A Congenital Muscular Dystrophy with Mitochondrial Structural Abnormalities Caused by Defective De Novo Phosphatidylcholine Biosynthesis

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Congenital muscular dystrophy is a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy. A number of genes harboring causative mutations have been identified, but several cases of congenital muscular dystrophy remain molecularly unresolved. We examined 15 individuals with a congenital muscular dystrophy characterized by early-onset muscle wasting, mental retardation, and peculiar enlarged mitochondria that are prevalent toward the periphery of the fibers but are sparse in the center on muscle biopsy, and we have identified homozygous or compound heterozygous mutations in the gene encoding choline kinase beta (CHKB). This is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. In muscle of three affected individuals with nonsense mutations, choline kinase activities were undetectable, and phosphatidylcholine levels were decreased. We identified the human disease caused by disruption of a phospholipid de novo biosynthetic pathway, demonstrating the pivotal role of phosphatidylcholine in muscle and brain.

A spontaneous mutant mouse with a neonatal-onset autosomal-recessive rostral-to-caudal muscular dystrophy (rmd mouse) due to a loss-of-function mutation in choline kinase beta (Chkb) was identified in 2006.1 Interestingly, rmd mice exhibit a unique mitochondrial morphology in muscle fibers, which show enlarged mitochondria at the periphery of the fiber but none at the center (Figure S1). These features are similar to those seen in a congenital muscular dystrophy (CMD) that we previously reported in four Japanese individuals.2 We therefore screened 15 genetically undiagnosed cases of CMD with fairly homogenous clinical features (Table 1) for mutations in choline kinase beta (CHKB); we included the four cases from our previous study in these 15 cases. Features included peculiar mitochondrial changes in muscle as well as motor delay followed by the appearance of severe mental retardation and microcephaly without structural brain abnormalities (Figure 1 and Table 1).

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethical Committee of the National Center of Neurology and Psychiatry. All mouse protocols were approved by the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry. For muscle pathology, samples of skeletal muscle were obtained from biceps brachii or quadriceps femoris in humans and from quadriceps femoris muscle in 8-week-old rmd mice. Muscles were frozen and sectioned at a thickness of 10 μm according to standard procedures, and a battery of routine histochemical stains, including hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), and Oil Red O, were analyzed. For electron microscopic analysis, muscles were fixed as previously described,3 and ultra-thin sections were observed at 120kV or 80kV. All affected individuals exhibited nonspecific dystrophic features (Figure 1A). However, in mGT, NADH-TR, SDH, and COX staining, prominent mitochondria at the periphery as well as central areas devoid of mitochondria were seen (Figures 1B and 1C). Oil Red O staining was unremarkable (data not shown). Electron microscopy confirmed enlarged mitochondria (Figure 1D).

We directly sequenced all exons and their flanking intronic regions in CHKB (MIM 612395, NM_005198.4, GenBank Gene ID 1120) in genomic DNA extracted from individuals’ peripheral lymphocytes. All 15 individuals in three different populations (Japanese, Turkish, and British) had homozygous or compound heterozygous mutations in...
Table 1. Summary of Clinical and Laboratory Features

<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Origin</th>
<th>Age at Last Follow-Up</th>
<th>Floppy at Birth</th>
<th>Walk Alone</th>
<th>Serum Creatine Kinase (IU/liter)</th>
<th>Head Circumference (percentile)</th>
<th>Mental Retardation</th>
<th>Seizure</th>
<th>Cardiomyopathy</th>
<th>Skin Change</th>
<th>Age at Muscle Biopsy</th>
<th>Necrotic Fiber</th>
<th>Regenerative Fiber</th>
<th>Endomyocardial Fibrosis</th>
<th>Mitochondrial Enlargement</th>
<th>Status</th>
<th>cDNA</th>
<th>Consequence</th>
<th>Exon</th>
<th>Literature ref. on phenotype</th>
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<tr>
<td>1 F</td>
<td>Japanese</td>
<td>died at 13 yr</td>
<td>+</td>
<td>2 yr 6 mo</td>
<td>370</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>7 yr 3 mo</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>homo</td>
<td>c.810T&gt;A</td>
<td>p.Tyr270X</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 M</td>
<td>Japanese</td>
<td>died at 23 yr</td>
<td>+</td>
<td>1 yr 9 mo</td>
<td>190-2676</td>
<td>25-50</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1 yr 2 mo</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>homo</td>
<td>c.810T&gt;A</td>
<td>p.Tyr270X</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 F</td>
<td>Japanese</td>
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<td>+</td>
<td>1 yr 6 mo</td>
<td>502</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>8 yr</td>
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<td>+</td>
<td>2 yr 6 mo</td>
<td>230</td>
<td>3-10</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4 yr 11 mo</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>het</td>
<td>c.458dup</td>
<td>p.Leu153PhefsX57</td>
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<td>2</td>
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<td></td>
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<td>5 M</td>
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<td>7 yr</td>
<td>-</td>
<td>2 yr 6 mo</td>
<td>843</td>
<td>&lt;3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>6 yr</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>homo</td>
<td>c.611_612insC</td>
<td>p.Thr205AsnfsX57</td>
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<tr>
<td>6* M</td>
<td>Turkish</td>
<td>died at 2 yr 6 mo</td>
<td>+</td>
<td>no</td>
<td>258</td>
<td>&lt;3</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>1 yr 3 mo</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.922C&gt;T</td>
<td>p.Gln308X</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7 F</td>
<td>Turkish</td>
<td>2 yr</td>
<td>-</td>
<td>no</td>
<td>368</td>
<td>3-10</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>9 mo</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.847G&gt;A</td>
<td>p.Glu283Lys</td>
<td>8</td>
<td></td>
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<tr>
<td>8 M</td>
<td>Turkish</td>
<td>13 yr</td>
<td>ND</td>
<td>2 yr</td>
<td>1122</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>12 yr 10 mo</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.1130G&gt;T</td>
<td>p.Arg377Leu</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 F</td>
<td>Turkish</td>
<td>17 yr</td>
<td>+</td>
<td>3 yr</td>
<td>2669</td>
<td>&lt;3</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>17 yr</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.554_562del</td>
<td>p.Pro185_Trp187del</td>
<td>4</td>
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<tr>
<td>10 F</td>
<td>Turkish</td>
<td>16 yr</td>
<td>+</td>
<td>3 yr</td>
<td>1103</td>
<td>&lt;3</td>
<td>+</td>
<td>-</td>
<td>2</td>
<td>+</td>
<td>3 yr</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.677+1G&gt;A</td>
<td>ND</td>
<td>5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>11 F</td>
<td>Turkish</td>
<td>3 yr 3 mo</td>
<td>+</td>
<td>no</td>
<td>497</td>
<td>10-25</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>3 yr</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.677+1G&gt;A</td>
<td>ND</td>
<td>5</td>
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<tr>
<td>12 F</td>
<td>Turkish</td>
<td>5 yr</td>
<td>-</td>
<td>3 yr 6 mo</td>
<td>467</td>
<td>25-50</td>
<td>+</td>
<td>-</td>
<td>2</td>
<td>+</td>
<td>4 yr 6 mo</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.677+1G&gt;A</td>
<td>ND</td>
<td>5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>13 M</td>
<td>Turkish</td>
<td>3 yr 6 mo</td>
<td>+</td>
<td>no</td>
<td>428</td>
<td>&lt;3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>3 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>homo</td>
<td>c.1031+1G&gt;A</td>
<td>aberrant splicing</td>
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<tr>
<td>14 F</td>
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<td>6 yr 4 mo</td>
<td>-</td>
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<td>1606</td>
<td>3-10</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>4 yr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>homo</td>
<td>c.1031+1G&gt;A</td>
<td>ND</td>
<td>9</td>
<td></td>
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</tr>
<tr>
<td>15 M</td>
<td>British</td>
<td>died at 8 yr</td>
<td>-</td>
<td>3 yr 4 mo</td>
<td>607-1715</td>
<td>&lt;3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>2 yr 2 mo</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>homo</td>
<td>c.852_859del</td>
<td>p.Trp284X</td>
<td>8</td>
<td></td>
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</table>

Detailed clinical information for individual 1 to 4 was previously described (2). Eleven CHKB mutations were identified in 15 affected individuals. All exhibited generalized muscle hypotonia and weakness from early infancy. Ambulation was delayed, and gait in those who achieved walking was limited. In addition, all displayed marked mental retardation, and most never acquired meaningful language. Microcephaly with head circumferences at or below the 3rd to 10th percentile was observed in most cases. Cranial magnetic resonance imaging showed no developmental brain defects. Six individuals had dilated cardiomyopathy, and two had cardiac anomaly. Individuals 1, 2, 6, and 15 died from cardiomyopathy at ages 13 yr, 23 yr, 2 yr 6 mo, and 8 yr, respectively. No one had respiratory insufficiency. Ichthyosiform skin changes were frequent. All showed mildly to moderately elevated serum creatine kinase (CK) levels. Individuals 7 and 9 also had homozygous single-nucleotide substitutions, c.902C>T (p.Trp301Leu) and c.983A>G (p.Gln328Arg), respectively. CHK activities of recombinant CHK-B proteins with p.Trp301Leu and p.Gln328Arg were only mildly decreased (Figure S2), suggesting these are likely to be neutral polymorphisms or only mildly hypomorphic mutations. Individuals 10, 11, and 12, who have same c.677+1G>A mutation, and individuals 13 and 14, who have same c.1031+1G>A mutation, are not siblings. Abbreviations are as follows: ND, not determined; p, percentile; F, female; and M, male.

* An affected sibling had ichthyosis and died at age 6 years with cardiomyopathy.

+ Patent ductus arteriosus.
+ Atrial septal defect.
+ Mitral valve prolapse.
CHKB (Table 1). Among a total of 11 mutations identified, six were nonsense, two were missense, one was a 3 amino acid deletion, and two were splice-site mutations. The six nonsense mutations, c.116C>A (p.Ser39X), c.458dup (p.Leu153PhefsX57), c.611_612insC (p.Thr205AsnfsX5), c.810T>A (p.Tyr270X), c.852_859del (p.Trp284X), and c.922C>T (p.Gln308X), were predicted to truncate the protein and eliminate highly conserved domains of CHK.5 Individuals 1 and 2 (unrelated, Japanese) had the same homozygous nonsense mutation of c.810T>A (p.Ser39X). Individual 2’s mother, who was healthy, had the heterozygous c.810T>A (p.Ser39X) mutation. Unfortunately, a DNA sample from the father of individual 2 was not available. DNA samples from other family members of individual 1 and 2 were not available. Individuals 3 and 4 (siblings, Japanese) had the same compound heterozygous mutation c.116C>A (p.Ser39X) and c.458dup (p.Leu153PhefsX57). Both parents were healthy, and the father was heterozygous for mutation c.116C>A (p.Ser39X), whereas the mother was heterozygous for mutation c.458dup (p.Leu153-PhefsX57), thus confirming a recessive inheritance pattern. These mutations cosegregated with the disease phenotype in all family members tested.

We therefore measured CHK activity in biopsied muscle. For all biochemical analyses, because of the limiting amounts of remaining tissue, biopsied muscle samples were available only from individuals 2, 3, and 4. Biopsied muscle samples from these three individuals were homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.5), 154 mM KCl, and 1 mM phenylmethanesulfonyl fluoride with a sonicator (MISONIX), and supernatants (105,000 × g, 60 min) were prepared and analyzed for CHK activity as previously described.6 Similar to muscles of md mice,1 muscles from individuals 2, 3, and 4, who carried homozygous or compound heterozygous nonsense mutations, did not have any detectable CHK activity (Figure 2A). Individuals 7, 8, and 9 had homozygous missense mutations c.847G>A (p.Glu283Lys) and c.1130 G>T (p.Arg377Leu) and a homozygous 3 amino acid deletion, c.554_562 del (p.Pro185_Trp187del), respectively. We screened 210 control chromosomes for the identified missense mutations and small in-frame deletion by direct sequencing or single-strand conformation polymorphism (SSCP) analysis. SSCP was performed with Gene Gel Excel (GE Healthcare) as previously described.7 These missense mutations and this small in-frame deletion were not identified in control

**Figure 1. Muscle Pathology of the Affected Individuals**

Cross-sections of muscle fiber from a human control and individual 4.

(A) On H&E staining, nonspecific dystrophic features with necrotic and regenerating fibers, internalized nuclei, and endomysial fibrosis are seen. The scale bar represents 25 μm.

(B) On cytochrome c oxidase staining, enlarged mitochondria at the periphery and central areas devoid of mitochondria were seen. The scale bar represents 20 μm.

(C) On NADH-TR staining, the intermyofibrillar network was preserved even in the central areas that are devoid of mitochondria, suggesting the presence of myofibrils and only absence of mitochondria. The scale bar represents 20 μm.

(D) Electron microscopy confirmed enlarged mitochondria. The scale bar represents 1 μm.
Figure 2. Choline Kinase Activity and Phospholipid Analyses

(A) In muscle tissue from individuals 2, 3, and 4, CHK activity cannot be detected ($n = 3$). Data represent the mean of three individuals.

(B) PC and PE content in frozen biopsied muscle tissues from individuals 2, 3, and 4 and hindlimb muscles from 8-week-old rmd mice ($n = 4$) and control littermates ($n = 5$) were analyzed by thin-layer chromatography followed by phosphorus analysis. PC and the PC/PE ratio are significantly decreased in affected individuals and rmd mice ($n = 3$ for humans, $n = 4$ for rmd mice, $n = 5$ for littermates).

(C) Fatty acid composition of PC molecular species in muscles and isolated mitochondria from hindlimb muscles of rmd mice are determined by electrospray ionization mass spectrometry (ESI-MS). We observed that 34:1-PC (16:0-18:1), 36:4-PC (16:0-20:4), and 38:6-PC (16:0-22:6) species are significantly decreased, whereas 36:2-PC (18:0-18:2) is increased in rmd muscle. Similarly, in isolated mitochondria from hindlimb muscle, 36:4-PC (16:0-20:4) and 38:6-PC (16:0-22:6) species are decreased, whereas 36:2-PC (18:0-18:2) is increased.
It has been shown that phospholipids have tissue-specific fatty acid composition. For example, heart PC and muscle PC mainly contain docosahexaenoic acid (22:6) (Nakanishi et al. and Figure 2C), but liver PC includes various fatty acids. NanoESI-MS analyses of PC molecular species in muscle and isolated mitochondria were performed with a 4000Q TRAP (AB SCIEX, Foster City, CA, USA) and a chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA). Quadriceps femoris (hindlimb) and Triceps (forelimb) muscle from affected rmd mice and littermate controls were frozen with liquid nitrogen, and total lipid was extracted by the Bligh and Dyer method. The ion spray voltage was set at −1.25kV, gas pressure at 0.3 pound per square inch (psi), and flow rates at 200 nl/min. The scan range was set at m/z 400–1200, declustering potential at −100V, collision energies at −35−−45V, and resolutions at Q1 and Q3 “unit.” The mobile phase composition was chloroform: methanol (1/2) containing 5 mM ammonium formate and was normalized to the muscle weight. The total lipids were directly subjected by flow injection, and selectivity was analyzed by neutral loss scanning of the polar head.
group for PC in negative-ion mode.\(^1\) Interestingly, there was a 10-fold decrease (9.8%) in the 16:0-22:6-PC levels versus the control in \(mld\) hindlimb muscle and also in muscle mitochondria (Figure 2C), indicating the importance of the PC de novo synthesis pathway for maintaining not only PC levels but also fatty acid composition of PC molecular species. Similarly, in forelimb muscle 16:0-22:6 PC levels were also decreased in comparison to the control, but to a milder extent (18.2%), suggesting an association between severity of muscle damage and fatty acid composition alteration of PC (data not shown). In \(mld\) mice, it has been shown that muscle PC can be delivered from plasma lipoprotein,\(^1\) suggesting that non-decreased PC molecular species might be derived from the plasma, whereas 16:0-22:6 PC might be synthesized only in muscle (and possibly in brain). However, confirmation of this requires further studies.

Individuals with CHKB mutations have severe mental retardation in addition to the muscular dystrophy. Interestingly, polymorphisms near the CHKB locus and decreased CHKB expression have been associated with narcolepsy with cataplexy, suggesting a link between CHK-\(\beta\) activity and the maintenance of normal brain function in humans.\(^1\) Furthermore, brain damage in pneumococcal infection has been attributed to the inhibition of de novo PC synthesis, suggesting the importance of PC synthesis for the brain.\(^2\) Our data provide evidence that altered phospholipid biosynthesis is a causative agent for a human congenital muscular dystrophy, and further studies will elucidate the detailed molecular mechanisms of the disease in both muscle and brain.

**Supplemental Data**

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/AJHG/.

**Acknowledgments**

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**Web Resources**

The URLs for data presented herein are as follows:


Online Mendelian Inheritance in Man (OMIM), [http://www.omim.org](http://www.omim.org)

R software version 2.11.0, [http://www.r-project.org/](http://www.r-project.org/)

**References**

lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494–496.