Recessive MYL2 mutations cause infantile type I muscle fibre disease and cardiomyopathy

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A cardioskeletal myopathy with onset and death in infancy, morphological features of muscle type I hypotrophy with myofibrillar disorganization and dilated cardiomyopathy was previously reported in three Dutch families. Here we report the genetic cause of this disorder. Multipoint parametric linkage analysis of six Dutch patients identified a homozygous region of 2.1 Mb on chromosome 12, which was shared between all Dutch patients, with a log of odds score of 10.82. Sequence analysis of the entire linkage region resulted in the identification of a homozygous mutation in the last acceptor splice site of the myosin regulatory light chain 2 gene (MYL2) as the genetic cause. MYL2 encodes a myosin regulatory light chain (MLC-2V). The myosin regulatory light chains bind, together with the essential light chains, to the flexible neck region of the myosin heavy chain in the hexameric myosin complex and have a structural and regulatory role in muscle contraction. The MYL2 mutation results in use of a cryptic splice site upstream of the last exon causing a frameshift and replacement of the last 32 codons by 20 different codons. Whole exome sequencing of an Italian patient with similar clinical features showed compound heterozygosity
In conclusion, the mutations in the last exon of MYL2 are responsible for a novel autosomal recessive lethal myosinopathy known to cause dominant hypertrophic cardiomyopathy; however, none of the parents showed signs of cardiomyopathy. We propose ‘light chain myopathy’ as a name for this MYL2-associated myopathy.

**Keywords:** MYL2; myosin regulatory light chain; myosinopathy; type I hypotrophy; light chain myopathy

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**Introduction**

Infantile fibre-type disproportion with type I fibre hypotrophy, cardiomyopathy and myofibrillar lysis in type I muscle fibres was previously reported in three Dutch families (Barth et al., 1998) and described as a progressive myopathy with onset shortly after birth and a cardiomyopathy leading to death in all patients between 4 and 6 months of age. Muscle biopsies showed fibre-type disproportion with small type I and normal sized type II fibres. Ultrastructural analysis showed disorganization of sarcomeres, especially in the smallest fibres, without evidence of storage material (Barth et al., 1998). In all patients, a generalized tremor/clonus was present from birth, even before the cardiomascular symptoms became predominant. Patients ultimately died of heart failure and shock following progressive cardiac dilatation. Although signs of cardiac strain were present early in the course of the disease, decompensation only occurred in the terminal phase. Autopsies revealed no clues for CNS involvement, and a sural nerve biopsy of one patient was normal. Additional features in the first five patients were intermittent mild lactic acidosis not explained by cardiac failure and glycogen depletion in skeletal muscle at the terminal stage of the disease. Two of five patients from the first three described families showed highly increased plasma creatine kinase activities. Despite an extensive search, mitochondrial and fatty acid oxidation defects were not found (Barth et al., 1998). Because both sexes were affected, all parents were apparently healthy and some families had more than one affected child, the disease was thought to result from autosomal recessive mutations. We therefore set out to identify the gene involved in this disorder and succeeded in identifying three different mutations, all affecting the same exon of the same gene, myosin regulatory light chain 2 (MYL2), as the underlying genetic cause of this disorder. Dominant mutations in MYL2 were already known to cause hereditary ventricular hypertrophy or hypertrophic cardiomyopathy (Poetter et al., 1996; Flavigny et al., 1998; Andersen et al., 2003). Also, defects in the MYL3 and MYH7 genes that encode the other components of the β-cardiac myosin, the cardiac essential light chain and myosin heavy chain, respectively (CMH8; OMIM #608751; CMH1; OMIM #192600), are known to cause hypertrophic cardiomyopathy. Here we report three different recessive mutations in MYL2, which are the cause of myopathy, fibre type I hypotrophy with myofibrillar disarray and cardiomyopathy.

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**Materials and methods**

**Patients**

All patients were referred to university centres for diagnosis and treatment. Genomic DNA was isolated from blood samples of patients and relatives according to standard diagnostic procedures. DNA analysis and further cardiological examinations were carried out after a genetic counselling procedure and consent of the relatives. Muscle biopsies were obtained for diagnostic reasons. Stored specimen from these biopsies, as well as frozen and paraffin embedded tissue of the deceased patients, were made available by the Departments of Pathology of the University Medical Centre Leiden, University Medical Centre Groningen and the Academic Medical Centre, Amsterdam.

**Enzyme and immunohistochemistry**

Immunohistochemical stainings were performed according to standard staining procedures. For the MYL2 stainings, paraffin sections of muscle tissue of deceased patients as well as control muscle tissue were deparaffinized in xylol, followed by rehydration in a series of ethanol solutions, after which antigen retrieval was carried out by boiling in 10 mM citrate buffer supplemented with 0.05% Tween-20 for 2 min in a microwave. After preincubation in a buffer containing 2% goat serum, 0.1% bovine serum albumin and 0.5% Triton X-100, incubation with the primary antibody was performed overnight at 4°C (Epitomics 17-1 and 42-2, 1:100) followed by blocking endogenous peroxidase (0.3% H2O2 in PBS supplemented with 0.1% Tween-20) before incubation with a peroxidase-coupled goat-anti rabbit secondary antibody (Brightvision, Immunologico) and detection using the NovaRED™ system (Vector Laboratories). Frozen tissue sections were fixed using methanol or acetone before primary antibody incubation, omitting the antigen retrieval step. Slides were counterstained using haematoxylin before rehydration and mounted onto slides using VectaMount™ (Vector Laboratories). Images were captured using an Olympus BX41 microscope, equipped with a DP25FW camera and CellID software. Slow (HybriDoma Bank; clone A 4.951, 1:100) and fast (Biogenex/MU109-UC, clone MY-32, 1:200) myosin staining was performed on paraffin sections according to routine diagnostic procedures. Frozen 6-μm thick sections prepared from muscle
biopsies were routinely stained for haematoxylin and eosin and Gomori trichrome, ATPase preincubated at pH 4.3, NADH, succinate dehydrogenase, periodic acid Schiff, periodic acid Schiff-diestase and Oil Red O. Frozen tissue sections stained for ATPase activity after preincubation at pH 4.3 were used for muscle morphometry as described (Dubowitz, 1985).

**Protein isolation and western blot analysis**

Proteins were extracted from minced tissues using ice-cold lysis buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1% TritonX-100 and 0.1% SDS, supplemented with protease inhibitor cocktail (Roche). After incubation of 2 min in a bath sonicator followed by incubation for 5 min at 95°C, the samples were mechanically homogenized as much as possible. Protein concentrations were determined using the Pierce BCA Protein Assay kit. Proteins were separated on a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl

**Linkage analysis**

DNA samples from six affected infants were genotyped using the Affymetrix GeneChip® Human Mapping 250 K Sty Array as described previously (Budde et al., 2008). The gender of samples was verified by counting heterozygous single nucleotide polymorphisms on the X chromosome. Relationship errors were evaluated with the help of the program Graphical Representation of Relationships (GRR) (Abecasis et al., 2001). For log of odds (LOD) score calculations, we assumed six consanguineous families with one child from second cousin marriages, an approach useful for identification of rare recessive disorders when consanguinity was not proven (Rutsch et al., 2009). Linkage analysis was performed assuming autosomal recessive inheritance, full penetrance and a disease allele frequency of 0.0001. Multipoint LOD scores were calculated using ALLEGRO (Gudbjartsson et al., 2000). Haplotypes were constructed with ALLEGRO and presented graphically with HaploPainter (Thiele and Nurnberg, 2005). All data handling was done using the graphical user interface ALOHOMORA (Ruschendorf and Nurnberg, 2005).

**Reverse transcriptase polymerase chain reaction**

Total RNA was isolated from frozen diaphragm tissue of N-C2 using TRIzol® according to the manufacturer’s protocol (Invitrogen). A first strand reaction performed on 5µg of total RNA primed with oligo-dT-VN was used for a reverse transcriptase reaction using SuperScript® II (Invitrogen) followed by heat inactivation and RNase H treatment before amplification using a touchdown PCR program and intron-spanning primers (see Supplementary Table 1 for sequences).

**Cardiological examination**

Parents who consented to evaluation were interviewed for signs of neuromuscular weakness or cardiac complaints, including rhythm disturbances, and underwent cardiological evaluation by 12-lead ECG and echocardiography. Specific attention was given to signs indicative of any type of inherited cardiomyopathy (i.e. hypertrophic, dilated, restrictive or non-compaction cardiomyopathy).

**Results**

**Clinical characteristics of patients**

We identified 11 affected infants from eight different Dutch families and two affected infants from one Italian family with similar clinical and biopsy findings (Table 1). All patients were born at term after uncomplicated pregnancies, with normal birth weight and length, and no outward signs of malformation. The only sign of distress at birth was a coarse clonus/tremor. This generalized high amplitude tremor or clonus was present in all
Table 1: Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Patient; number of affected siblings</th>
<th>Sex</th>
<th>Monthb</th>
<th>Tremor/ clonus</th>
<th>Progressive myopathy; creatine kinase (U/l)</th>
<th>Cardiomyopathy</th>
<th>Autopsy</th>
<th>Fibre-type disproportion</th>
<th>Additional data: electron microscopy of small fibres; respiratory chain in skeletal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-A1; 2/2</td>
<td>M</td>
<td>6</td>
<td>Yes</td>
<td>Generalized, tented mouth, ptosis; 90 U/l</td>
<td>Acute decompensation following infection shortly before death</td>
<td>General cardiac dilatation with slight hypertrophy</td>
<td>Quadiceps m. I: 6.0 ± 3.3 μm, II: 17.8 ± 3.6 μm; FSD 66; 70% type I; paucity type 28 fibres</td>
<td>EM: severely disorganized sarcomeres, partial loss of myofilibrin, no loss of mitochondria; RC: no abnormalities</td>
</tr>
<tr>
<td>N-A2; 2/2</td>
<td>F</td>
<td>6</td>
<td>Yes</td>
<td>Generalized, tented mouth; 26 U/l</td>
<td>Dilated, decompensation</td>
<td>General cardiac dilatation with slight hypertrophy</td>
<td>Quadiceps m. I: 4.9 ± 3.3 μm, II: 13.0 ± 1.6 μm; FSD 62; 69% type I; paucity type 28 fibres</td>
<td>EM: severely disorganized sarcomeres, partial loss of myofilibrin, no loss of mitochondria; RC: no abnormalities</td>
</tr>
<tr>
<td>N-B10; 2/10</td>
<td>F</td>
<td>5</td>
<td>Yes</td>
<td>Generalized, tented mouth; 134 U/l</td>
<td>Dilated, decompensation</td>
<td>ND</td>
<td>ND</td>
<td>EM: severely disorganized sarcomeres, partial loss of myofilibrin, no loss of mitochondria; RC: no abnormalities</td>
</tr>
<tr>
<td>N-C2; 2/2</td>
<td>F</td>
<td>5</td>
<td>Yes</td>
<td>Generalized, terminal rhabdomyolysis; 1733 U/l</td>
<td>Dysfunction both ventricles, F5 11%, cardiogenic shock and multorgan failure</td>
<td>Cardiac dilatation, hypoxic–ischaemic brain damage</td>
<td>Quadiceps m. I: 7.6 ± 3.5 μm, II: 14.0 ± 2.8 μm; FSD 79; 79% type I; paucity type 28 fibres</td>
<td>EM: severely disorganized sarcomeres, partial loss of myofilibrin, no loss of mitochondria; RC: no abnormalities</td>
</tr>
<tr>
<td>N-D1; 1/1</td>
<td>F</td>
<td>4</td>
<td>Yes</td>
<td>Generalized, tented mouth; 712 U/l</td>
<td>Restrictive, cardiogenic shock</td>
<td>Cardiac hypertrophy and dilatation, interstitial fibrosis, subendocardial fibrosis LA</td>
<td>Quadiceps m. I: 6.7 ± 2.5 μm type 2A: 15.2 ± 2.5 μm; FSD 56; 70% type I</td>
<td>EM: severely disorganized sarcomeres, partial loss of myofilibrin, no loss of mitochondria; RC: no abnormalities</td>
</tr>
<tr>
<td>N-E1; 3/4</td>
<td>M</td>
<td>5</td>
<td>Yes</td>
<td>Generalized; ND</td>
<td>Acute decompensation</td>
<td>Dilated cardiomyopathy, fibroelastosis LA and RV</td>
<td>Present, no quantitative data</td>
<td></td>
</tr>
<tr>
<td>N-E2; 3/4</td>
<td>M</td>
<td>5</td>
<td>Yes</td>
<td>Generalized; 9 U/l</td>
<td>Decompensation</td>
<td>ND</td>
<td>ND</td>
<td>EM: severely disorganized sarcomeres, no loss of mitochondria</td>
</tr>
<tr>
<td>N-F1; 1/1</td>
<td>M</td>
<td>5</td>
<td>Yes</td>
<td>Generalized, tented mouth; 46 U/l</td>
<td>Dilated, restrictive and slightly hypertrophic LV, decreased contractility</td>
<td>Dilated cardiomyopathy, subendocardial, interstitial and perivascular fibrosis</td>
<td>Quadiceps m. I: 6.2 ± 1.3 μm, II: 15.5 ± 2.2 μm; FSD58; 59% type I</td>
<td>EM: disorganized sarcomeres; RC: decreased oxidation of radiolabelled substrates and pyruvate oxidation</td>
</tr>
<tr>
<td>N-G1; 1/1</td>
<td>F</td>
<td>1</td>
<td>Yes</td>
<td>Generalized from birth; 32–204 U/l (n = 7)</td>
<td>Non-compaction</td>
<td>Cardiac non-compaction</td>
<td>Quadiceps m. I: 6.4 ± 2.8 μm, II: 12.5 ± 3.5 μm; FSD 49; 45% type I</td>
<td>RC: no abnormalities</td>
</tr>
<tr>
<td>N-H1; 2/2</td>
<td>F</td>
<td>6</td>
<td>Unknown</td>
<td>Generalized, proximal &gt; distal, diaphragmatic respiration, tented mouth; 106–5116 U/l (n = 8)</td>
<td>Dilated</td>
<td>Cardiac dilatation, thinning RV</td>
<td>Quadiceps m. I: 6.1 ± 1.0 μm, II: 12.8 ± 1.9 μm; FSD 53; 57% type I</td>
<td>EM: disorganized sarcomeres; RC: decreased oxidation of radiolabelled substrates and pyruvate oxidation, slight reduction activity complex II</td>
</tr>
<tr>
<td>N-H2; 2/2</td>
<td>M</td>
<td>5</td>
<td>Yes</td>
<td>Generalized, tented mouth; 41–425 U/l (n = 6)</td>
<td>RV and septal hypertrophy, insufficiency mitral valve, restrictive cardiomyopathy</td>
<td>ND</td>
<td>Quadiceps m. I: 7.2 μm ± 3.0 μm, II: 14.5 ± 3.0 μm; FSD 90; 67% type I</td>
<td>EM: severely disorganized sarcomeres</td>
</tr>
<tr>
<td>I-A2; 2/3</td>
<td>M</td>
<td>6</td>
<td>Yes</td>
<td>Generalized weakness, facial muscles affected; 450 U/l</td>
<td>Dilated, slightly hypertrophic</td>
<td>ND</td>
<td>Quadiceps m. I: 4.5 ± 1.9 μm, II: 11.2 ± 2.4 μm, 28: 11.5 ± 2.7; FSD 53; 51% type I</td>
<td>EM: myofibrillar disarray; RC: no abnormalities</td>
</tr>
<tr>
<td>I-A3; 2/3</td>
<td>M</td>
<td>6</td>
<td>Yes</td>
<td>Generalized weakness, facial muscles affected; 412 U/l</td>
<td>Normal at birth, markedly dilated at 5 months, slightly hypertrophic LV, with decreased contractility F5 18%, evidence of LV non-compaction</td>
<td>ND</td>
<td>Present, no quantitative data</td>
<td></td>
</tr>
</tbody>
</table>

a Patients previously published by Barth et al., 1998.

b Died in months (mo) after birth.

CK = creatine kinase; EM = electron microscopy; FS = fractional shortening; LA = left atrium; LV = left ventricle; ND = not done; RA = right atrium; RC = respiratory chain; RV = right ventricle.

FSD = fibre size disparity = μ2 − μ1/μ2 × 100 where μ2 and μ1 represent mean diameters of type II and type I fibres.
patients before the onset of cardiac or neuromuscular symptoms. It was present while awake, absent during sleep and strikingly resembled the hyperexcitation syndrome known in distressed newborns. However, none of the affected babies had perinatal problems. Hypoglycaemia and hypocalcaemia were excluded. The tremor slowly abated over a period of weeks. No other symptoms pertaining to the CNS were present. No cerebral abnormalities were found in routine autopsies, except one case with hypoxic–ischaemic damage due to postnatal circulatory failure. Patients were vigorous at birth without signs of muscle weakness or other signs of prenatal muscle involvement such as contractures or polyhydramnios. All patients had rapidly progressive generalized muscle weakness starting within weeks after birth, and the majority had a tented mouth or global facial muscle involvement (Fig. 1A and B). All patients died of heart failure due to cardiomyopathy, which was mainly dilated, although some patients had features of other forms of cardiomyopathy (hypertrophic, restrictive or non-compaction) (Table 1). All had similar muscle biopsies morphologically characterized by fibre-type disproportion with small type I fibres and normal-sized type II fibres. Fibre size disparities ranged between 48 and 79% (n < 12%) (Table 1 and Fig. 1C and D). Electron microscopy of muscle biopsies showed abnormalities varying between loss of register between adjacent sarcomeres in normal or small fibres to severe disorganization and loss of myofibrils in the smallest fibres without storage material (Fig. 1E). No loss of mitochondria was seen in affected fibres with myofibrillar disorganization (Fig. 1D and E). Plasma creatine kinase levels were increased and very high in several patients. This finding mirrors the rapid clinical progression and the observed histopathological changes, such as subendocardial fibrosis and myofibrillar disorganization in skeletal muscle (Table 1).

Identification of the genetic cause

Based on the rare occurrence of this disorder, we assumed a recessive monogenic cause for the disease in the Dutch families, and performed a genome-wide search for regions of homozygosiy shared between the six Dutch patients (Patients N-A1, N-B10, N-C2, N-D1, N-E1 and N-F1) from six different families, using a 250K Affymetrix® Sty1 single nucleotide polymorphism microarray. We assumed consanguinity as well as full penetrance, an autosomal recessive mode of inheritance and a disease allele frequency of 0.001. By multipoint parametric linkage analysis using a reduced panel of single nucleotide polymorphisms (~20000), we identified a single peak on chromosome 12 with a maximum LOD score of 9.8. Analysis of the critical region with all markers gave a
maximum LOD score of 10.8, indicating that this region must harbour the disease-causing mutation. An identical haplotype for this region could be constructed for all Dutch patients defined by the limiting single nucleotide polymorphisms rs10849988 and rs10849946 located at a distance of 2.1 Mb (Fig. 2).

We set out to identify the pathogenic mutation by sequencing of the 2.1 Mb region. We designed a custom 2.3 Mb Nimblegen array for sequence capture that covered the entire linkage region (chr12:108,060,000–110,350,000 NCBI36/hg18) and used genomic DNA from N-D1 to enrich for these target sequences. Analysis on an FLX Titanium 454 sequencer (Roche) yielded 1.1 million filter-passed sequences with a median length of 370 bp, which were mapped to the human consensus genome (hg18) with Newbler software. Twenty-six per cent of the reads mapped to the captured region, which contained 31 annotated genes, one hypothetical locus, one mitochondrial RNA locus and three pseudogenes. More than 95% of all exons were sequenced with coverage of more than eight times. Analysis yielded 1192 reliable nucleotide changes. Because the mode of inheritance is autosomal recessive, the expected disease-causing mutation would be a homozygous change. Within the coding regions of annotated genes and adjacent intronic sequences (within 20 nucleotides of the exon–intron boundaries), we found 49 homozygous nucleotide variations, most of which were located in the non-coding 3’ tails of the last exons. Fifteen variations were located in the coding regions, and eight of these were predicted to lead to an amino acid change; however, these were all annotated as known polymorphisms (www.ncbi.nih.gov).

A homozygous splice site mutation (c.403-1G>C) was detected in the last acceptor splice site of the MYL2 gene when the threshold was reduced to six-times coverage. Sanger sequencing was used to obtain sufficient coverage of all coding regions that did not reach the level of eight-times coverage. This analysis confirmed the presence of the found splice site mutation as the only mutation not annotated as a polymorphism in the entire linkage interval. Additional sequence analysis of the other five Dutch patients showed the same homozygous mutation in all patients. Three additional Dutch patients (Patients N-G1, N-H1 and N-H2), with similar clinical characteristics that were not included in the linkage analysis, also carried the same homozygous mutation. Of seven families, DNA from the parents was available and on testing, they were all shown to be heterozygous carriers.

Owing to the location of the mutation, just before the last exon, the mutant messenger RNA is not expected to be prone to nonsense-mediated decay, and whole exon skipping also cannot occur within the normal transcript. Because the mutation most probably will affect splicing, we screened the genomic sequences of intron 6 for the presence of cryptic splice sites using splice prediction program NNSplice and, consequently, designed several reverse transcriptase PCRs (based on the use of these sites). Analysis of the messenger RNA from muscle tissue (diaphragm) of Patient N-C2 using a forward primer spanning the exon 5–6 junction and one of a number of reverse primers downstream of the predicted sites, showed that a cryptic splice site 23 nucleotides upstream of the original splice site is used (Fig. 3 and 4).
Supplementary Fig. 1). This results in a frameshift mutation and replacement of the last 32 codons by 20 different codons and thus alters the C-terminal part of the protein.

Whole exome sequencing of the Italian patient (Patient I-A2) demonstrated compound heterozygosity for two other mutations, c.431delC; p.Pro144LeufsX2 and c.432delT; p.Asp145ThrfsX2, affecting two adjacent nucleotides in the last exon of the MYL2 gene. Sanger sequencing of the parents indeed demonstrated that the patient had inherited one defective allele with a deletion of one nucleotide from each parent (Fig. 4). Both changes result in a frameshift and premature termination in the third codon of the shifted open reading frame (original codon 146), leading to mutant truncated proteins that are 20 amino acids shorter than the normal protein.

**Expression of the protein**

The use of two commercially available antibodies (Epitomics), one that recognizes an epitope in the C-terminal tail (antibody 2742-1) and another more centrally located (antibody 2917-1), enabled us to discriminate between normal and mutant protein. In control muscle tissue, normal protein was detected at a high level in the muscle fibres that were identified as type I fibres by additional staining against slow and fast myosin. In patients, the level of expression was lower, ranging from moderate to hardly detectable with antibody 2917-1, which should detect both normal and mutant protein. Moreover, fibre type specificity had disappeared (Fig. 5). The second, C-terminal MYL2 antibody (2742-1), showed the same fibre type l-specific pattern in normal muscle tissue, while normal MYL2 was absent in all seven patients examined (Fig. 5 and Supplementary Fig. 2), which is in line with the predicted loss of the epitope detected by antibody 2742-1. The loss of the C-terminal epitope was confirmed by western blot analysis of post-mortem muscle tissue of Patients N-C2 and N-F1 (Fig. 6). Biopsy muscle tissue from Patients N-H1 and N-H2 gave the same result (not shown). Owing to poor performance of antibody 2917-1, which was raised against an epitope present in both mutant and normal MYL2, a band of the same size was only seen as a faint signal in control tissue, whereas it was below detection level in the patients despite loading of comparable amounts of protein.

**Cardiological evaluation of heterozygous carriers**

Because dominant missense mutations in MYL2 have been reported in adults with hypertrophic cardiomyopathy, all available parents \((n = 14)\) were subjected to cardiological evaluation. No indications for cardiomyopathy were found. Only a structural variation consisting of an accessory papillary muscle in the left ventricle apex and on the septum was seen in one of the parents, which is not indicative of a cardiomyopathy.

**Discussion**

We identified three different mutations in MYL2 as the causative gene defects causing the present disease, thereby enabling prenatal molecular screening for this fatal disorder.

Heterozygous dominant MYL2 mutations were previously described in patients with hypertrophic cardiomyopathy, but no reports have linked mutations in this gene with skeletal myopathies. All Dutch patients shared the homozygous c.403-1G>C mutation, indicative of a founder mutation, whereas in an Italian family, two compound heterozygous mutations were found. All three mutations target the last exon of the gene.

MYL2, also known as MLC-2V, encodes the cardiac, ventricular isoform of myosin regulatory light chain, which is part of the slow-twitch skeletal muscle myosin complex as well as the \(\beta\)-cardiac complex. Myosins, encoded by a multigene family, are large hexameric motor proteins that bind actin and convert energy from ATP hydrolysis into mechanical force. They consist of two
heavy chains, two essential light chains and two regulatory light chains. The heavy chains consist of a long rod-like tail, the flexible neck-region and the head region where binding to actin as well as hydrolysis of ATP occurs. Heavy chain isoform profiles determine the specific physiological properties of the muscle fibres (Weiss and Leinwand, 1996). In mammalian skeletal muscles, four major fibre phenotypes are distinguished: slow-twitch or type I fibres (MYH7) and three types of fast-twitch types (MYH2, MYH1 and MYH4): type IIa, IIb and IIx/d fibres (summarized in Schiaffino and Reggiani, 2011; see Table 2). In humans, three fibre types exist: slow/type I fibres (MYH7) and two types of fast fibres, type 2A (myosin Ila/MYH2) and type 2B (myosin IIX/ MYH1). Although MYH4 is present in the human genome and MYH4 is the predominant heavy chain in mouse type IIb fibres, the human protein is absent despite expression at the RNA level in foetal muscle and certain conditions such as Duchenne muscular dystrophy (Harrison et al., 2011). Both (essential/alkali and regulatory) light chains bind to the neck region that functions as a lever arm in the myosin complex and have a structural role in actomyosin sliding velocity (Lowey et al., 1993; Uyeda and Spudich, 1993). Essential light chains have been shown to be essential for full force production (VanBuren et al., 1994) by altering actomyosin cross-bridge kinetics, as has been demonstrated in transgenic mice carrying a human pathogenic mutation that causes hypertrophic cardiomyopathy (Muthu et al., 2010). In contrast to the essential light chains, the regulatory light chains can be phosphorylated leading to a conformational change, which consequently affects muscle contraction. More isoforms of (regulatory) light chain genes also exist that encode different light chain isoforms, possibly fulfilling different physiological requirements (Table 2). The light chains that predominate in slow twitch-muscle are the same as those in the cardiac ventricle. In contrast, the atrial form of myosin regulatory light chain (MYL7/MLC-2a/ MYL2A) is not expressed during skeletal myogenesis or in skeletal muscle (Hailstones et al., 1992).

The MYL2 mutations that we identified all cause frame shifts either directly as a consequence of the mutation or through use of an alternative splice site that will consequently lead to mutant proteins with altered C-terminal tails. Normal MYL2 was undetectable in tissue sections of skeletal muscle of seven Dutch patients from six families, whereas mutant protein expression was weak, diffuse and without the expected type I-specific expression. Subsequent western blot analysis indeed showed absence of wild-type MYL2 in the patients when using the C-terminal antibody. Although mutant messenger RNA was clearly present in muscle post-mortem tissue from Patient N-C2, this apparently
myosins and major muscle fibre types in adult human skeletal muscle and heart

<table>
<thead>
<tr>
<th>Myosin</th>
<th>Adult heart</th>
<th>Myosin gene</th>
<th>Adult skeletal muscle</th>
<th>Myosin gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin heavy chain</td>
<td>Type-α, atrial isoform</td>
<td>MYH6</td>
<td>Type 2A: myosin Ila, fast</td>
<td>MYH2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type 2B: myosin IIX/d, fast</td>
<td>MYH1</td>
</tr>
<tr>
<td>Myosin essential/alkal light chain</td>
<td>Type-β, ventricular isoform</td>
<td>MYH7</td>
<td>Type 1, slow</td>
<td>MYH7</td>
</tr>
<tr>
<td>Myosin regulatory light chain</td>
<td>Atrial isoform</td>
<td>MYL1 (MYL4)</td>
<td>Fast</td>
<td>MYL1</td>
</tr>
<tr>
<td></td>
<td>Ventricular isoform</td>
<td>MYL3</td>
<td>Slow</td>
<td>MYL3 (MYL6B)</td>
</tr>
<tr>
<td></td>
<td>Atrial isoform</td>
<td>MYL7</td>
<td>Fast</td>
<td>MYLPF</td>
</tr>
<tr>
<td></td>
<td>Ventricular isoform</td>
<td>MYL2</td>
<td>Slow</td>
<td>MYL2</td>
</tr>
</tbody>
</table>

Myosins expressed in highly specialized tissue and embryonal and perinatal isoforms are not included in this table except for the embryonic/atrial isoform MYL4/MLC1A (between brackets) which is also expressed in the atrium. MYL6A (between brackets), also known as MLC1a, is also expressed in non-muscle cells. MYL-PF is also known as MLC2B or HUMMLC2B. MYL1 encodes two isoforms, MLC1F and MLC3F. Regarding the composition/expression, in addition to ‘pure’ fibres, many hybrid fibres exist owing to anatomical location and interindividual variability.

Interestingly, mutations in MYH7, which encodes the β-cardiac myosin heavy chain (the heavy chain partner of MYL2), are accompanied by fibre-type disproportion in a few cases. Mutations in the rod region are mostly associated with myosin storage myopathy or Laing’s myopathy, whereas MYH7 mutations in the globular region, which contains the binding site for the light chains, mostly cause hypertrophic cardiomyopathy without skeletal muscle involvement (Kelly and Strauss, 1994; Laing et al., 1995; Tajsharghi et al., 2003; Walsh et al., 2010). Two cases with mutations in the rod region were reported with type I hypotrophy together with or without a myosin storage myopathy in one case each (Muelas et al., 2010; Ortolano et al., 2011). One report described a mutation in the neck region of MYH7 in a patient with type I hypotrophy as well as a cardiomyopathy (Darin et al., 2007). No previous reports have linked MYL2 to fibre type I hypotrophy. Our study shows that C-terminal recessive mutations in MYL2 result in a cardiomyopathy and skeletal myopathy with morphological features of type I hypotrophy in addition to myofibrillar disorganization, leading to a severe disease phenotype.

MYL2 specifically binds to the myosin heavy chain (Wadgaonkar et al., 1993), and its phosphorylation was reported to be important in regulation of myosin ATPase activity in smooth muscle cells and non-muscle myosin (Macera et al., 1992). In striated muscle, an increase in calcium concentration triggers contraction via the troponin/tropomyosin pathway, which controls the actin–myosin interaction. The identification of MYL2 mutations in adults with hypertrophic cardiomyopathy demonstrated their relevance in striated muscle as well. A spatial gradient of phosphorylated MYL2 across the heart has been described in human, mouse and rabbit cardiac tissue to facilitate cardiac contraction (Davis et al., 2001). MYL2 belongs to the EF-hand superfamily of proteins, with two EF-hand motifs capable of calcium-binding. EF-hands are found in pairs or higher copy numbers and form a structure implied to function as a calcium sensor and modulator of calcium signalling. The binding of calcium can cause a conformational change and may affect the structure of both EF-hands (Wimberly et al., 1995), thus affecting interaction with downstream targets (Grey et al., 2005). Ten different MYL2 mutations have been reported to date as the probable cause for hypertrophic cardiomyopathy, six of which are dominant missense mutations.

did not lead to a protein expression level comparable to that in control tissue, which suggests that the pathogenic mechanism is one of (partial) loss of function which would be expected for a recessive mutation. Loss of fibre type-specific expression may result in a compensation mechanism as has been described for the heavy chain of myosin as a result of physical stress or disease. In heart muscle in rodents, a shift takes place from the predominant α-type (Myh6) towards the foetally expressed β-type isoform (Myh7) through a microRNA-dependent mechanism (van Rooij et al., 2007). In contrast, human adult healthy hearts are mostly composed of the β-type (MYH7) in addition to a small fraction of α-fibres (MYH6). In failing human hearts, a shift toward more myosin heavy chain β-fibres is also observed due to downregulation of the α-isoform (Nakao et al., 1997; Hang et al., 2010). Downregulation of MYL2 was found to be associated with chronic heart failure (Li et al., 2011). In heart tissue sections of two of our patients, staining was hardly seen if not absent at all (Supplementary Fig. 3), whereas normal expression was clearly visible in the control sections.

Because we could demonstrate the presence of the mutant MYL2 messenger RNA, we expected to see some expression of the mutant MYL2 protein in muscle. The diffuse staining seen in the skeletal muscle paraffin sections without fibre-type specificity that was lacking in control muscle sections must be due to residual expression of mutant MYL2 regardless of the fibre type or to upregulation of a cross-reacting protein, which may be a highly homologous myosin light chain member. In comparison to control tissue, the staining is strongly reduced in patient tissue.

Selective type I hypotrophy occurs as part of many congenital myopathies. In congenital fibre-type disproportion, hypotrophy of type I fibres is the single remaining histological abnormality without more specific findings (Clarke and North, 2003). Sarcomere gene mutations constitute a growing part of disorders, previously labelled as congenital fibre-type disproportion. In addition to nemaline myopathies (Sanoudou and Beggs, 2001), hypotrophy of type I fibres can be caused by dominant mutations in tropomyosins TPM2 and TPM3 (Brandis et al., 2008; Clarke et al., 2008) or skeletal α-actin (ACTA) (Laing et al., 2004) and recessive mutations in the genes coding for ryanodine receptor RYR1 (Clarke et al., 2010) and selenoprotein (SEPN1) (Clarke et al., 2006).
The location of dominant missense mutations in patients with hypertrophic cardiomyopathy is indicated by the black triangles (A13T, F18L, E22K, N47K, R58Q, P95A and D166V) and (G–I) the recessive MYL2 mutations in patients with light chain myopathy by the red triangles. The EF-hand domains are indicated by red bars, the green bar indicates the phosphorylation site (ph), the turquoise bar at the left indicates the calcium-binding site (Ca) and at the right site predicted calcium-binding sites are shown (www.ncbi.nlm.nih.gov).

Figure 7 Distribution of dominant MYL2 mutations in hypertrophic cardiomyopathy and currently described recessive MYL2 mutations. (A–F, J) The location of dominant missense mutations in patients with hypertrophic cardiomyopathy is indicated by the black triangles (A13T, F18L, E22K, N47K, R58Q, P95A and D166V) and (G–I) the recessive MYL2 mutations in patients with light chain myopathy by the red triangles. The EF-hand domains are indicated by red bars, the green bar indicates the phosphorylation site (ph), the turquoise bar at the left indicates the calcium-binding site (Ca) and at the right site predicted calcium-binding sites are shown (www.ncbi.nlm.nih.gov).

Located in or near the region containing the N-terminal EF-hand or phosphorylation-binding site (Kabaeva et al., 2002; Poetter et al., 1996; Flavigny et al., 1998; Andersen et al., 2001; Richard et al., 2003; Szczesna-Cordary et al., 2004, 2005; Kerrick et al., 2009) (Fig. 7). All six dominant pathogenic mutants were functionally tested and all affected myosin function (Szczesna et al., 2001). In a transgenic mouse model where ~10% of cells expressed mutant A13T instead of endogenous Myl2, abnormal modelling of the heart and abnormal cross-bridge functions were found despite the low percentage of mutant cells and a poison-peptide mechanism was therefore suggested (Kazmierczak et al., 2012), which is in line with the dominant nature of these missense mutations. The other four reported variations, two missense and two splice site mutations, affect the C-terminal part of MYL2, which contains the second EF-hand motif. A functional effect was only shown for the mutation affecting the very last codon (D166V) of MYL2. Myofilibr preparations of D166V transgenic mice showed altered cross-bridge kinetics with actin after induced contraction (Muthu et al., 2010). Actomyosin cross-bridges deliver cyclic impulses to actin, resulting in periodic fluctuations of orientation. An irregular pattern of such fluctuations was observed in fibres carrying the mutation compared with the wild-type controls, possibly bearing relevance to the origin of myofibrillar disorganization. Myofilibrar disarray, which is a relatively rare phenomenon in paediatric neuromuscular disorders, is a common finding in heart muscle in hypertrophic cardiomyopathy. Myofilibrillar disarray in type I muscle fibres in the present disease carries morphological similarity to the myofilibrillar disarray in myocardial muscle cells, and the same mechanism may cause the micro-architectonic disturbance in both cell types.

The splice site mutation we identified here was reported previously in a patient with hypertrophic cardiomyopathy in a heterozygous state, in combination with another heterozygous variation leading to a K104E change in a family with hypertrophic cardiomyopathy. The K104E variation was regarded as a likely benign variant, as all four carriers of this change only were asymptomatic with normal electrocardiograms, even late in adulthood (Andersen et al., 2001). In retrospect, the compound heterozygosity may have been responsible for the disease, with one mutant allele only partially complementing the other mutant allele. The parents of the present patients were all heterozygous carriers of the MYL2 splice site mutation and their ages ranged from 30–69 years at the time of examination, but on cardiological examination, only a structural variation of unknown clinical significance was found in one case. To date, no sudden cardiac deaths have been reported in carriers with the splice site mutation (Andersen et al., 2001). Although based on a limited number of families, mutations in this part of the protein may be considered more benign and may only lead to severe problems in a homozygous state indicative of a loss of function mechanism. A long-term follow-up is needed to establish the potential risk for carriers of a C-terminal truncating mutation of the MYL2 protein.

A complete loss of function has been reported in a severely affected homozygous Myl2−/− mouse model (Chen et al., 1998) whereas heterozygous animals were completely normal (Minamisawa et al., 1999). The mice died at embryonic day 12.5 with dilated cardiomyopathy bearing a resemblance to the human cardioskeletal myopathy reported here. Moreover, ultrastructural defects in sarcomeric assembly of embryonic cardiomyocytes were also present. In the embryonic ventricle of the mutant Myl2−/− mice, expression of the atrial form of myosin regulatory light chain was found to be significantly higher while in their normal counterparts it was downregulated from this point in development. Analogous to this situation, in the human heart of our patients, MYL2 may have been partially replaced by MYL7, a human cardiac-specific atrial isoform with a weak ventricular expression. However, after birth, the expression of MYL7 sharply declines (Hailstones et al., 1992), which coincides with the onset of cardiac problems. In muscle, a similar replacement may theoretically also have occurred by other regulatory light chains, which, inadequately, have taken over the role of MYL2. Alternatively, if there is residual mutant MYL2 in the myosin complex, it probably affects myosin function. Because neuromuscular and cardiac functioning were apparently normal in all patients at the time of birth, failing adaptation to increased postnatal force requirements may also explain the onset and progression of muscular pareses after birth.

Because the N-terminal EF-hand structure and the consensus calcium-binding site, as well as the phosphorylation site, are still intact in our patients, the exact mechanisms that lead to the disease are currently unknown. Except for the interaction with the myosin heavy chain, MYL2 associations have also been described with three E3 ubiquitin ligases, MURF1/TRIM63/RNF28 (Kedar et al., 2004), MURF2/TRIM55/RNF29 and LRSAM1, a ubiquitously expressed E3 ligase involved in a hereditary motor and sensory neuropathy (Weterman et al., 2012). MURF proteins are thought to be involved in triggering protein degradation during pathophysiological muscle wasting. Ubiquitylation is a process relevant for the balance of hypertrophy and atrophy. A search for MURF1 interactors identified muscle-type creatine kinase, an
essential enzyme for energy metabolism (Koyama et al., 2008), and troponin, leading to the suggestion of a role in the balance of hypertrophy and atrophy as well as contractility responses during heart failure. Yeast two-hybrid screens identified more interactions with 11 enzymes required for ATP/energy production in muscle. This clearly shows a link with the coordination of energy metabolism (Witt et al., 2005), which may explain some of the findings that were suggestive of a metabolic defect in the first five patients (Barth et al., 1998). During embryonic development, Murf2 is specifically expressed at the very onset of cardiac development, and small interfering RNA knockdown experiments of Murf2 in neonatal rat cardiomyocytes disrupts post-translational microtubule modification and myofibril assembly (Perera et al., 2011). Disturbance of interactions of MYL2 with the MURF proteins may well be reflected in the clinical phenotype we observe in our patients who have no normal MYL2 protein.

In conclusion, identification of several recessive mutations affecting the same exon of MYL2 with similar effects on the protein has solved the genetic basis of this disorder that presented with clinical findings suggestive of a metabolic disorder rather than a primary cardiomyopathy. Its hallmarks are a rapidly progressive myopathy with hypotrophy of type I fibres, myofibrillar disarray and cardiomyopathy. This study defines this disorder as a new hereditary myosinopathy and extends the clinical range of symptoms due to mutations in myosins. In the case of the currently reported MYL2 mutations, they cause a very severe phenotype with morphological and structural skeletal abnormalities in addition to a cardiomyopathy that leads to early infant death.

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Supplementary material

Supplementary material is available at Brain online.

References


