikangas et al. 1999) and γ-filamin (van der Ven et al. 2000*b*). The threonine 57 to isoleucine (T57I) mutation in family 39 occurs in a region of unknown function, so the mechanism by which it causes disease is not apparent.

We report here the screening of 86 additional pedigrees to find additional *myotilin* mutations that might provide further insight into possible structure-function relationships in the myotilin protein. LGMDs are characterized by a large degree of variation in clinical presentation, between, and often within families. Individuals with novel *myotilin* mutations may well present with a significantly different set of symptoms from those previously seen, especially if the mutations are located in different protein domains. Therefore, mutation analysis was performed on at least one affected individual from each of 44 families with LGMD type 1 (autosomal dominant), 14 families with LGMD type 2 (autosomal recessive), 24 families with facioscapulohumeral muscular dystrophy, 2 families with scapuloperoneal muscular dystrophy, and 2 families with unclassified dominant myopathies. Informed consent was obtained from all human subjects.

Mutation analysis was performed on genomic DNA isolated from whole blood using the Puregene kit (Gentra Systems). The primers in table 1 were used to PCR amplify exons 2–9 and part of exon 10 (the full coding region) of the *myotilin* gene from genomic DNA in pools of five unrelated individuals. These PCR products were analyzed using the Transgenomic WAVE denaturing high-performance liquid chromatography system. Several different temperatures were tested for each PCR amplicon to optimize mutation detection sensitivity. All observed changes were confirmed by sequencing both DNA strands using the Beckman CEQ2000 capillary electrophoresis sequencer or the Beckman 3700 DNA sequencer using Big Dye chemistry.

A single Argentinian sample exhibited a C444→T missense mutation in exon 2 of the *myotilin* gene, predicted to result in a change of residue 55 from serine to phenylalanine (S55F). This change is found in a single allele, consistent with the observed pattern of autosomal dominant inheritance, and was not detected in 392 control chromosomes. DNA samples from eight additional members of this Argentinian pedigree (family 2654) were then screened, and the missense mutation was found to segregate with disease status. The FASST method (Vance and Ben Othmane 1998) was used to genotype family 2654 with two polymorphic microsatellite repeat markers (D5S479 and D5S178) flanking the *myotilin* gene (fig. 1). These markers establish a disease-associated haplotype entirely different from that seen in our original LGMD1A pedigree (Duke family 39).

The identification of two independent pedigrees with the same disease, each bearing a different mutation in the same gene, has long been the gold standard for establishing a causal relationship between defects in a gene and the resultant disease. As a description of the second known pedigree with LGMD1A, this finding constitutes such gold-standard proof that mutations in the *myotilin* gene cause LGMD1A. The clinical presentation of affected individuals in family 2654 is quite similar to that of the LGMD1A pedigree we reported elsewhere. They exhibit proximal leg and arm weakness by 42–58 years of age, which later progresses to include distal weakness. Serum CK levels are elevated 5-fold to 15-fold. Two of the four living affected individuals exhibit a distinctive dysarthric pattern of speech. All members of family 2654 have been examined by two of the authors (A.L.R. and G.Z.). The similarity in clinical presentation in the two families may be related to the proximity of the observed mutations, which are located 2 aa apart in the unique N-terminal domain of the protein, immediately adjacent to a 20-aa hydrophobic stretch.

There are striking similarities between LGMD1A and

Table 1

Primers Used to Amplify *Myotilin* Coding Sequence

Exon	Primer		Product Size (bp)
	Forward	Reverse	
2a	CAGATCTGAAAGATGTCAAATAAACAA	GTTGTAACCCTTTGGCCTGG	546
2b	CTCAACAAGGAAGAGCAGAC	TACTGCTATTGTAATCAGGC	481
2c	GCTCCAGATTGCAGCCTCCT	CCAGTACCCTGGTTCAGCAT	476
3	ATTTGCAAAATGAGGCCAAG	GGGCCCAAATATTCCTTCTT	373
4	TGTCTCAATAAATTCTCTAAAGCG	GTGGATGGAACTGACCGACT	273
5	CTGGGCTTCTTGCTAGAGTGGTAG	GATCCTGGCTTATTTGACC	462
6	CTCCTGCCTTAGCCTCCTGAG	GGAGGATGGCAGAGCCAGAATT	471
7	TCTGCCATCTCCTTGTGTTTT	TGAAGTCTGCTGGGCTTTTC	330
8	GGTATAACAAAATAGTACTGCATGTC	AACTGGATTCACCCAAATAAAC	380
9	TGGTCAGAGACATCCACTTCA	TTTTATACTCTGCTGGGATTTTCA	286
10a	CCAATTTGGTTAGAACAGGTTT	GTAGGCTTCACAAATCGGAG	420
10b	TACCAACATTGGAAAACAG	TCATAGGTTTTGCTGAGTGGAG	522

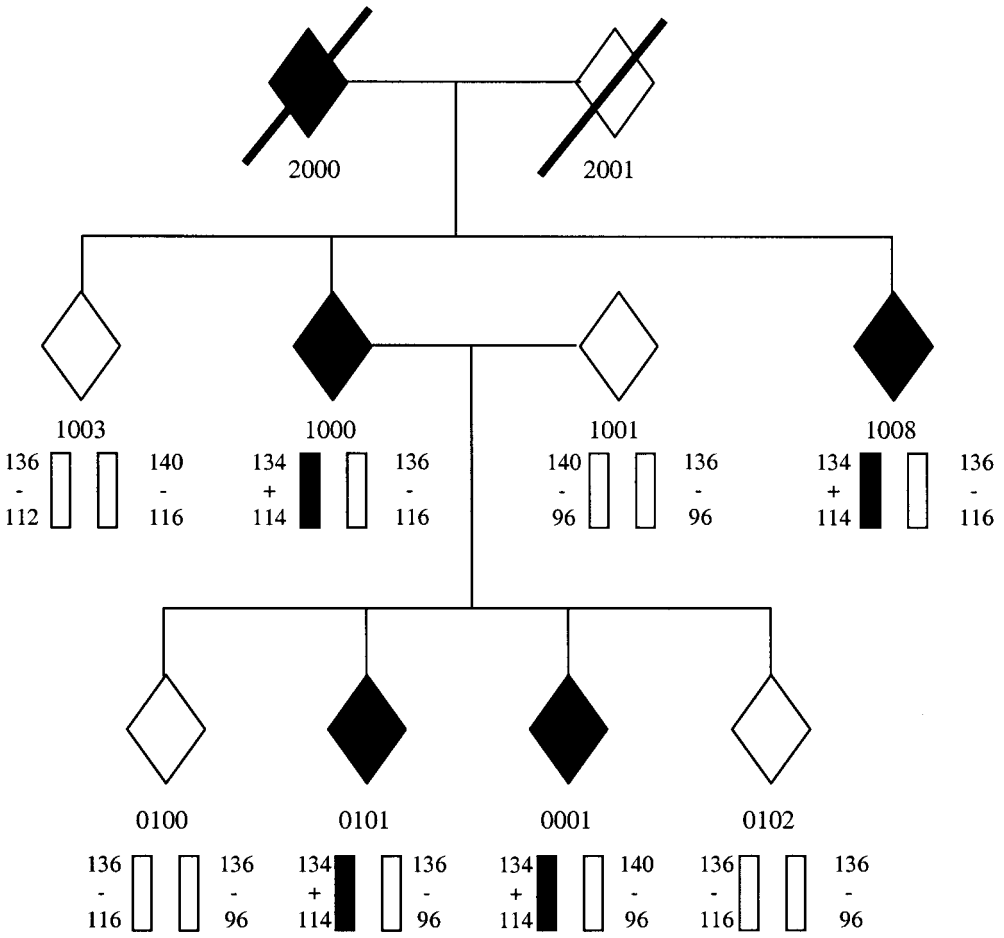

Figure 1 Segregation of 5q31 microsatellite markers and the C444T missense mutation in Family 2654. Affected individuals and the disease-bearing haplotype are indicated in solid black. From top to bottom, the alleles shown are for D5S479, the S55F mutation, and D5S178.

the nemaline myopathies, suggesting possible mechanisms by which *myotilin* mutations may give rise to disease. Missense mutations in α-tropomyosin (NEM1) give rise to nemaline myopathy with Z-line streaming similar to that seen in LGMD1A (Laing et al. 1995), and both myotilin protein and α-tropomyosin bind to α-actinin. One of the primary roles of α-actinin is to tether actin filaments to the Z-line, and missense mutations in α-actin also give rise to nemaline myopathy (Nowak et al. 1999). These observations suggest that the observed *myotilin* mutations might act by disrupting the tethering of actin filaments through their interactions with α-actinin. However, we were unable to detect any effect of the T57I mutation on α-actinin binding by means of a yeast two-hybrid assay. Further, this assay identified a minimum α-actinin binding domain extending from residues 79–150, well separated from the observed mutations (Hauser et al. 2000).

It has also been reported that myotilin protein binds to γ-filamin, a form of filamin specifically expressed in striated muscle, and does so through a region spanning its 78-aa muscle-specific domain (van der Ven et al. 2000*b*). Several filamin isoforms bind to α-actin and are thought to regulate its polymerization (Wang and Singer 1977). This network of actin filaments is then anchored to the plasma membrane by filamin's interactions with a number of other cytoskeletal proteins, including β1-integrin (Loo et al. 1998), β2-integrin (Sharma et al. 1995), and, in the case of γ-filamin, γ- and δ-sarcoglycan (Thompson et al. 2000). Further, γ-filamin colocalizes with α-actinin in the Z-disk in striated muscle and has been implicated in Z-disk assembly and myofibrillogenesis (van der Ven et al. 2000*a*). Through its binding interactions with both α-actinin and γ-filamin, myotilin protein may play a role in colocalizing α-actinin and γ-filamin to the Z-disk. This model is supported by the observation that transfection of differentiating C2C12 myoblasts with a truncated *myotilin* gene encoding just

the C-terminal Ig domains disrupts Z-disk formation, whereas introduction of the entire *myotilin* gene does not (van der Ven et al. 2000*b*). This would seem to indicate that severing the connection between the N- and C-terminal domains creates a dominant negative protein in much the same way that the isolated Z1 and Z2 domains of titin have been reported to disrupt sarcomere assembly through their interactions with telethonin (Peckham et al. 1997; Mues et al. 1998). It is unlikely that the reported T57I and S55F *myotilin* mutations directly disrupt γ-filamin binding: yeast two-hybrid experiments have demonstrated that the γ-filamin binding site is located within the C-terminal Ig domains of myotilin protein (residues 215–493), well removed from the T57I and S55F mutations (van der Ven et al. 2000*b*). Although it is conceivable that the yeast two-hybrid system is not sufficiently sensitive to detect the subtle effects on tertiary structure caused by the *myotilin* mutations and that these mutations actually do disrupt binding with either α-actinin or γ-filamin, a more likely explanation is that there is another, as-yet-undetermined binding partner for myotilin protein and that the observed mutations disrupt this interaction or disrupt myotilin's ability to colocalize this protein with γ-filamin.

The N-terminal region of myotilin protein that contains the T57I and S55F mutations is not homologous to any other protein. It includes a 23-residue hydrophobic domain and a region rich in serine (27/96 residues). Although the function of this domain is unknown, it is possible that the hydrophobic stretch mediates the localization of small amounts of myotilin protein to the sarcolemmal membrane. Both of the observed *myotilin* mutations would have the effect of elongating this hydrophobic stretch, possibly disturbing its interactions with the sarcolemmal membrane or with a novel protein-binding partner. A better understanding of the biological function of the N-terminus will be necessary to fully appreciate the effects of the two observed *myotilin* mutations. Further, mutations in a different portion of the protein might well result in a significantly different phenotype. For example, myotilin protein is expressed at relatively high levels in the heart, and, although there is no evidence of a cardiac defect in either known pedigree, novel mutations might give rise to a cardiac phenotype. We are currently testing this hypothesis by expressing synthetic *myotilin* mutations in transgenic mouse models. These experiments may suggest alternate groups of human patients who could be screened for defects in *myotilin*.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/ (for *myotilin, TTID*, or *MYOT* [accession number NM_006790])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for LGMD1A [MIM 159000])

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