Hereditary myopathy with early respiratory failure associated with a mutation in A-band titin

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Hereditary myopathy with early respiratory failure and extensive myofibrillar lesions has been described in sporadic and familial cases and linked to various chromosomal regions. The mutated gene is unknown in most cases. We studied eight individuals, from three apparently unrelated families, with clinical and pathological features of hereditary myopathy with early respiratory failure. The investigations included clinical examination, muscle histopathology and genetic analysis by whole exome sequencing and single nucleotide polymorphism arrays. All patients had adult onset muscle weakness in the pelvic girdle, neck flexors, respiratory and trunk muscles, and the majority had prominent calf hypertrophy. Examination of pulmonary function showed decreased vital capacity. No signs of cardiac muscle involvement were found. Muscle histopathological features included marked muscle fibre size variation, fibre splitting, numerous internal nuclei and fatty infiltration. Frequent groups of fibres showed eosinophilic inclusions and deposits. At the ultrastructural level, there were extensive myofibrillar lesions with marked Z-disc alterations. Whole exome sequencing in four individuals from one family revealed a missense mutation, g.274375T > C; p.Cys30071Arg, in the titin gene (TTN). The mutation, which changes a highly conserved residue in the myosin binding A-band titin, was demonstrated to segregate with the disease in all three families. High density single nucleotide polymorphism arrays covering the entire genome demonstrated sharing of a 6.99 Mb haplotype, located in chromosome region 2q31 including TTN, indicating common ancestry. Our results demonstrate a novel and the first disease-causing mutation in A-band titin associated with hereditary myopathy with early respiratory failure. The typical histopathological features with prominent myofibrillar lesions and inclusions in muscle and respiratory failure early in the clinical course should be incentives for analysis of TTN mutations.

Keywords: myopathy; respiratory failure; exome sequencing; titin; mutation

Abbreviations: HMERF = hereditary myopathy with early respiratory failure; SNP = single nucleotide polymorphism
Introduction

Respiratory failure as an early symptom in ambulant patients with inherited primary muscle diseases is uncommon. However, early respiratory failure is a frequent complication in a group of myopathies with characteristic myofibrillar lesions and cytoplasmic bodies (Jerusalem et al., 1979; Patel et al., 1983; Winter et al., 1986; Chapon et al., 1989; Edström et al., 1990; Chinnery et al., 2001; Tasca et al., 2010). These myopathies have more recently been referred to as hereditary myopathy with early respiratory failure (HMRF; OMIM #603689). Previous studies have indicated that they are genetically heterogeneous. The family described by Chapon et al. (1989) demonstrated linkage to 2q21 (Xiang et al., 1999) whereas two of the families that Nicolao et al. (1999) described were linked to 2q31. Sequence analyses in these two families demonstrated a heterozygous mutation (R279W) in the protein kinase domain of titin (TTN) (Lange et al., 2005). No other causative mutations have been demonstrated.

We describe the clinical, histopathological and muscle MRI findings in eight individuals from three apparently unrelated Swedish families with a dominantly inherited adult-onset myopathy characterized by proximal and distal muscle weakness and early respiratory failure compatible with a diagnosis of HMRF. Muscle histopathological features included extensive myofibrillar changes, fiber size variability, prominent fibre splitting, increased internal nuclei, rimmed vacuoles and frequent eosinophilic inclusions. Genetic analysis in our patients by whole exome sequencing disclosed a missense mutation in the C-terminus of the myosin binding A-band protein, titin, which was the apparent cause of disease in these families. This was further supported by a unique common haplotype using high-density single nucleotide polymorphism (SNP) arrays covering the entire genome.

Materials and methods

Patients and muscle biopsy

This study was approved of by the Regional Ethics Committee. Eight patients were examined clinically, six patients from Family A, one patient from Family B and one patient from Family C (Fig. 1). Clinical data of deceased patients were obtained from case records and relatives. Muscle weakness was evaluated according to the Medical Research Council scale (MRC). Investigations of the patients included serum levels of creatine kinase, 12-lead electrocardiogram, echocardiogram and pulmonary function tests. Electromyography was performed in three patients. MRI was performed in two patients from Family A (Patients III:14 and IV:6) of pelvic and lower limb on a 1.5 T magnetic resonance scanner.

Muscle biopsy was performed in seven of the affected patients, and in four of these a second biopsy was obtained after 7–10 years. Specimens were frozen in propane chilled by liquid nitrogen for histochemical analysis and fixed in glutaraldehyde for electron microscopy. Standard techniques were applied for histochemical staining of cryostat sections and for electron microscopy. Immunohistochemical analysis was performed with antibodies against neural cell adhesion molecule (CD56; Becton Dickinson), desmin (Dako), dystrophin (Novoceastra), titin (Biocytex), alpha-B-crystalline (Novoceastra) and myotilin (Novoceastra). EnVision™ Flex (Dako) was used to visualize immunoreactive material. Analysis of actin was performed by incubation of sections with phallolidin–rhodamine and fluorescence microscopy.

Genetic analysis

Exome sequencing

Genomic DNA extracted from blood samples from two affected (Patients III:14 and III:16) and two healthy (Subjects IV:2 and IV:5) members of Family A were analysed. Enrichment of coding exons and flanking intronic regions totalling 50 Mb was performed with the SureSelect human all exon kit following the manufacturer's standard protocol (Agilent). The DNA library consisting of paired-end reads was sequenced on an Illumina HiSeq™ 2000 instrument following the manufacturer’s standard protocol.

Base calling was performed by the Illumina pipeline with default parameters. All the raw reads were aligned to the reference human genome (UCSCg19) using the Burrows–Wheeler alignment (Li and Durbin, 2009). SNPs and indels were identified using the Genome Analysis Tool Kit (GATK) (DePristo et al., 2011). SNPs and indels with a Phred-like variant quality score of at least 30 were filtered against dbSNP131 and the 1000 Genome project to exclude previously identified SNPs. Novel insertions/deletions, splice-site, missense and nonsense variants were excluded if detected in any of the two healthy alleles.

Figure 1 Pedigree of Families A, B and C. Black solid symbols = definitely affected, proven by characteristic muscle biopsy findings. Grey solid symbol = probably affected, clinical signs and symptoms of hereditary myopathy with early respiratory failure.
individuals. Filtering against other in-house sequenced exomes was then performed to further reduce the number of candidate mutations. Functional consequences of the remaining non-synonymous variants were predicted using the SIFT algorithm and information regarding conservation for the variants among vertebrate species (phyloP score) by applying the UCSC genome browser. Variants that were predicted to be tolerated and/or not conserved among vertebrates were excluded.

**Sanger sequencing**

Sanger sequencing was performed using standard techniques of polymerase chain reaction amplicons with primers from genomic DNA to confirm the presence and identity of the variants in the ABCB11 and TTN genes after they had been identified by exome sequencing (primer sequences are available on request).

**Array analysis**

SNP array analysis was performed as previously described (Caren et al., 2008). Affymetrix 250K SNP arrays (Affymetrix) were used and data analysis was performed using GDA (GeneChip DNA Analysis software) and GTYPE (Affymetrix) for extraction of genotype calls. SNP genotype data for the individuals were analysed to identify regions free from incompatibilities. Data for six affected family members of Pedigree A (Patients III:14, III:16, IV:1, IV:4, IV:6 and IV:7), one in Pedigree B (Patient II:1) and one in Pedigree C (Patient II:1) were analysed to identify genomic regions fitting an autosomal dominant genetic model. The method is indirect in that it scores loci where the dominant gene cannot be localized, with the aim of finding a region where no such incompatibilities occur, i.e. the inferred disease locus. For each SNP locus, individuals can have either genotype calls ‘AA’, ‘AB’, ‘BB’ or a ‘NoCall’. A locus where at least one affected individual is ‘AA’ and at least one other affected individual is ‘BB’, is scored as an incompatibility. Such a locus is therefore an ‘incompati-bility’ and can by definition not be included in the correct disease gene haplotype. In contrast, a continuous region of SNP loci, without any incompatibilities among affected individuals, may include a unique disease haplotype and, consequently, also the disease genotype. Genotypes for all analysed affected individuals were compared and incompatibilities as defined above for all the 260,000 SNP loci were scored and plotted against the genome position for each locus. Corresponding genotype data generated by Affymetrix 250K array for 25 healthy control individuals corresponding to 50 haplotypes were included in the analyses. All genomic positions for SNPs are given relative to the February 2009 – GRCh37/hg19 genome assembly.

**Results**

**Clinical features**

**Family A**

All patients had a normal perinatal period and motor development. Six patients were clinically investigated (Table 1). Age at onset of disease in these patients varied from age 18 to 40 years old. The muscle weakness was slowly progressive and symmetrically distributed. Muscle atrophy was not a prominent finding. In two patients, myalgia was an early symptom. Muscle weakness in the pelvic girdle was found in all patients, and in three patients muscle weakness was also found in the shoulder girdle. There was no winging of scapula. The neck flexors and trunk muscles were severely affected in all patients, and they had a prominent calf hypertrophy. Knee flexor and ankle dorsiflexor muscle weakness was seen in five patients. All patients were ambulatory but showed signs of respiratory insufficiency with decreased vital capacity (range 20–74% of predicted value) (Table 2). Three patients needed a ventilator at night, from age 35, 37 and 55 years, respectively. Serum creatine kinase was either normal or slightly elevated (analysed in three patients). EMG was performed in one patient and showed myopathic changes. Electrocardiography and echocardiography were performed in all six patients and no signs of cardiomyopathy were found.

**Family B**

The two affected patients from Family B had a normal birth and neonatal period. The motor development was normal. Patient B II:1 died by respiratory failure at the age of 51 years. Patient B II:1 first noticed muscle weakness in the ankle dorsiflexors with frequent falls at the age of 22 and mild muscle atrophy was noticed after a few years. Clinical examination at the age of 30 years showed moderate to severe weakness in both pelvic and shoulder girdle muscles (MRC grades 2 and 3). There was no scapular winging. The neck flexor, trunk and ankle dorsiflexor muscles were more severely affected (MRC grades 1 and 2). Weakness was also seen in the elbow extensor and flexor muscles (MRC grade 4) as well as in finger extensor and flexor muscles (MRC grades 2 and 3). There was prominent calf hypertrophy. At the age of 30 years, he was able to walk up to 5 km but shortness of breath and myalgia were frequent symptoms. EMG showed myopathic changes. Electrocardiography and echocardiography showed no signs of cardiomyopathy.

The muscle weakness has been markedly progressive and from the age of 36 years, he had lost ambulation and needed a wheelchair. He had respiratory muscle weakness with shortness of breath from the age of 30 years and needed a ventilator at night from the age of 39 years. Clinical examination at the age of 43 years showed severe loss of function in most of the muscles in the lower extremities (Table 1) and severe muscle weakness in most of the muscles in the upper extremities. Blood carbon dioxide partial pressure was 8.1 kPa (normal value 4.4–6 kPa).

**Family C**

The two affected patients from Family C had normal motor development. Patient C I:2, a male patient, died by respiratory insufficiency at the age of 73 years. He suffered from a myopathy, which was diagnosed at the age of 55 years but was never further classified. In his daughter, Patient C II:1, transient respiratory insufficiency was for the first time observed at the age of 36 years when her youngest child was born. Approximately at the age of 40 years, she began to notice proximal muscular weakness in her upper and lower extremities. There was also weakness in her foot extensors but not in her hands. Because of gradual progress of the muscular weakness, the patient was referred for neurological examination at the age of 45 years. It demonstrated generalized muscular atrophy with corresponding weakness but sparing the facial muscles (Table 1). There was no winging of scapula. Cardiac examination by electrocardiography and
Table 1 Summary of clinical characteristics

<table>
<thead>
<tr>
<th>Patient (sex)</th>
<th>All:14 (F)</th>
<th>All:16 (M)</th>
<th>AIV:1 (F)</th>
<th>AIV:4 (F)</th>
<th>AIV:6 (M)</th>
<th>AIV:7 (F)</th>
<th>BII:1 (M)</th>
<th>CII:1 (F)</th>
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<tr>
<td>Age at onset (years)</td>
<td>30</td>
<td>35</td>
<td>30</td>
<td>40</td>
<td>20</td>
<td>18</td>
<td>22</td>
<td>36</td>
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<td>Initial symptoms</td>
<td>Weakness in shoulder girdle</td>
<td>Weakness in ankle dorsiflexors</td>
<td>Myalgia and weakness in pelvic girdle</td>
<td>Weakness in shoulder and pelvic girdle</td>
<td>Asymptomatic</td>
<td>Myalgia</td>
<td>Weakness in ankle dorsiflexors</td>
<td>Respiratory insufficiency</td>
</tr>
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<td>40</td>
<td>35</td>
<td>Asymptomatic</td>
<td>20</td>
<td>20</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>Age at investigation (years)</td>
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<td>66</td>
<td>56</td>
<td>49</td>
<td>41</td>
<td>37</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
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<td>Mild weakness in m. frontalis</td>
<td>Normal</td>
<td>Normal</td>
<td>Mild weakness in m. frontalis</td>
<td>Normal</td>
<td>56</td>
<td>Normal</td>
<td>56</td>
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<td>4</td>
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<td>2</td>
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<td>5</td>
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<tr>
<td>Wrist extension</td>
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<td>5</td>
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<td>5</td>
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<td>3</td>
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<td>5</td>
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<td>Finger extension</td>
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<td>Hip flexion</td>
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<td>3</td>
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<td>3</td>
<td>2</td>
<td>3</td>
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<td>2</td>
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<tr>
<td>Knee extension</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Ankle dorsiflexors</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ankle extensors</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Calf hypertrophy</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>nd</td>
</tr>
</tbody>
</table>

Muscle strength graded (0–5) according to Medical Research Council scale (MRC). The muscle strength were symmetrically distributed and MRC grades refer to both left and right side except in hip abduction in Patient AIV:6. F = female; M = male; nd = not determined; m. = musculus.
ultrasound was normal. EMG showed generalized myopathy in all extremities, most pronounced in the proximal muscles. Pulmonary function test showed decreased vital capacity to 70% of the expected normal value.

Magnetic resonance imaging

MRI was performed in two patients from Family A (Patients III:14 and IV:6). On T1-weighted sequences the fatty degenerative changes were symmetric and showed a unique distribution. In the pelvic region, severe replacement was present in the iliopsoas, rectus abdominis, obturatorius, gluteus minimus and the proximal parts of the gluteus maximus muscles. In the thighs, there was an unusual gradient with proximal parts being more replaced than distal parts of the individual muscles. The vastus lateralis, intermedius and medialis, as well as the sartorius, gracilis and semitendinosus muscles were severely involved, whereas the adductor longus muscles were relatively spared (Fig. 2). All distal parts of the thighs showed only mild fatty infiltration. In the lower legs, there was marked fibre size variability with atrophic and hypertrophic fibres in all biopsy specimens. Many fibres showed numerous internally located nuclei and there were focal areas with frequent split fibres (Fig. 3). The characteristic pathological changes were focal, frequently with groups of several fibres showing marked alterations, whereas other regions were less affected or even normal. There was also great variation between different muscle biopsy samples with regard to degree of pathological changes. Some of the biopsy specimens revealed marked fatty infiltration. No association between fibre type and histopathological changes was found. Eosinophilic inclusions or deposits were seen in all samples. The deposits were red or dark green in trichrome stained sections and some of them had the appearance of cytoplasmic bodies (Fig. 3). The cytoplasmic body-like inclusions were also variable accumulation of myotilin and \(-\)C11 accumulation of desmin, dystrophin and titin (Fig. 5B). There were several affected fibres with structural abnormalities, there were occasional cytoplasmic bodies surrounded by a halo of radially arranged thin filaments were found. Accumulation of granulo-filamentous material or dappled dense bodies as can be seen in desminopathy and some other myofibrillar myopathies were not identified. Structural alterations corresponding to rimmed vacuoles showed degradation products and lamellate myeloid structures. Collections of 15–20 nm tubulofilaments were observed in one patient. Most mitochondria were structurally normal but a few contained paracrystalline inclusions.

Muscle pathology

There was marked fibre size variability with atrophic and hypertrophic fibres in all biopsy specimens. Many fibres showed numerous internally located nuclei and there were focal areas with frequent split fibres (Fig. 3). The characteristic pathological changes were focal, frequently with groups of several fibres showing marked alterations, whereas other regions were less affected or even normal. There was also great variation between different muscle biopsy samples with regard to degree of pathological changes. Some of the biopsy specimens revealed marked fatty infiltration. No association between fibre type and histopathological changes was found. Eosinophilic inclusions or deposits were seen in all samples. The deposits were red or dark green in trichrome stained sections and some of them had the appearance of cytoplasmic bodies (Fig. 3). The cytoplasmic body-like inclusions were positive for F-actin using phalloloidin–rhodamine fluorescence analysis (Fig. 4). Rimmed vacuoles were seen in all patients to some degree. Interstitial inflammatory cells were rare and seen in only one biopsy specimen. In NADH-tetrazolium reductase stained sections, fibres with unstained; ‘rubbed-out’ regions were seen in more than half of the biopsies. No ragged red fibres were identified. In two patients, repeat biopsy was performed in the same muscle (tibialis anterior) (Table 3, Patients A IV:4 and A IV:6). In both patients, the second biopsy showed advanced atrophy with fat tissue replacement.

Immunohistochemical analysis revealed neural cell adhesion molecule upregulation in many of the muscle fibres (Fig. 5A). In several affected fibres with structural abnormalities, there were accumulation of desmin, dystrophin and titin (Fig. 5B). There was also variable accumulation of myotilin and \(\alpha\)-B crystallin.

In electron microscopy, Z-disk alterations were very frequent with Z-disk streaming and regions with extensive dispersion of semi-dense Z-disk material over the entire sarcomere (Figs 6 and 7). There were also large regions with myofibrillar disruption and irregular electron-dense deposits (Figs 6 and 7). Most of these deposits did not show the typical structure of cytoplasmic bodies but occasional cytoplasmic bodies surrounded by a halo of radially arranged thin filaments were found. Accumulation of granulo-filamentous material or dappled dense bodies as can be seen in desminopathy and some other myofibrillar myopathies were not identified. Structural alterations corresponding to rimmed vacuoles showed degradation products and lamellate myeloid structures. Collections of 15–20 nm tubulofilaments were observed in one patient. Most mitochondria were structurally normal but a few contained paracrystalline inclusions.

<table>
<thead>
<tr>
<th>Patient (sex)</th>
<th>Age (years)</th>
<th>Respiratory symptoms</th>
<th>VC standing (litres)</th>
<th>VC in per cent of predicted normal</th>
<th>VC supine (litres)</th>
<th>P(_{\text{CO}_2}) at day-time (normal 4.4–6 kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIII:14 (F)</td>
<td>67</td>
<td>Shortness of breath during exercise from age 55 years</td>
<td>1.2</td>
<td>50</td>
<td>1.1</td>
<td>5.18</td>
</tr>
<tr>
<td>AIII:16 (M)</td>
<td>66</td>
<td>Shortness of breath during exercise from age 40 years, nocturnal NIV from age 55 years</td>
<td>1.05</td>
<td>30</td>
<td>0.75</td>
<td>6.2</td>
</tr>
<tr>
<td>AIV.1 (F)</td>
<td>56</td>
<td>Asthma, shortness of breath during exercise from age 35 years</td>
<td>2</td>
<td>74</td>
<td>1.92</td>
<td>nd</td>
</tr>
<tr>
<td>AIV.4 (F)</td>
<td>49</td>
<td>Asymptomatic</td>
<td>1.8</td>
<td>58</td>
<td>1.76</td>
<td>4.9</td>
</tr>
<tr>
<td>AIV.6 (M)</td>
<td>41</td>
<td>Shortness of breath during exercise from age 20 years, nocturnal NIV from age 35 years</td>
<td>2.2</td>
<td>54</td>
<td>1.6</td>
<td>6.4</td>
</tr>
<tr>
<td>AIV.7 (F)</td>
<td>37</td>
<td>Shortness of breath during exercise from age 20 years, nocturnal NIV from age 37 years</td>
<td>0.73</td>
<td>20</td>
<td>0.54</td>
<td>9.7</td>
</tr>
</tbody>
</table>

F = female; M = male; nd = not determined; NIV = non-invasive ventilation; P\(_{\text{CO}_2}\) = carbon dioxide partial pressure; VC = vital capacity.
of Family A. Overall, ~43.4 million sequencing reads were produced for each of the samples and ~99% of these were aligned to the human reference genome (hg19) and 64% of these fell onto targeted and enriched exons (Table 4).

The exome coverage distribution showed that 80% of the exome had a read depth of 8× and 50% of the exome had a read depth of 30×. In each individual, between 38,300 and 40,200 variants were detected. In each individual, 4,500 to 5,040 variants (SNPs and indels) were not present in dbSNP131 or the 1000 Genome project, and of these 2,386 to 2,508 were nonsense, missense, framshift or splice site variants (Table 5). After exclusion of variants found in healthy individuals, 147 SNPs and 21 indels remained (Table 5). By further filtering of these against data from other in-house sequenced exomes and detection of possible pathogenic SNPs using SIFT algorithms, only four variants remained.

Two of these four variants were found in the gene ZNF594 (Homo sapiens zinc finger protein 594, NM_032530) located on chromosome 17. These two missense variants had a non-conserved region and therefore unlikely to be causative of the disease. The third variant was in exon 18 of the ABCB11 gene (ATP-binding cassette sub-family B member 11; NM_003742). It was a heterozygous mutation c.2093G>A, p.Arg698His, affecting a residue that is conserved among vertebrates but was recently reported in dbSNP134 as a polymorphism (rs138642043). The fourth variant was a heterozygous mutation g.274375T>C (AJ277892) in exon 343 of the TTN gene (Bang et al., 2001), resulting in a novel, missense mutation p.Cys30071Arg (Q8WZ42), changing a residue that is highly conserved among vertebrates (Fig. 8 and Supplementary Fig. 2). This was the obvious best candidate as a disease-causative mutation in Family A.

Sanger sequencing
Sanger sequencing to study the identified TTN and ABCB11 gene variants demonstrated that only the heterozygous TTN g.274375T>C mutation co-segregated with the disease in all three families. The mutation was not identified in any of 400 control chromosomes using Sanger sequencing.

Array analysis
DNA from selected members of the kindreds (Patients A III:14, A III:16, A IV:1, A IV:4, A IV:6, A IV:7, B II:1 and C II:1)
were subjected to analysis with Affymetrix 250K (260,000 SNPs) SNP arrays and subsequent data analyses. Analysis of the SNP array genotype data disclosed a region free of incompatibilities in chromosome region 2q (Fig. 9). In this region that spanned 769 SNP loci, there were very few incompatibilities, likely to be only rare genotyping errors. No other region in the genome came close in length to that on 2q. The region covers 6.99 Mb of DNA on chromosome region 2q, from marker rs6708551 in position 177,367,950 to marker rs2196690 in position 184,353,132 (Fig. 10 and Supplementary Fig. 1). Healthy control individuals

**Figure 3** Muscle biopsy sections of the vastus lateralis muscle from Patient B II:1. (A and B) Groups of fibres show marked structural alterations with splitting and numerous red or purple inclusions/deposits in trichrome stained sections (arrows). (C) The deposits are partly eosinophilic and there are numerous internalized nuclei (haematoxylin–eosin). (D) NADH-terazolium reductase stained sections show pronounced structural alterations, including rubbed out areas in some fibres (arrow).

**Figure 4** Serial sections of muscle biopsy from Patient B II:1 demonstrating that the purple or red deposits in trichrome staining contain actin. (A) Trichrome and (B) phalloidin–rhodamine fluorescence.
Table 3 Summary of muscle biopsy findings

<table>
<thead>
<tr>
<th>Patient</th>
<th>AllI:14</th>
<th>AllI:16</th>
<th>AIV:1</th>
<th>AIV:4</th>
<th>AIV:6</th>
<th>BII:1</th>
<th>CII:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at biopsy</td>
<td>56/66</td>
<td>58/58/65</td>
<td>43</td>
<td>41/48</td>
<td>33/40</td>
<td>29</td>
<td>44</td>
</tr>
<tr>
<td>Muscle</td>
<td>TA/QVL</td>
<td>TA/D/QVL</td>
<td>TA</td>
<td>TA/TA</td>
<td>TA/TA</td>
<td>TA</td>
<td>QVL</td>
</tr>
<tr>
<td>Type 1 fibres (%)</td>
<td>40/50</td>
<td>60/40/30</td>
<td>80</td>
<td>90/90</td>
<td>80/80</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>Fat and fibrous tissue replacement</td>
<td>–</td>
<td>–</td>
<td>Almost total</td>
<td>Marked/almost total</td>
<td>–/almost total</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Internal nuclei</td>
<td>+/+</td>
<td>++/+/+/+/+/+</td>
<td>+</td>
<td>++/+/+/+/+</td>
<td>++/+/+/+/+</td>
<td>++/+/+/+</td>
<td>++/+/+/+</td>
</tr>
<tr>
<td>Fibre splitting</td>
<td>+/+</td>
<td>++/+/+</td>
<td>+</td>
<td>++/+</td>
<td>++/+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rimmed vacuoles</td>
<td>++/–</td>
<td>++/–</td>
<td>+</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
<td>++/–</td>
</tr>
<tr>
<td>Eosinophilic inclusions/cytoplasmic bodies</td>
<td>++/+</td>
<td>++/+/+/+</td>
<td>+</td>
<td>++/+</td>
<td>++/+</td>
<td>++/+</td>
<td>++/+</td>
</tr>
<tr>
<td>Necrotic fibres</td>
<td>–/–</td>
<td>–/–</td>
<td>–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>Interstitial inflammatory cells</td>
<td>–/–</td>
<td>–/–</td>
<td>–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>Fibres with large, unstained regions in NADH-TR staining</td>
<td>–/+</td>
<td>++/+</td>
<td>+</td>
<td>++/+</td>
<td>++/+</td>
<td>++/+</td>
<td>++/+</td>
</tr>
</tbody>
</table>

D = deltoideus; QVL = quadriceps vastus lateralis; TA = tibialis anterior; – = not found; + = found but not abundant; ++ = abundant; +++ = in majority of muscle fibres.

Figure 5 Immunohistochemical analysis. (A) Neural cell adhesion molecule is expressed in the majority of muscle fibres (Patient A III:16). (B) Titin appears to be accumulated in some regions of abnormal fibres (arrows) (Patient A IV:4).

Figure 6 Electron micrographs. (A) In the periphery of the fibre, there is an elongated deposit of dense and partly fibrillar material (arrow) with connection with altered Z-disks. There is also Z-disk streaming (Patient A III:14). (B) Irregular electron-dense inclusions with partly fibrillar structure (Patient A III:14).
were analysed (one at a time) together with the set of affected individuals. For all of these individuals, the region on chromosome region 2q became covered with incompatibilities when they were analysed one by one, indicating that the haplotype shared by the affected were not present in any of the 25 control individuals (50 possible healthy haplotypes). The shared haplotype was either present in one of the individuals previously demonstrated with the mutation, R279W, or in the protein kinase region of TTN reported by Lange et al. (2005). The data strongly indicate that the depicted region represents a disease containing shared haplotype among the affected members of Families A, B and C.

**Discussion**

We describe eight individuals from three Swedish families with an adult-onset autosomal dominant myopathy demonstrating
characteristic myofibrillar lesions. Respiratory insufficiency, with shortness of breath during exercise and decreased vital capacity, was present early in the clinical course and most patients who needed ventilator at night were ambulatory.

The clinical and morphological characteristics are compatible with so-called HMERF (Edstro¨m et al., 1990; Lange et al., 2005). By whole exome sequencing, we identified the apparent causative mutation in the titin gene (TTN). Several lines of evidence support this conclusion: (i) this was the best candidate mutation remaining after our filtering of all identified variants; (ii) the mutation segregated with all affected individuals in the families; (iii) the mutation is located in the gene encoding the giant muscle protein titin, containing 363 exons, which was already shown to be mutated in other patients with HMERF just 15 exons from the present mutation (Lange et al., 2005); (iv) the mutation changes the highly conserved, small, uncharged and hydrophobic cysteine in position 30071 to the large, positively charged arginine; (v) it was not found in any of 400 control chromosomes; and (vi) genetic analysis in our three families with array data using SNP markers demonstrated that the affected individuals shared only one large haplotype on chromosome 2q31 corresponding to a 6.99 Mb region. This region encompasses 19 known genes including the TTN gene.

Titin is >1 µm long and spans half the sarcomere, from the Z-disk to the M-line. It is known to contribute to the myofibril assembly providing specific attachment sites for different sarcomeric proteins and it has an important role in the mechanism of muscle elasticity (Labeit and Kolmerer, 1995; Krüger and Linke, 2011). The mutated residue in our three families is located in a myosin-binding fibronectin-III (FnIII) domain of A-band titin (A150; Bucher et al., 2010). The previously described HMERF mutation, R279W, in the close but more C-terminal kinase domain of titin was considered to act by disrupting the mechanosensor signalling complex associated with the mutated regulatory tail of the kinase (Lange et al., 2005). We excluded a mutation in the protein kinase domain in our patients by exome sequencing as well as by Sanger sequencing. Our findings of a mutation in a relatively close but functionally completely different FnIII domain of the protein, causing an identical phenotype, raise questions concerning the downstream molecular pathogenesis. Perturbed interaction with
other sarcomeric proteins and possibly misfolding of defective titin protein may be common important factors leading to the same clinical and pathological phenotype. The variable penetrance and diversity among affected individuals may be explained by a variable expression of the mutated allele and variations in the structure and composition of proteins interacting with titin.

The histopathological hallmark in our patients included eosinophilic inclusions or deposits that were red or dark green in trichrome staining. As in the original description of the patients, later demonstrated to carry a mutation in the kinase domain of titin, the inclusions were stained intensely with rhodamine-conjugated phalloidin, which is a specific marker for F-actin (Edström et al., 1990). Thus different TTN mutations cause similar pathological changes. Irregular electron-dense deposits were found by electron microscopy but they only rarely had the typical structure of cytoplasmic bodies with a halo of radially arranged thin filaments around the deposits. There were very frequent Z-disk alterations with Z-disk streaming and regions with extensive dispersion of semi-dense Z-disk material over the entire sarcomere and large regions with myofibrillar disruption. In addition, there was muscle fibre necrosis and progressive replacement of muscle tissue by fibrous connective and fat tissue. Many of these features are typically found in myofibrillar myopathies (Selcen, 2011) and therefore, in terms of histopathological classification, HMERF should be considered to belong to this group of pathologically defined myopathies. An interesting finding was the variable degree of pathological changes in different parts of the same muscle biopsy. This is possibly due to variable expression of the mutated allele in different cells.

MRI examinations demonstrated selective involvement of specific muscles. In the pelvic region, our patients showed fatty replacement mainly of the iliopsoas, rectus abdominis, obturatorius and gluteus minimus muscles. Severely affected muscles in the thighs were the semitendinosus, gracilis, sartorius muscles and the vastus lateralis, intermedius and medialis muscles, whereas the adductor longus muscles were relatively spared. In the lower legs, there was fatty replacement predominantly in the anterior and lateral compartments. Muscle MRI findings (Birchall et al., 2005) in the family described by Chinnery et al. (2001) together with another British family showed a similar pattern of involvement; and in one patient, described by Tasca et al. (2010), a very similar pattern of muscle involvement was shown in different families suggesting that they may share a common pathogenetic mechanism.

Compared to the previously reported skeletal muscle titinopathies, HMERF is very dissimilar. The only other autosomal dominant disease is tibial muscular dystrophy, a late onset distal myopathy without clinical involvement of respiratory functions or muscles in the upper limb/body regions (Udd et al., 1993).
caused by missense or nonsense mutations in the last two exons (362 and 363) of titin and the only similarity with HMERF is rimmed vacuolar pathology and involvement of anterior lower leg muscles. In homozygous state tibial muscular dystrophy mutations cause a very different early onset severe recessive LGMD2J (Udd et al., 2005). Recessive nonsense mutations in the exons 358 and 360 are known to cause severe lethal cardiomyopathy with generalized skeletal myopathy with no clinical or pathological similarity to HMERF (Carmignac et al., 2007).

The affected individuals shared a haplotype on chromosome 2q31 corresponding to a 6.99 Mb region indicating a common ancestry and a founder mutation in these apparently unrelated three families. Since the penetrance is variable and some individuals do not experience any symptoms until an older age, it is possible that many other familial and apparently sporadic cases share this mutation.

In conclusion, we have identified three different Swedish HMERF families with a new mutation in the A-band titin. Further studies will show if this mutation and other TTN mutations cause most or all cases of HMERF.

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Supplementary material

Supplementary material is available at Brain online.
References


