OLAYINKA RAHEEM

Molecular Basis of Myotonic Disorders and New Diagnostic Techniques

ACADEMIC DISSERTATION
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Finland

Supervised by
Professor Bjarne Udd
University of Tampere
Finland

Reviewed by
Professor Basiel van Engelen
Radboud University Nijmegen
The Netherlands
Docent Minna Pöyhönen
University of Helsinki
Finland

Distribution
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33014 University of Tampere
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II. Tajsharghi H, Hilton-Jones D, Raheem O, Saukkonen AM, Oldfors A, Udd B. Human disease caused by loss of fast IIa myosin heavy chain due to recessive MYH2 mutations. Brain 2010; 133(Pt 5): 1451-1459.

III. Raheem O, Olufemi SE, Bachinski LL, Vihola A, Sirito M, Holmlund-Hampf J, Haapasalo H, Li YP, Udd B, Krahe R. Mutant (CCTG)n expression causes abnormal expression of zinc finger protein 9 (ZNF9) in myotonic dystrophy type 2. American Journal of Pathology 2010; 177(6): 3025-3036.

IV. Raheem O, Penttilä S, Suominen T, Kaakinen M, Burge J, Haworth A, Sud R, Schorge S, Haapasalo H, Sandell S, Metsikkö K, Hanna M, Udd B. New immunohistochemical method for improved myotonia and chloride channel mutation diagnostics. In press. Neurology.

ABBREVATIONS

ATP	adenosine-5´-triphosphate
ATPase	adenosine-5´-triphosphatase
cDNA	complementary deoxyribonucleic acid
CISH	chromogen *in situ* hybridization
ClC-1	chloride channel 1 protein
CLCN1	chloride channel 1 gene
CMD	congenital muscular dystrophy
DAB	3,3´-diaminobentzidine
DM	myotonic dystrophy
DM1	myotonic dystrophy type 1
DM2	myotonic dystrophy type 2
DMPK	dystrophia myotonica protein kinase
DNA	deoxyribonucleic acid
EMG	electromyography
FDB	flexor digitorum brevis
FIGE	field-inversion gel electrophoresis
FISH	fluorescent in *situ* hybridization
H&E	haematoxylin and eosin
IF	immunofluorescence
ISH	*in situ* hybridization
MTJ	myotendous junction
mRNA	messenger ribonucleic acid
MyHC	myosin heavy chain
NMJ	neuromuscular junction
NTB	nitro tetrazolium blue
pAb	polyclonal antibody
PCR	polymerase chain reaction
PROMM	proximal myotonic myopathy

PTC	premature termination codon
RA	repeat assay
RNA	ribonucleic acid
RP-PCR	repeat primed polymerase chain reaction
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
T-tubule	transverse tubule
WB	western blot
WT	wild type
ZNF9	zinc finger 9

ABSTRACT

Myotonic disorders are primary diseases of the muscle characterized by myotonia, the delayed relaxation of skeletal muscles after voluntary contraction. Myotonic disorders include the myotonic dystrophies (DMs) and non-dystrophic myotonias. Myotonic dystrophies type 1 (DM1) and type 2 (DM2) are multisystemic disorders caused by tri- (CTG)n or tetranucleotide (CCTG)n repeat expansion mutations in transcribed but not translated regions of the genes *DMPK* and *ZNF9*, respectively. Adult onset DM1 and DM2 share some features in the clinical presentation as well as in the molecular genetics and pathomechanisms. However, they also show distinct differences, including disease severity and involvement of muscles and muscle fiber types. So far, over 300 DM2 patients have been identified in Finland, which is in contrast to the prevalence estimate of $1/10^5$ (corresponding to 50 patients) reported previously. DM2 is of particular interest, because it shows a wide range of clinical manifestations and therefore a makes clinical diagnosis extremely difficult. Regarding molecular pathogenesis it has been suggested that *ZNF9* is of no significance for the disease pathogenesis, and that the disease is caused solely by RNA toxicity as a result of the underlying repeat expansion mutation. Such an exclusive explanation does not however explain the higher amount of toxic RNA in DM2 than DM1 muscle still resulting in milder clinical manifestations in DM2 compared to DM1. In this thesis work, we were able to show that ZNF9 expression in DM2 patients is in fact altered at multiple levels. While toxic RNA effects likely explain overlapping phenotypic manifestations between DM1 and DM2, abnormal ZNF9 levels in DM2 may account for at least some of the differences.

It is important to improve diagnostic accuracy in order to efficiently identify symptomatic patients for correct final diagnosis and appropriate medical attention and management. The different enzyme histochemical ATPase properties of myosins to separate the muscle fiber types have been utilized in diagnostic muscle

biopsy routine for more than four decades. The ATPase staining method is rather laborious and has several disadvantages, such as weakening of staining over time and non-specific staining of capillaries, making the distinction of extremely atrophic muscle fibers difficult. Extremely small atrophic type 2/IIA fibers are characteristic for DM2 and usually remain undetected using the ATPase staining method. A reliable and advanced immunohistochemical myosin double staining method for the identification of fiber types, including these highly atrophic type IIA fibers in routine diagnostics was developed in this thesis work. With this double staining method, it is easily possible to distinguish all different fiber types using a one slide technique.

In addition to the obvious usefulness in DM2 diagnostics we were able to identify a completely new disease with this technique, because of the absence of fast type IIA fibers in patients' muscle biopsies. The disease is caused by disruptive recessive mutations in the *MYH2* gene resulting in total absence of MyHC IIA protein and correspondingly total lack of fast type IIA muscle fibers.

Myotonia congenita is a non-dystrophic myotonia disease caused by mutations in the chloride channel gene (*CLCN1).* Currently, final diagnosis of patients with symptoms is frequently obtained by molecular genetic DNA testing. However, the increased use of genetic testing also results in many cases where the genetic results do not provide full clarification of the clinical disease.

In this thesis work, the developed immunohistochemical staining method for chloride channel protein (ClC-1) in muscle fibers proved to be a robust method for the assessment of sarcolemmal ClC-1 protein on muscle sections. This method provided means to identify new mutations, to reclassify the W118G *CLCN1* change as a moderately pathogenic mutation, and to clarify the final diagnosis in myotonia patients in whom only one recessive mutation had been identified by genetic testing.

The methods developed in this thesis work combined with genetic testing are powerful approaches to achieving final diagnosis in patients with myotonic disorders. Comprehensive understanding of the molecular pathomechanisims of genetic diseases is also one of the pre-requisites for the future development of therapeutical options.

TIIVISTELMÄ

Myotoniset sairaudet ovat lihastauteja, joihin liittyy myotoniaa. Myotonia on tila, jossa lihaksen tahdonalaisen supistuksen jälkeinen rentoutuminen viivästyy. Myotoniset sairaudet käsittävät myotoniset dystrofiat sekä ei-dystrofiset myotoniat. Tyypin 1 (DM1) ja tyypin 2 (DM2) myotoniset dystrofiat ovat monielinsairauksia, jotka johtuvat DNA-mutaatioista, kolmen (CTG)n tai vastaavasti neljän (CCTG)n emäksen toistojaksojen laajentumista vastaavissa geeneissä *DMPK* ja *ZNF9*. Aikuisiällä alkavalla DM1-taudin muodolla ja DM2-taudilla on joitakin yhtäläisyyksiä kliinisissä oireissa sekä mutaatiotyypin kautta tautien molekyyligenetiikassa ja patomekanismissa. Tautien välillä on kuitenkin selviä eroavaisuuksia kuten taudin vaikeusasteessa sekä eri lihaksien ja lihassyytyyppien vaurioitumisessa. DM2-taudin esiintyvyys Suomessa on taudin tunnistamisen alkuaikoina arvioitu olevan $1/10^5$, joka vastaa noin 50 potilasta, mutta tähän mennessä on jo yli 300 DM2-potilasta tunnistettu Suomessa. DM2-taudin kliininen taudinmääritys on erittäin vaikeaa, koska oireet ovat hyvin vaihtelevat, eikä niiden raja-alueita tarkkaan tunneta. Taudin molekyylipatogeneesiin liittyen, ZNF9-proteiini on ajateltu olevan vailla merkitystä taudissa ja taudin aiheuttajamekanismina on pidetty toistojakson tuottaman RNA:n haitallisuutta. Tämä yksinomainen selitys taudille ei kuitenkaan selitä sitä, että DM2-tauti on ilmiasultaan lievempi kuin DM1-tauti vaikka haitallista RNA:a on enemmän DM2-taudin solutumissa. Tässä väitöskirjatyössä pystyimme todistamaan, että ZNF9-ilmentymä DM2-potilaissa on poikkeava monella eri tasolla. Vaikka haitallisen RNA:n vaikutus voi hyvinkin selittää DM1- ja DM2-tautien ilmiasun yhtäläisyyksiä, poikkeavat ZNF9-ilmentymistasot DM2-taudissa voi ainakin osittain selittää joitakin tautien välisiä eroja.

Diagnostiikan tarkkuuden parantaminen on tärkeää, jotta oireellisten potilaiden oikea lopullinen diagnoosi ja asianmukainen hoito toteutuisivat. Myosiinien eri entsyymikudoskemiallisia ATPaasi-ominaisuuksia on käytetty lihassyytyyppien

erotteluun ja sitä on hyödynnetty lihasnäytteiden rutiinidiagnostiikassa yli neljän vuosikymmenen ajan. ATPaasi-värjäysmenetelmä on jokseenkin työläs ja värjäysmenetelmässä on monia haittapuolia, kuten ajan myötä tapahtuva värjäyksen haalistuminen ja epäspesifinen hiussuonten värjäytyminen, joka tekee erittäin pienten surkastuneiden lihassyiden erottelun kudoksessa vaikeaksi. Joidenkin tyypin 2A/IIA–lihassyiden surkastuminen on ominainen piirre DM2-taudille ja jäävät pääsääntöisesti tunnistamatta ATPaasi-värjäysmenetelmällä. Tässä väitöskirjatutkimuksessa kehitettiin uusi luotettava immunokudoskemiallinen myosiinien raskasketjujen kaksoisvärjäysmenetelmä eri syytyyppien erotteluun sekä kyseessä olevien hyvin surkastuneiden IIA lihassyiden tunnistamiseen. Tällä kaksoisvärjäysmenetelmällä voidaan helposti ja luotettavasti erotella kaikki lihassyytyypit yhdeltä näytelasilta.

DM2 diagnostiikan lisäksi pystyimme tällä uudella menetelmällä tunnistamaan täysin uuden taudin, jossa nopeat tyypin IIA lihassyyt puuttuivat potilaan lihasnäytteestä kokonaan. Taudin aiheuttaa resessiiviset mutaatiot *MYH2*-geenissä joiden seurauksena MyHC IIA-proteiini näillä potilailla puuttuu täysin ja vastaavasti heidän lihaksistaan puuttuu nopeat tyyppi IIA-lihassyyt.

Kongenitaalinen myotonia on ei-dystrofinen myotonia -sairaus, jonka aiheuttaa mutaatiot kloridikanavageenissä (*CLCN1*). Nykyään oireellisten potilaiden lopullinen diagnoosi saavutetaan usein molekyyligeneettisellä DNA-testauksella. Geenitestien lisääntyneen käytön seurauksena esiintyy myös monia tapauksia, joissa geenitulokset eivät tarjoa täyttä selvyyttä kliiniselle taudille.

Tässä väitöskirjatutkimuksessa kehitetty immunokudoskemiallinen värjäysmenetelmä kloridikanava-proteiinin (ClC-1) tunnistamiseen tarjosi hyödyllisen menetelmän sarkolemmaaliseen ClC-1 proteiinin arviointiin lihasleikkeistä. Tämän uuden menetelmän avulla löytyi uusia mutaatioita ja *CLCN1*-geenin W118G muutos voitiin luokitella haitalliseksi. Lisäksi pystyimme menetelmän avulla täsmentämään lopulliset diagnoosit kaikille myotoniapotilaille, joiden kohdalla lopullinen kannanotto oli aiemmin jäänyt auki kun oli löydetty vain yksi resessiivinen mutaatio geenitestien avulla.

Tässä väitöskirjatutkimuksessa kehitetyt uudet menetelmät yhdistettynä geenitestaukseen ovat tehokas tapa saavuttaa lopullinen diagnoosi myotoniaa sairastaville potilaille. Lisäksi diagnostiset mahdollisuudet ovat tarkentuneet myös muiden lihastautien osalta. Kattava ymmärrys geneettisten sairauksien

molekyylitason patomekanismeista ja näiden osoittaminen uusilla menetelmillä on paitsi diagnostiikan kannalta tärkeä, mutta myös perusedellytys mahdollisten tulevaisuuden terapiavaihtoehtojen kehittämiselle.

INTRODUCTION

Skeletal muscles are composed of long, multinucleated cells called myofibers, which are highly differentiated and are therefore unique in structure. Myotonic disorders are diseases of the muscle cell and they can be divided into myotonic dystrophies and non-dystrophic myotonias. This thesis focuses on myotonic dystrophy type 2 and chloride channel non-dystrophic myotonia, myotonia congenita. The final diagnosis of these diseases is based on mutation verification by genetic testing. However, clinical assessment, electrophysiological studies and muscle histopathology still have important roles in the diagnostics of muscle diseases and are combined to complement each other.

The clinical symptoms of DM2 are very variable between individuals, and myotonia can be present to variable degrees, clinically or electrophysiologically, or even totally absent. In addition to similarities of findings with adult onset myotonic dystrophy type 1 (DM1), there are significant differences making the two types of myotonic dystrophy clearly different diseases also by clinical presentation. As will be detailed later, both diseases are considered to be caused by toxic mutant RNA. However, the variability of symptoms in DM2 and the differences to DM1 are not easily explained by the reported pathomechanisim of RNA toxicity only. This is shown by the fact that the ribonuclear inclusions formed by the toxic mutant RNA are much more marked in DM2 than in DM1, whereas the myotonia considered to be the downstream effect of toxic RNA misplicing of *CLCN1* gene is much more severe in DM1 than DM2. The search for additional molecular mechanisms is a major part of current research.

Myotonia congenita is caused by primary mutations in the chloride channel gene (*CLCN1*). Numerous mutations causing the disease have been identified in the gene. Molecular genetic diagnosis is the gold standard for having full understanding of the underlying mechanisms explaining the symptoms in the patients. However, identified and known mutations do not always explain the disease in all situations.

Patients may have only one heterozygous mutation identified or may have mutations of uncertain pathogenic significance. Sequencing the whole gene in all situations is rather expensive and still it is not always clear if the changes identified in the gene cause the disease. For practical diagnostic purposes additional tools in the assessment of myotonia patients are needed.

Fiber type distribution, fiber size and shape of individual fiber types are important diagnostic tools in skeletal muscle histopathology. The observations based on fiber type composition can help in guiding towards the correct diagnosis. Comprehensive fiber typing also serves as an important tool for differentiating whether the disease is neurogenic or myopathic. In addition, it may also help in understanding disease pathomechanisims. The main technique for differentiating fiber types has been based on ATPase enzyme histochemistry. Due to methodological drawbacks this technique does not provide identification of extremely atrophy fibers which are one hallmark of histopathological changes in DM2. Again, new tools for fiber typing in muscle pathology was needed and the new techniques developed in the present research proved to be very effective, not only for the identification of correct pathology in DM2, but also for the identification of a totally new previously unknown disease.

REVIEW OF LITERATURE

1. The skeletal muscle

Muscle cells and tissues have the capacity of contraction. This generates a mechanical force that is needed for many different bodily functions, including locomotion, maintaining posture, breathing, heart beat and digestion, among others. There are three main muscle types, all composed of elongated cells. The main muscle types are the smooth muscle, striated skeletal muscle and striated heart muscle. The heart muscle is an involuntary striated muscle present only in the heart. The involuntary smooth non-striated muscle is present mainly in the walls of internal organs such as blood vessels, bladder, uterus, respiratory and digestive tracts. The skeletal muscle is the most abundant tissue in the human body. There are more than 450 different skeletal muscles which differ in size and shape and are organized to cause voluntary movement (Torta and Garbowski 2003; Alberts et al. 1994).The skeletal muscle cell, the myofiber is one single large cell up to several centimeters in length and surrounded by connective tissue, endomysium, and capillaries. Bundles of muscle fibers form fascicles enveloped by the connective tissue perimysium. The muscle is composed of bundles of fascicles that are enclosed in a strong connective tissue sheath epimysium called the muscle fascia. At the ends of one muscle, the fascial tissue continues as a tendon or some other arrangement of collagen rich connective tissue that attaches the muscle to other body structures, typically skeletal bones, forming a myotendous junction (MTJ) (figure 1) (Torta and Garbowski 2003; Dubowitz and Sewry 2007). Axons of motor nerves innervate the muscle fibers. A motor unit is composed of a single parent motor neuron with its axon and all the muscle fibers it innervates with its branches. Depending on the need

for fine control of movements, one motor unit consists of just a few muscle fibers, or if force is more relevant, several hundred fibers. The branched axons form nerve terminals located in grooves on the surface of the muscle fiber, invaginations of the sarcolemma (see below), and form a synapse called the neuromuscular junction (NMJ).(Engel 2004).

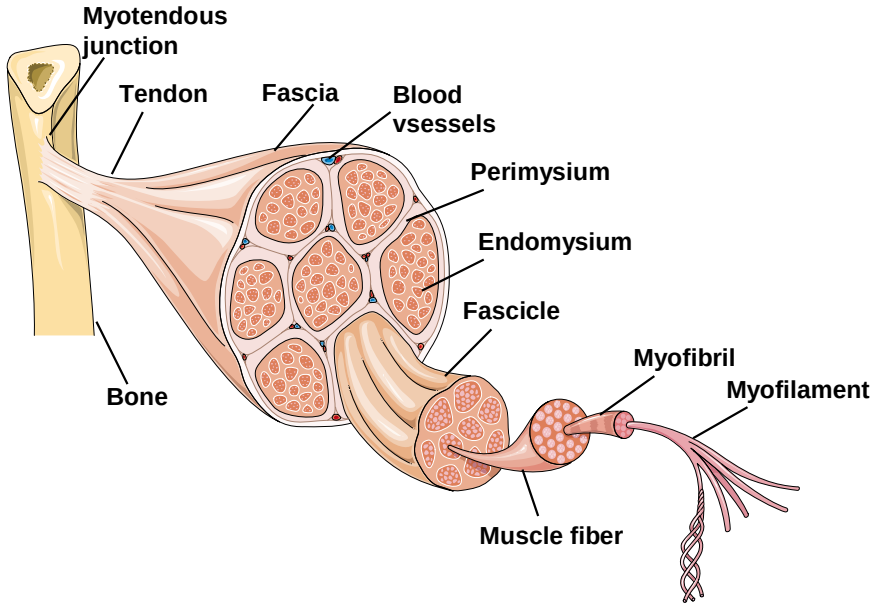


Figure 1. *Structure of the skeletal muscle. (The figure was generated using Servier Medical Art at http://www.sevier.com)*

1.1 Myogenesis

Skeletal muscles are derived from progenitor cells present in somites. Each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome, from which myogenic precusors originate. The embryonic tissue, which develops into skeletal muscle is called the myotome. It divides into epaxial and hypaxial myotome, developing into axial and limb muscles respectively (Deries et al. 2010; Biressi et al. 2007). Only a fraction of myogenic progenitor cells terminally differentiate during primary myotome formation. These developmental steps involve different types of embryonic and fetal myoblasts and satellite cells (Biressi et al 2007). Myoblasts fuse into myotubes in which the assembly of muscle

specific contractile proteins begins. During differentiation, the organization of the cellular organelles and the plasma membrane of the myoblasts changes dramatically. The process involves recognition of the microtubular network characterized by the re-localization of microtubule nucleating sites at the surface of the nuclei in myotubes, in contrast with the classical pericentriolar localization observed in myoblasts (Tassin et al 1985). Also new muscle specific organelles such as the sarcoplasmic reticulum (SR) and transverse (T-) tubules are formed, The plasma membrane of the fused myoblasts is together with basal lamina and transformed into the sarcolemma of the muscle fiber (Flucher et al. 1992).

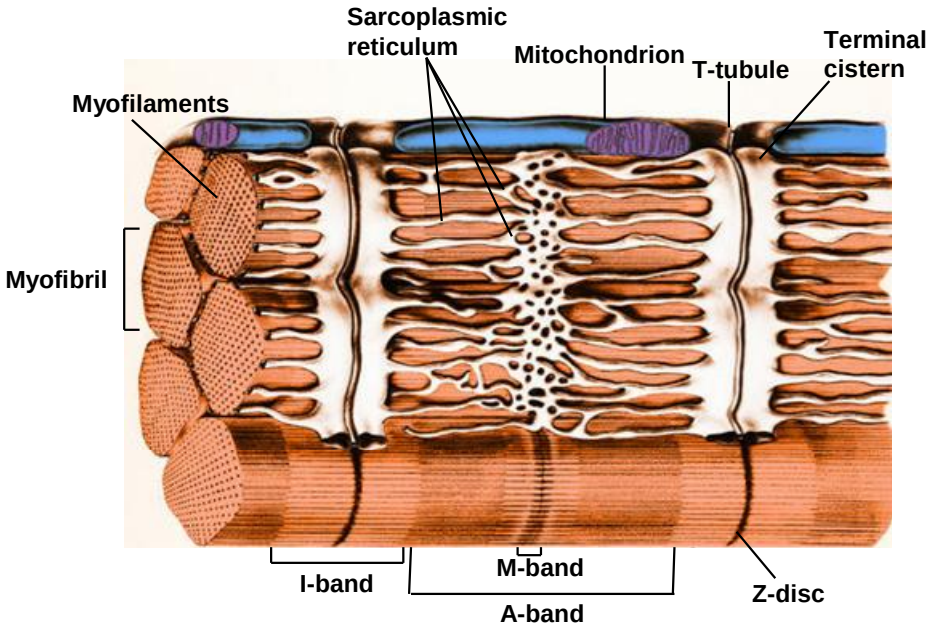


Figure 2*. The structure of the contractile machinery of the skeletal muscle. Myofibrils are surrounded by the sarcoplasmic reticulum. The triad structure consists of a T-tubule surrounded by terminal cisternae of the sarcoplasmic reticulum near the A-I junction. (Drawing modified from science photo library at http://www.sciencephoto.com)*

1.2 Structure and function of myofibers

Myofibers are highly heterogeneous because of different anatomical, physiological and biochemical features. Myofibers are large cylindrical skeletal muscle cells surrounded by the sarcolemma composed of the plasma membrane and basal lamina. They consist of multiple nuclei scattered along the fiber length just beneath the sarcolemma. Myofibers are 10 μm to 100 μm in diameter and can be up to several centimeters long. The myofibers are composed of contractile myofibrils and the cytoplasm, called the sarcoplasm. The sarcoplasm contains an elaborate membrane system consisting of an extensive SR and T-tubular system (Figure 2), besides organelles such as mitochondria, Golgi, lysosomes, etc. The basic unit of the contractile myofibrils is the sarcomere, which is composed of myosin-containing thick filaments in the A-band, of actin-containing thin filaments that span the I-band, of a dense Z-disc (also called the Z-band or Z-line) constituting lateral boundaries of the sarcomere and connecting thin filaments from neighboring sarcomeres as well as the backbone of the sarcomeric structure: the titin based third filament system. Titin is the largest known protein in nature and one single molecule spans half the sarcomeres from the Z-disc to the center of the A-band: the M-band. Another giant protein, nebulin, spans the length of the thin filaments and forms the fourth type of filament structure in the sarcomere (figure 3). (Dubowitz and Sewry 2007; Clark et al. 2001).

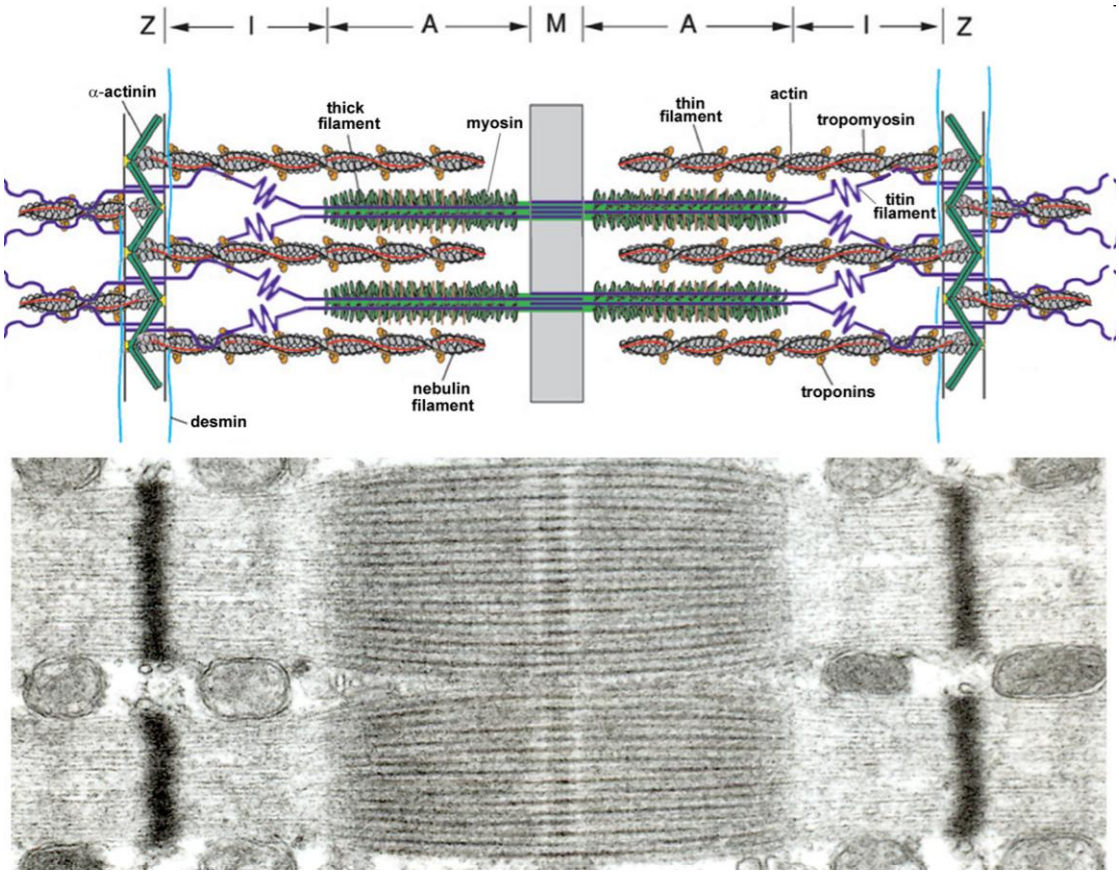


Figure 3. Schematic representation of the major structural components of the sarcomere (top). Electron micrograph of the corresponding ultrastructure, showing mitochondria between the myofibrils. (Ottenheijm et al. Respiratory Reseach 2008).

1.2.1 Myosin

In the muscle fiber, actomyosin filaments are the principal structural contractile proteins of the sarcomere. (Rayment et al. 1993). Myosin is a molecular motor that converts chemical energy into movement. This is established by the sliding of actin and myosin filaments over each other after hydrolysis of ATP providing energy for this process. (Ruppel et al. 1996). The myosin thick filament consists of hexameric myosin molecules formed by two heavy chains (MyHC) and four associated light chains. The N-terminal part of the myosin heavy chain protein forms a globular head which is the site of the myosin ATPase, an enzyme that hydrolyzes ATP required for the actin and myosin cross-bridge formation. These heads interact with

a binding site on actin. (Ruppel et al. 1996). Myosin heavy chain proteins are encoded by several different genes and thus exist in several different isoforms, which are expressed in a tissue-specific and developmentally regulated manner. More than one MyHC isoform may be expressed in each myofiber at developmental stages, but in the single adult mature myofiber only one isoform is expressed. (Izumo et al. 1986).

1.2.2 Myosins in relation to muscle fiber type distribution and myosinopathies

Skeletal muscles are composed of variable proportion of different fiber types that have been determined as fast and slow types based on their physical properties of appropriate force and duration of their contraction. The different fiber types, slow type1 and fast types 2A and 2B, were first identified on muscle histological sections in the 1960's by histochemical ATPase reaction differences at different pH levels, a technique that is still in use for routine diagnostic fiber typing purposes (Dubowitz and Sewry 2007). These differences in the fiber types are based the molecular fact that the different myofibers express different MyHC isoforms.

In adult human skeletal muscle fibers the MyHC isoform expressed in slow aerobic, type 1, fibers is encoded by the *MYH7* gene on chromosome 14, which is also the main isoform of cardiac muscle. In the fast aerobic type 2A fibers the corresponding MyHC isoform IIA is encoded by the *MYH2* gene in chromosome 17. (Weiss et al 1999). Mutations in *MYH7* gene have been reported to cause both skeletal and cardiac or combined myopathies (Dye et al.2006; Oldfors 2005;,Tajsharghi et al. 1997; Meredith et al. 2004; Dubourg et al 2011), whereas mutations in *MYH2* were previously reported in rare families with skeletal myopathy (Martinsson et al. 2000; Oldfors et al. in Karpati et al. 2002). In the ultrafast glycolytic type 2B fibers the corresponding MyHC IIX is expressed by the *MYH1* gene, but so far no human disease was associated with mutations in the gene.

Hybrid fibers expressing both fast and slow myosin heavy chains occur in most pathological state as a result of reprogramming in altered muscle fibers. Such secondary changes in the expression of MyHC genes are also useful for the assessment of muscle biopsies and reflect the plasticity of muscle. In harmful events

in the muscle fiber, irrespective of due to disease, toxic or mechanical injury, a process of reprogramming of the gene expression to overcome the alteration is turned on which includes the expression of developmental MyHC isoforms, fetal and neonatal isoforms, in adult muscle tissue. Although the exclusive expression of one MyHC gene per fiber is pre-programmed (Meredith et al. 2004; Martinsson et al. 2000; Oldfors et al. 2002; Laing et al. 1995), various exogenic influences can modulate the expression, such as thyroid hormone, and innervation can also influence and induce isoform transitions. (Mahadavi et al. 1986; Pette and Vrbova 1992).

1.3 Muscle biopsy

The main indication for a muscle biopsy are the clinical findings suggesting neuromuscular disease which would not be possible to clarify without a muscle biopsy. The selection of the site of the biopsy may be crucial to show the relevant abnormalities in the biopsy.

Muscle biopsies are immediately snap frozen, in isopentane cooled with liquid nitrogen to prevent formation of ice crystals. When used for histology, the biopsy should be oriented for cross sections of muscle fibers and embedded in a supporting material, an O.C.T compound before freezing. Freezing the tissue prevents degradation and in this form it is suitable for different types of analysis, including enzyme histochemistry, immunohistochemistry, in situ hybridization, protein extraction, DNA and RNA extraction (Meola et al. 2012).

1.4 Muscle biopsy histology

For diagnostic purposes, sections of suitable thickness usually 6-10 µm are cut in a cryostat (cryomicrotome) from frozen muscle biopsies. The sections may be used for histological stainings that help in observing different structural components in the muscle tissue and identifying pathological changes. Routine histological stainings performed vary between different laboratories. However, one of the most

widely used staining method is the haematoxylin and eosin (H & E) staining. This shows the overall morphology of the tissue. Haematoxylin stains acidic molecules blue, such as nucleic acids in the nuclei. Also, regenerating fibers stain bluish due to the large amount of RNA molecules in these fibers. Eosin stains muscle fibers pink and connective tissues a lighter shade of pink. The modified Gomori trichrome staining in which the muscle fibers stain greenish-blue stains the mitochondria red, resulting in a darker staining of slow oxidative fibers. Rimmed vacuoles and the presence of nemaline rods are also revealed in red. Abnormal cytoplasmic bodies appear more intensely stained red-blue. The oxidative enzyme histochemical staining, reduced nicotinamide adenine dinucleotide dehydrogenase-tetrazoleum reductase (NADH-TR) staining identifies the mitochondrial pool and also T-tubules and the sarcoplasmic reticulum (SR). Oxidative enzymes of the mitochondria, cytochrome c-oxidase (COX) and succinate dehydrogenase (SDH) can also be stained. They are often combined in the staining where SDH and COX positive fibers are brown and COX negative fibers appear blue in case of compensatory mitochondrial increase in molecular defects of the mitochondria involving COX deficiency. In routine diagnostic histopathology it is important to be able to differentiate fiber types. This has conventionally been achieved by the ATPase staining, however, immunohistochemical myosin heavy chain staining for different fiber types is shown to be even more reliable. (publication I in this study; Dubowiz and Sewry 2007). In figure 4, examples of histochemical stainings are shown. Targeted immunohistochemical stainings also performed from frozen sections provide a wider opportunity in the histopathologic diagnostics of muscular disorders (Meola et al. 2012).

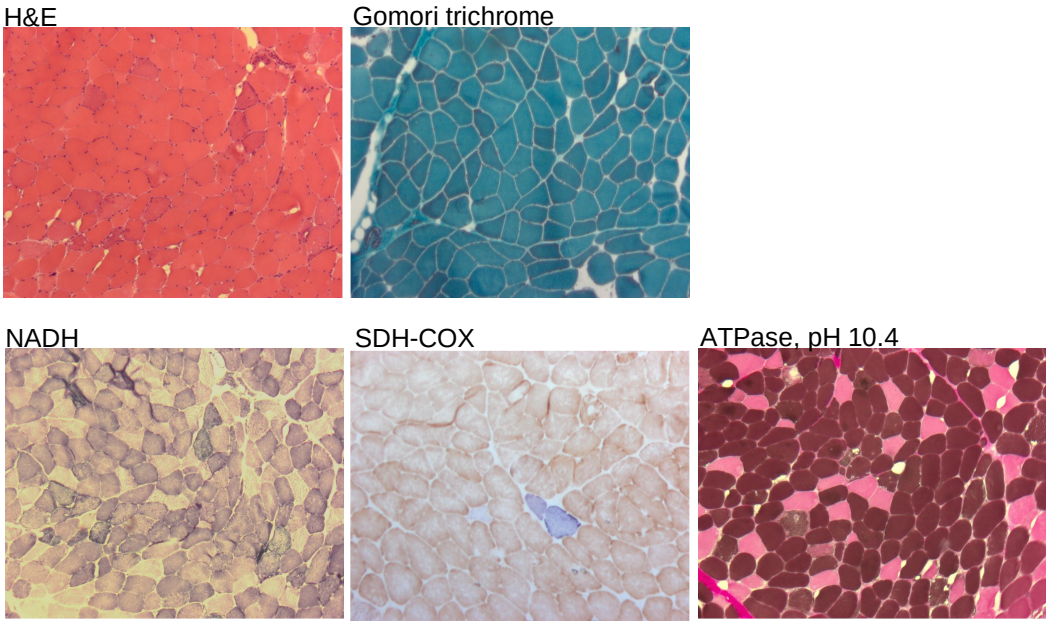


Figure 4. *Histochemical and enzyme histochemical stainings of the skeletal muscle. H&E, haematoxylin and eosin; NADH-TR, nicotinamide adenine dinucleotide dehydrogenase-tetrazoleum reductase; SDH-COX, succinate dehydrogenase-cytochrome c-oxidase; ATPase, adenosine-5´-triphosphatase. Combined SDH-COX staining showing one blue SDH positive COX negative fiber.*

2. Muscular dystrophies

Neuromuscular disorders can be divided into 1) myopathies, primary disease of the muscle fiber, 2) myasthenias, diseases caused by defects of the neuromuscular junction and 3) neurogenic muscular atrophies, caused by the defects of the motor nerve.

Muscular dystrophies are a heterogeneous group of myopathies. They are genetic disorders caused by muscle fiber degeneration often causing progressive weakness and wasting (modified from: Karpati 2001; Anthony et al. 2010).

Muscular dystrophies can be further divided into the following:

- Myotonic dystrophies – DM1 and DM2
- Dystrophinopathies - DMD, BMD
- Facioscapulohumeral - FSHD
- Limb-girdle – LGMD subtypes
- Distal muscular dystrophies
- Congenital dystrophies - CMD
- Oculofaryngeal - OPM, OPDM
- Emery-Dreifuss - X-EMD, AD-EMD
- Other and unclassified muscular dystrophies

3. Myotonic disorders

Myotonia is the delayed relaxation of skeletal muscle fibers after voluntary contraction (Harper 2001). Patients with myotonia may report painless muscle stiffness immediately upon initiating movement after a period of rest. For example, the inability to release handgrip after a strong handshake or difficulties rising from the chair or climbing stairs after a period of sitting (Heatwole and Moxley 2007).

Clinical myotonia is the cumulative result of electrical hyperexcitability of individual muscle fibers. Needle electromyography (EMG) reveals spontaneous runs of motor unit potentials with a characteristic waxing and waining of the frequency and the amplitude (Streib et al. 1987).

Myotonic disorders include the myotonic dystrophies (DM1 and DM2) and non-dystrophic myotonias. Persistent depolarization of the myotonic muscle is due to abnormal function or amount of ion channels in the muscle membrane. Mutations in the genes encoding the voltage-gated alpha-subunit of sodium channel (*SCN4A*) or the voltage gated chloride channel (*CLCN1*) expressed in skeletal muscle result in myotonia without muscular atrophy or degeneration, these are the non-dystrophic myotonias (George et al. 1994; Lerche et al. 1996; Heatwole and Moxley 2007; Ryan et al. 2007). By contrast, myotonic dystrophies cause muscle degeneration

with weakness and atrophy, and are caused by a DNA repeat expansion mutations in dystrophia myotonica protein kinase (*DMPK*) coding gene in DM1, and in zinc-finger 9 (*ZNF9*) gene in DM2. (Liquori et al. 2001; Brook et al. 1992; Fu et al. 1992; Mahadevan 1992).

Co-segregation of DM2 with *CLCN1* mutations have also been reported with clear myotonia as a symptom (Suominen et al. 2008, Cardani et al. 2012).

3.1 Myotonic dystrophies DM1 and DM2

Myotonic dystrophy (Dystrophia myotonica, DM) is the most common inherited muscular dystrophy in adults. Two different types of myotonic dystrophy have been identified. Both myotonic dystrophy type 1 (DM1, Steinert´s disease [OMIM #160900]) and type 2 (DM2, [OMIM #602668]) are autosomally dominantly inherited disorders caused by repeat expansion mutations. The estimated prevalence of DM1 is 1/8000 (Harper. 2001), while in DM2 the prevalence has not been established, but is considered to be even as common as DM1 in many European populations (Udd et al. 2011; Suominen et al. 2011).

DM1 was described one hundred years ago and the (CTG)n trinucleotide repeat expansion mutation in the 3´untranslated region (UTR) of the *DMPK* gene was identified in 1992. The gene is located in chromosome 19q13.3 (Brook et al. 1992; Fu et al. 1992; Mahadevan et al. 1992). The mutation underlying DM2 disease is a (CCTG)n tetranucleotide expansion located in the first intron of *ZNF9* gene on chromosome 3q21 (Liquori et al. 2001) and a single founding mutation of European origin has been suggested (Bachinski et al. 2003; Coenen et al. 2011).

The repeat expansion size in DM1 may vary from more than 50 to more than 3000 repeats and there is a gross correlation between repeat length and disease severity. The number of repeats in the expansion mutation causing DM2 varies from 75 to 11000. No correlation in the disease severity and the size of the expansion mutation has been shown in DM2. There is no clear evidence for anticipation in DM2 as there is in DM1. Due to this, successive generations inherit increasing disease severity with decreasing age of onset due to increased size of the repeat expansion (Day et al 2003). In both DM1 and DM2 the molecular pathomechanism

is based on RNA gain-of-function. Transcription of the repeats into mutant $(CUG)_{DM1}$/$(CCUG)_{DM2}$-containing RNAs is both necessary and sufficient to cause disease by formation of ribonuclear foci and interference of the splicing of downstream "effector" genes through trans-acting splicing factors, namely muscleblind 1 (*MBLN1*) (Osborne et al. 2006; Ho et al. 2005) and CUG binding protein 1 (CUGBP1) (Timchenko et al. 1996). Several 'effector' genes including, *CLCN1* (chloride channel-1), *INSR* (insulin receptor), *TNNT2* (cardiac troponin T), *TNNT3* (skeletal fast troponin T), *ZASP*, *ATP2A1* (*SERCA1*) and *MAPT* (microtubule-associated protein tau) show aberrant splicing in DM1, and in DM2. (Charlet-B N et al. 2002; Mankodi et al 2002; Maurage et al. 2005; Savkur et al. 2004; Vihola et al 2010)

3.1.1 Symptoms of DM2

The major symptoms of DM2 include late-onset proximal muscle weakness, myalgic muscle pain and/or stiffness, cataracts, myotonia, tremors, cardiac conduction defects and endocrinological abnormalities (Udd et al. 2003). In DM1, the muscle weakness and wasting is more severe, prominently distal and facial, with ptosis, late dysphagia and respiratory failure. Involvement of the brain has also been reported in both DM1 and DM2 (Meaola 2010; van Engelen et al. 2010; Tieleman et al. 2011). Unlike DM1, DM2 has no congenital or childhood onset form of the disease, with developmental multisystem abnormalities. Compared to adult-onset DM1, clinical symptoms are generally milder with normal life expectancy, more inconsistent and extremely diverse in DM2 (Udd et al. 1997; Udd et al. 2003).

In any given DM2 patient, any of the core symptoms may be absent, and myotonia may be variable over time in the same individual. A number of less consistent findings are occasionally associated with this disorder, making the clinical diagnosis a challenge (Udd et al. 1997; Udd et al. 2003; Udd et al. 2011; Schneider et al. 2000; Meola et al. 2004; Tieleman et al 2009; Auvunen et al 2008; Krahe et al 2006).

3.1.2 Muscle histopathology, differences between DM1 and DM2

The only common histological feature in both DM1 and DM2 is the increased variation in fiber size and number of internal nuclei (figure 5). Ring fibers and sarcoplasmic masses are characteristic features only in DM1, whereas nuclear clump fibers without neurogenic changes are prominent in DM2 and are present even before clinical muscle weakness in proximal lower limb muscles (Vihola et al 2003; Schoser BG, et al. 2004). In DM1 mild type 1 fiber hypotrophy can be present, whereas in DM2 invariably a subpopulation of type 2A fibers are extremely atrophic, including the nuclear clump fibers. These are not detected by conventional ATPase staining, and have therefore not been observed in the early descriptions of the disease. Extremely atrophic type 2A fibers characteristic for DM2 are shown in figure 6.

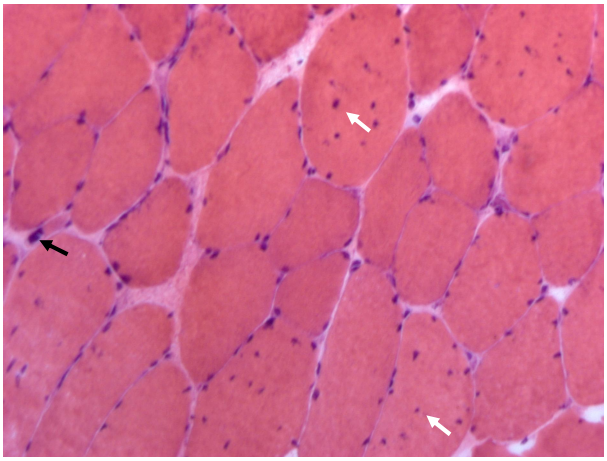


Figure 5*. H & E staining of DM2 muscle showing fiber size variation, several internal nuclei (white arrows) and nuclear clump fibers (black arrows).*

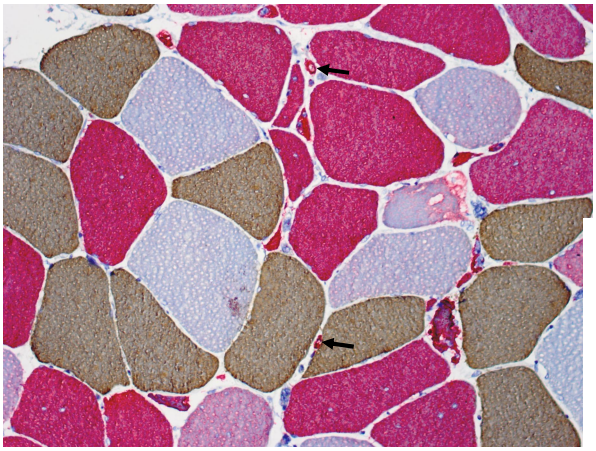


Figure 6. *Myosin heavy chain double staining of DM2 muscle showing several highly atrophic type 2A(IIA) fibers stained red (arrows).*

3.1.3 Diagnostics of DM1 and DM2

Mutation verification by genetic testing is the gold standard in all genetic diseases. In adult onset DM1 symptoms are usually very clear and distinctive enough to make clinical diagnosis and the mutation can be confirmed most commonly with a direct PCR across the mutation locus or using a Southern blot analysis for repeats larger than 100 (Ashizawa et al. 2000; Brook et al. 1992; Mahadevan et al 1992). However, because symptoms in DM2 are inconsistent and more variable, clinical diagnosis is much more difficult, and proceeding with genetic testing in patients where DM2 is not excluded is essential for correct diagnosis. Due to somatic instability and the extremely large amount of repeats, Southern analysis and direct PCR have proven to be insufficient for mutation detection in DM2 (Day et al. 2003). A DM2 repeat assay (RA) (Day et al. 2003) and a repeat primed PCR (RP-PCR) method (figure 7).(Bachinski et al. 2003) are currently used methods for adequate diagnosis. A two step molecular diagnostic procedure is convenient but any diagnostic laboratory needs to have access to different methods to confirm equivocal results (Udd et al. 2011). First, PCR-based allele sizing across the DM2 (CCTG)n for the exclusion of DM2 in individuals with two normal amplifiable alleles. The second step is the RP-PCR with 99% accuracy (Bachinski et al. 2003). In addition to RA and RP-PCR, other methods can also be used to genetically verify DM2. Methods such as Long-range PCR (Bonifazi et al. 2004) and a tetraplet-primed PCR (TP-PCR) (Catalli et al. 2010) have been used. The modified Southern method using field-inversion gel electrophoresis (FIGE-S) is efficient in determining mutation length in addition to the mutation verification. In addition, in situ hybridization protocols either using fluorescent (FISH) or chromogen (CISH) (figure 8) labels on muscle sections for the direct detection of the genomic expansion mutation and the mutant RNA foci in the nuclei of affected individuals have been shown to be effective (Sallinen et al. 2004).

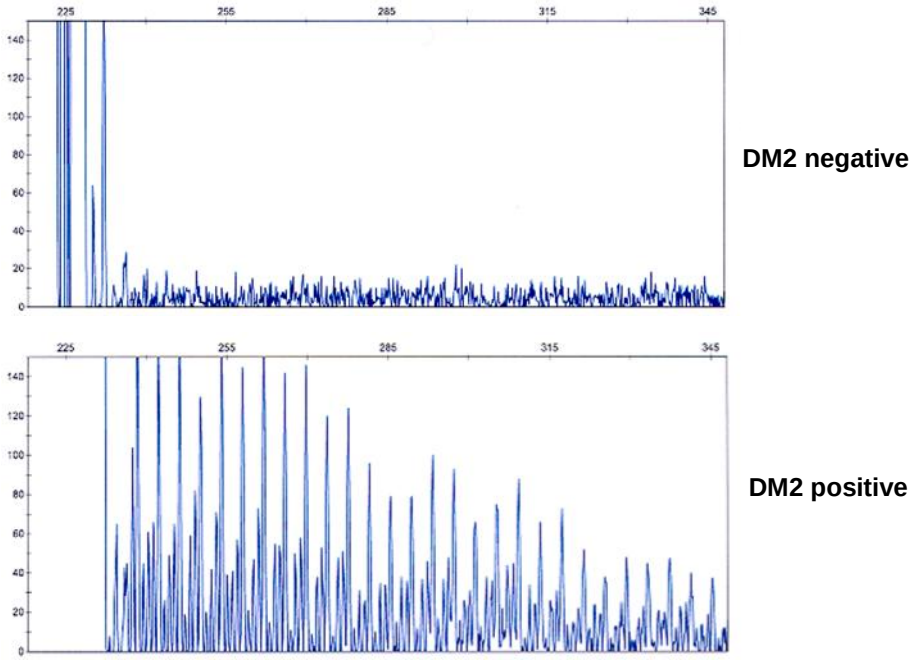


Figure 7. *RP-PCR analysis of DM2 negative (top) and DM2 positive (bottom) patient samples (Picture kindly provided by Tiina Suominen, Neuromuscular Research Unit, Tampere).*

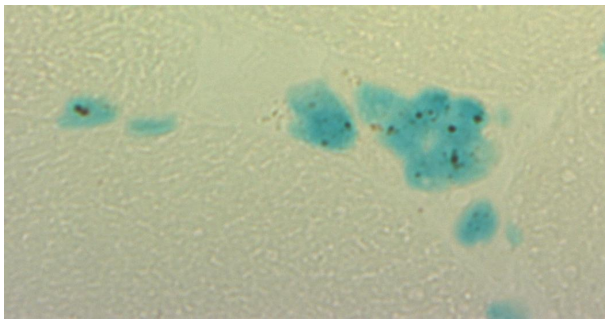


Figure 8*. CISH analysis of DM2 patient muscle showing nuclear RNA foci using antisense* $(CAGG)_8$ *probe. Foci not present in DM2 negative patient muscle.*

3.2 Non-dystrophic myotonias

The two most common non-dystrophic myotonias are the chloride channelopathies and the sodium channelopathies. The term myotonia congenita is usually used for chloride channel myotonias. Paramyotonia congenita, potassium-aggravated myotonia and hyperkalemic periodic paralysis with myotonia and their

variants comprise the non-dystrophic sodium channel myotonias (Rudel et al. 1995; Heatwole and Moxley 2007; Meola et al. 2009).

3.2.1 Chloride channelopathies -myotonia congenita

Autosomal recessive Becker (OMIM #255700) and autosomal dominant Thomsen congenital (OMIM #160800) myotonia are non-dystrophic disorders of the skeletal muscle characterized by myotonia (Becker PE 1977 Myotonia congenital and syndromes associated with myotonia) due to decreased chloride current in the defect chloride channel (Pusch 2002, Wu 2002). They are caused by mutations in *CLCN1* on chromosome 7q35 (Koch 1992). More than 100 different *CLCN1* mutations, comprising missense, nonsense, insertions and deletions, as well as splice mutations have been identified causing congenital myotonia (Matthews et al. 2010). The majority of these mutations are recessive. At present it is not generally possible to predict from the sequence alone whether a certain mutation will cause a dominant or a recessive phenotype.

3.2.1.1 Autosomal dominant form: Thomsen´s myotonia congenita

A dominant form of myotonia was described in the 1870s by Dr. Thomsen a Danish physician who himself and his family members had an autosomal dominant inheritance pattern of the disease. The prevalence of Thomsen´s disease is approximately 1/400 000 (Jukratt-Rott et al. 2010). Clinical diagnosis of Thomsen´s disease is obtained by careful clinical examination, family history and electrophysical examinations, but the final diagnosis relies on the molecular genetic verification. Prognosis in Thomsen´s myotonia is favourable with no reduction in life expectancy (Gutmann and Philips 1991). Patch clamp conductivity measurements of the chloride current have shown a lack of function for dominant mutations. Therefore, the dominant Thomsen´s disease is thought to result from a dominant negative effect in the dimeric structure of the chloride channel formation. (Duffield et al 2003)

3.2.1.2 Autosomal recessive form: Becker ´s myotonia congenita

Although being much more common than Thomsen disease the recessive form of myotonia congenita was first described by Dr. Becker in 1966. The prevalence without complete genetic ascertainment has been reported to be about 10/100 000 has been reported in Northern Scandinavia and Northern Finland (Baumann et al 1998; Sun et al. 2001). Besides myotonia with warm-up phenomenon transient weakness has been reported in some Becker patients, whereas it has not been reported in patients with dominant myotonia (Deymeer et al 1998). Overall, the prognosis in Becker´s myotonia congenita is favourable, with no reduction in life expectancy. Some recessive *CLCN1* mutations are more common than others. R894X (c.2680C>T) located in exon 23 is the most common single mutation, estimated with a carrier frequency around 1 % in European populations. In the Finnish population the mutation F413C (c.1238T>G) located in exon 11 is almost as frequent (Papponen et al. 1999), but these two mutations explain only about half of the congenital myotonias. Some myotonia patients remain without final genetic diagnosis due to only one mutation identified when screening for the common mutations.

3.2.2 Voltage gated chloride channel CLC1

The voltage dependent ClC-1 regulates the electric excitability of the skeletal muscle membrane by stabilizing the resting membrane potential. It is a member of the ClC family which consists of nine members in mammals (Jentsch 1999). Skeletal muscle has an unusually high resting Cl(-) conductance. Reduction of this high Cl(-) current causes a less stable resting membrane potential which leads to electrical instability and increased induction of involuntary action potentials which is myotonia (Barchi et al. 1975). *CLCN1* mRNA is expressed mainly in skeletal muscle, with weak expression in kidney, liver, heart and smooth muscle tissue (Steinmeyer et al. 1991). ClC-1 protein expression appears to be limited to skeletal

muscle. In vitro expression studies combined with electrophysiological measurments showed that ClC-1 forms a plasma membrane channel (Steinmeyer et al. 1991; Fahlke et al. 1996). Also, expression of myc-tagged ClC-1 isolated from myofibers gave similar result (Chen and Jockusch 1999). Furthermore, using an antibody against a C-terminal synthetic peptide on skeletal muscle cryosections indicated that the main portion of ClC-1 is located on the sarcolemma (Gurnett et al. 1995; Papponen et al. 2005). However, some studies show that ClC-1 may also be present in the T-tubular system (DiFranco et al. 2010; Lamb et al. 2011).

AIMS OF THE STUDY

The overall purpose of the study was to gain insight in molecular mechanisms of DM2 and other myotonias in general.

The specific aims of this study were:

1. To improve routine histopathological diagnostics by developing an easy and reliable method for the differentiation of muscle fiber types, including the detection of highly atrophic type 2 fibers characteristic for DM2.

2. To characterize and describe a new disease and the mutations causing total lack of type IIA fibers in muscle biopsies, identified by the use of the new method in Aim 1.

3. To gain further understanding of the molecular pathomechanisims underlying DM2, i.e. the role of ZNF9, in order to identify explanations for the differences in muscle and fiber type involvement between DM1 and DM2, and the large variability of symptoms observed in DM2.

4. To improve differential diagnostics and possibilities to reach final genetic diagnosis in myotonic channelopathies by developing a screening method for the assessment of ClC-1 protein on the sarcolemma of muscle samples in order to guide further genetic testing.

SUBJECTS AND METHODS

1. Subjects

1.1. Patient and control samples

In total samples of fifty-one genetically verified DM2 patients were included in this study. Thirty-one samples were from Finnish patients and the rest were from patients in other European countries (sample sharing: European Neuromuscular Centre (ENMC) consortium) (publications I, III, IV). Samples of seventy-four Finnish myotonia patients were also included in the study and samples of 261 myotonia British patients were screened for one *CLCN1* mutation (publication IV). Samples of twenty-two DM1 patients were used as comparable disease controls (publications I, III, IV). Samples of five patients with neurogenic disorders, patients with polymyositis, LGMD2I and *MYH7* mutated Laing myopathy were used for comparison (publication I). In addition, samples of thirty-seven healthy controls were used including healthy family members of myotonia patients (publications I-IV). Samples of five patients with facial weakness and marked ophthalmoplegia were studied in publication II. Patient and control samples used are also summarized in table 1.

The majority of all muscle biopsy samples were initially obtained for diagnostic purposes. All studies were approved by the ethical committees of the respective universities and university hospitals and samples (muscle biopsy and blood) were used after obtaining written informed consent from the patients.

Disorder	Number of patients	Origin	Used in publication
DM2	51	FIN, ENMC	I, III, IV
DM1	22	FIN, SWE	I, III, IV
Myotonia	335	FIN, UK	IV
Nerogenic disorder	5	FIN	I
Polymyositis	1	FIN	I
Laing myopathy MYH7	1	FIN	I
LGMDI	1	FIN	I
Facial weakness and ophtalmoplegia	4	FIN, SWE	II
Healthy controls	37	FIN	I, II, III, IV

Table 1: Patients and control samples used in the thesis. *FIN, Finland; SWE, Sweden; UK, United Kingdom; ENMC, European Neuromuscular Center.*

1.2. Population control samples

A cohort of 100 Finnish population samples from the general population, 100 Finnish population samples from a genetically isolated island region of western Finland, Larsmo, and 64 UK population control samples were used for genetic testing of *CLCN1* mutations (publication IV).

2. Methods

Method	Used in publication
Cell culture	III, IV
Myoblast-myotube cell cultures	III
Immunofluorescence	III, IV
Immunohistochemistry	I, II, IV
Enzyme histochemistry	I, II
Microscopy (Bright field, fluorescent, confocal)	I, II, III, IV
Sequencing	II, III, IV
PCR	II, III, IV
DNA extraction	II, III, IV
RNA extraction	II, III, IV
Microarray expression profiling	III
Patch clamp analysis	IV
Electroporation	IV
SDS-PAGE	II, III, IV
Western blotting	II, III, IV
Cloning and sequence analysis	III
Statistical analysis	III, IV

Table 2: Experimental methods used in the thesis

2.1. Immunohistochemistry and immunofluorescence (I, II, III, IV)

All muscle biopsies were snap frozen with isopentane chilled in liquid nitrogen and stored in -80 ºC until use. Sections of 6 µm thickness were cut on objective slides using a cryomicrotome. If needed, the sections were stored in -20 ºC or -80 ºC until use.

Air dried tissue sections were stained by immunohistochemical procedures using the BenchMark automated immunostainer (Roche Tissue Diagnostics, Ventana Medical Systems, Tucson, AZ 85755, USA) according to manufacturer's instructions. Double stainings were performed either by using two different detections for different antigens using A DAB (UltraView^{TM} Universal DAB detection kit, Roche Tissue Diagnostics, Ventana Medical Systems Inc.,) and an alkaline phosphatase (UltraView^{TM} Universal Alkaline Phosphatase Red detection kit, Roche Tissue Diagnostics, Ventana Medical Systems Inc) based detection kits (publication I and II) or by mixing two different antibodies and using only a DAB(UltraView^{TM} Universal DAB detection kit, Roche Tissue Diagnostics, Ventana Medical Systems Inc.,) based detection for both antigens (publication IV).

For manual immunofluorescent staining of tissue sections, unspecific proteins were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) before primary antibody incubation. Antibody incubations were performed at RT for 1 hr and the sections were rinsed with PBS. Sections were counterstained with DAPI and mounted with a mounting media containing an anti-fading agent (publication III). Cultured cells were pre-treated by fixing with methanol at -20˚C and permeabilized using 0.2% triton-X 100 with 0.2% BSA in PBS. After blocking with 0.2% BSA in PBS, antibody incubations were performed at RT for 80 min, coverslips rinsed with PBS, counter stained and mounted (publication III).

2.2. Histochemistry and enzyme histochemistry (I, II)

H & E staining was performed on muscle sections by first incubating in Mayer´s haematoxylin solution to stain nucleic acids. After washing the sections were incubated in eosin solution to stain other components of the tissue (publication II).

The myosin adenosine triphosphatase (ATPase) properties of different myosin isoforms is widely used as the main routine diagnostic method for fiber type separation. The enzyme histochemical method is based on the release of phosphate, the capture of phosphate by calcium resulting in calcium phosphate and substitution of calcium by cobalt (Bancroft and Cook 1994). Enzyme histochemical ATPase stainings for fiber type distribution with pH 4.3, 4.6 and 10.4 were performed by

pre-incubating 10µm sections in sodium barbiturate and calcium chloride solution of pH 10.4, barium acetate and hydrochloric acid solution of pH 4.3 and pH 4.6. The sections were then incubated in a solution of sodium barbiturate pH 9.4 containing adenosine triphosphate for 30 minutes in +37 ˚C. After incubation in 1 % calcium chloride and 2 % cobalt chloride respectively the sections were dipped into a solution of 0.01 M sodium barbiturate. The sections were washed and then dipped in to a solution of 0.2 % ammonium sulphide to form the black precipitate of cobalt sulphide. Fibers without reaction and connective tissue were stained using a Van Gieson solution. The Van Gieson staining also changes the appearance of the black precipitate of cobalt sulphide into shades of brown. Nuclei were stained using weigert-hematoxylin (publication I).

NADH-TR staining was performed on sections by incubating sections in a solution containing nitro blue tetrazoleum (NBT) and nicotinamide adenine at 37 °C for 30 min. The sections are then dipped in acetone and mounted (publication II).

2.3. Microscopy (I, II, III, IV)

All stained slides and coverslips were viewed under a light microscope (Leica DM 2000, Leica Microsystems CMS GmbH, Wetzlar, Germany), fluorescent microscope (Zeiss Axioplan 2, Carl Zeiss, Göttingen, Germany) or confocal (Olympus 1X70, Olympus Corporation, Tokyo, Japan).

2.4. Myoblast-myotube cell cultures (III)

Myoblast cell lines were established from skeletal muscle samples. The muscle sample was cut into very small pieces in a trypsin solution, stirred and centrifuged. The pellet was then re-suspended in a skeletal muscle cell growth media with a supplement mix (Promo Cell GMBH, Heidelberg, Germany) and the cells were allowed to adhere in a CO_2 incubator in 37 °C for 2-3 days. The cells were maintained in Skeletal Muscle Cell Growth Medium (PromoCell) supplemented with the supplement mix (PromoCell), 10% FBS, Gentamicin (Gibco, Carlsbad, CA,

USA) and Gluta-MAX-1 (Gibco) in 5% CO_2 at 37 °C. The cells were differentiated in D-MEM containing Gentamicin, Gluta-MAX and 10 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO, USA). At different time points of differentiation (0, 3 and 7 days) cells on glass coverslips were fixed with methanol at -20˚C and immunofluorescent staining was performed.

2.5. SDS-PAGE and Western blotting (II, III, IV)

Frozen muscle biopsies were used for SDS-PAGE and Western blotting. Samples were prepared by mechanical homogenization and sonication in lysis buffer (RIPA buffer, Sigma-Aldrich, Saint Louis, MO, USA) containing protease inhibitors (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) before adding sample buffer containing beta-mercaptoethanol as a reducing agent and sodium dodecyl sulphate (SDS) for polypeptide linearization. Samples were then denaturated by heating.

To separate membrane components of muscle cells (publication IV), the homogenized tissue was centrifuged to separate the cytoplasm components and the pellet was dissolved in a buffer containing 1 % Triton X-100, 1 % deoxylate and protease inhibitors. The suspension was further centrifuged to separate nuclear components and supernatant then precipitated with 10 % trichloroacetic acid. Alternatively, membrane components were extracted using a membrane extraction kit (ProteiJET Membrane Protein Extraction kit, Fermentas Life Science, MD, USA) according to manufacturers´ instructions.

Depending on the protein to be detected, 5-20 µl of sample were loaded on SDS-PAGE gels and separated electrophoretically. After SDS-PAGE, proteins were transferred onto PVDF membrane and immunolabeled with antibodies. Primary and secondary antibody incubations varied from 1 hr at room temperature to over night at +4 ºC, and enhanced chemiluminiscence (ECL) was used for detection of bands. To assess protein loads on blots, they were most often normalized to Coomassie brilliant blue stained MyHC bands in gels after blotting. Quantification of bands (publication III) was done using a densitometer (GS-700, Bio-Rad laboratories, CA,

USA) and results were analyzed for statistical significance using Mann-Whitney pair wise comparisons.

2.6. Microarray expression profiling (III)

Purified RNA extracted from frozen skeletal muscle biopsies was used. The quality and integrity of the RNA was analyzed on an Agilent BioAnalyzer using the RNA 6000 Nano LabChip (Agilent, Santa Clara, CA); samples with a RIN (RNA integrity number) >7 were used. cDNA was synthesised using total cellular RNA using the Superscript II system (GIBCO/BRL). *In vitro* transcription labelling with biotinylated UTP and CTP was performed according to the manufacturer's recommendations (Enzo Diagnostics, Farmingdale, NY, USA). The labelled and amplified cRNA was purified and the quality of the amplification was verified. Fragmented cRNAs were then hybridized to Affymetrix U133Plus2 GeneChips (Affymetrix, Santa Clara, CA, USA) and scanned according to the manufacturer's protocol.

Data analysis: Normalization was performed with the Invariant Set Normalization method (PM-only model with DChip default settings) on a normal sample as the reference; DChip model-based expression was applied to calculate the expression values for each probe set. Comparisons between groups were performed, using a fold-change (FC) cut-off FC $\geq$ 1.2, a lower bound (lb) limit lb = 90% (default), e-b, b-e difference thresholds of 100 (e=experiment, b=baseline), and a 50-permutations false discovery rate (FDR) calculation for each comparison.

2.7. Quantitative real time RT-PCR (III)

Skeletal muscle biopsies were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA). The samples were chilled on ice between runs and RNA was extracted according to manufacturer's instructions**.** The samples were further purified using the RNeasy kit (Qiagen, Valencia, CA, USA). All RNAs were DNase-1-treated using Ambion DNA-free according to the manufacturer's instructions (Applied

Biosystems Inc., Foster City, CA, USA). RNA amplifications were performed using RNA Amplification SenseAMP Plus Kit according to the manufacturer's instructions (Genisphere Inc., Hatfield, PA, USA). To avoid 3' bias random primers were used. The final RNA produced was in the sense orientation. cDNA produced as a by-product during the amplification was removed by one or, if necessary, more rounds of treatment with Ambion DNA-free according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA). Presence of DNA was tested with PCR from S15 primers that amplify a 361 bp band present in genomic and cDNA but not in RNA. cDNA was synthesized from the amplified RNA using 250 ng random hexamers and SuperScript II Reverse Transcriptase enzyme according to the manufacturers protocol (Invitrogen Carlsbad, CA, USA). Samples were run on an Applied Biosystems 7000 Real-Time PCR System.

2.8. Splice Variant and Allele-specific Transcript Analysis (III)

cDNA was used for RT-PCR. M13-tailed PCR products were cloned into pCR2.1 and transformed into *E. coli* according to the manufacturer's suggestions (Invitrogen Life Technology, Carlsbad, CA). Transformed cells were plated on a single 7-cm LB agar plate with kanamycin. Following incubation at 37°C for 18 hrs, 96 single colonies were picked and transferred to a 96-well PCR plate containing LB broth. Following an additional incubation at 37°C for 2 hrs, 5 µl of each micro-culture were dissolved in 50 µl H_2O. Each culture was then PCR amplified using M13F and M13R primers. Each PCR product was analyzed on a 3% standard TAE agarose gel. Amplicons were used for direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The sequencing products were subjected to post-sequencing clean-up by ethanol precipitation, containing 3 M NaAcetate (1/10 volume) and 100% ice-cold ethanol. Products were precipitated by centrifugation washed in ethanol, and dried in a vacuum isotemp oven. Following denaturation in Hi-Di formamide (Applied Biosystems) at 95 °C, samples were loaded on a 3100 Genetic Analyzer (Applied Biosystems). Sequences were analyzed with Sequencher 4.7 (Gene Codes, Ann Arbor, MI).

For allele specific analysis skeletal muscle cDNA from patients and controls were subjected to repeat primed PCR (RT-RP-PCR) and capillary electrophoresis. We used a quantitative allele-specific method where PCR amplification was carried out on both cDNA and genomic DNA using a three primer reaction to incorporate a 5' fluorescent (FAM) label. After digestion with *Hae*III, samples were subjected to capillary electrophoresis and the ratio of the peak heights of the two alleles in heterozygous individuals were used to calculate an allele specific expression index (ASEI) equal to [Intensity (A_{cDNA})/Intensity ($A_{cDNA} + C_{cDNA}$)] / [Intensity (A_{gDNA})/Intensity ($A_{gDNA} + C_{gDNA}$)].

2.9. Genomic DNA and cDNA sequencing (II, III, IV)

Genomic DNA was extracted from peripheral blood leucocytes by standard procedures. Primer sequences to the wanted exons were designed to include exons and exon-intron borders. The exons were amplified by polymerase chain reaction and sequenced using bidirectional fluorescent sequencing on an ABI3130xl automatic DNA sequencer system (Applied Biosystems, Forster City, CA, USA), with Big-Dye Version 3.1 chemistry.

Extracted RNA from frozen muscle biopsies was used for cDNA analysis. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene transcripts were sequenced using overlapping primer pairs. All sequences were analyzed with Sequencer software (Gene Codes Corporation, Ann Arbor, MI, USA).

2.10. Preparation of mammalian expression plasmids, in vivo electroporation and expression analysis of chimeric GFP constructs (IV)

Appropriate cDNA in Bluescript vector was digested with EcoR1 and SacII. The digestion products were separated on agarose gel electrophoresis. The fragment corresponding to the cDNA encoding the wanted gene was excised and blunted with

Klenow enzyme. The blunted fragment was ligated to pACGFP vector. A point mutation was created by QuickChange II site-directed in vitro mutagenesis kit (Stratagene, Agilent technologies Inc., CA, USA). All the sequences were verified with ABI PRISM™ 3130XL sequencer and BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

In vivo electroporation into the living rat flexor digitorum brevis (FDB) muscle was performed. The expression plasmids encoding the GFP combined gene were introduced into the FDB via a small incision in the footbad. Thereafter, electric pulses were applied on the footbad via custom made electrodes. To improve the electrical conductance fine hairs were first shaved from the dorsal side of the foot and conducting gel (Aquasonic, Parker Laboratories Inc., NJ, USA) was then mounted on each side of the foot. After 3 - 5 five days of the operation the rats were sacrificed the transfected muscles were excised and frozen in liquid nitrogen- cooled isopenthane and cryosectioned on objective slides.

2.11. Whole cell patch clamp analysis (IV)

Microelectrodes were pulled from borosilicate glass capillary tubes on a Stutter P97 pipette puller and backfilled with intracellular solution. For patch clamp recordings on HEK293T cells, intracellular solution contained (in mM): Cs-Aspartate 110, CsCl 30, MgCl2 5, EGTA 10, HEPES 10. Extracellular solution contained (in mM): TEA-Cl 145, CaCl2 10, HEPES 10. Both solutions were pH 7.4.

To obtain the voltage dependence of activation, the instantaneous current on stepping to -100 mV (tail current) was measured after pre-pulses to variable voltages from -140mV to +120mV. The full voltage protocol started from a holding potential of -40mV after which the voltage was first stepped to +60mV (which fully activates wild-type CLC-1 channels) before applying the variable pre-pulse voltage and then the step to -100mV. The normalized tail current, I, was plotted against pre-pulse voltage was fitted with a Boltzmann function ($y = I_{min} + [I_{max} – I_{min}]/[1 + \exp((V_{50} – V_{prepulse})/\text{slope})]$) to estimate the voltage of half maximal activation (V_{50}) and slope factor.

2.12. Antibodies used in the thesis (I, II, III, IV)

Antibody	Gene	Protein	Clone	Supplier	Ref.
MyHC A4.74	*MYH2*	MyHC IIa	A4.74	DSHB	
MyHC slow	*MYH7*	MyHC I / beta	WB-MHCs	NC, Leica	
ZNF9	*ZNF9 /CNBP*	ZNF9	mouse pAb	Abnova	
ZNF9	*ZNF9 /CNBP*	ZNF9	rabbit pAb	L. T.	Chen et al 2003
ClC-1	*CLCN1*	ClC-1	rabbit pAb	ADI	
ClC-1	*CLCN1*	ClC-1	rabbit pAb	K. M.	Papponen et al 2005

Table 3: Antibodies used in the thesis. *DSHB, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; NC, Novocastra, Leica Microsystems, Newcastle Upon Tyne, UK; Abnova Corporation, Taipei, Taiwan; L.T., A kind gift from Professor. Lubov Timchencko, Baylor Collage of Medicine, Houston, TX, USA, ADI, Alpha Diagnostics International, San Antonia, TX, USA; K.M., a kind gift from Professor Kalervo Metsikkö, University of Oulu, Oulu, Finland.*

For verification of each antibody recognizes its intended target, they have been carefully tested in immunohistochemistry and/or Western blotting using healthy control material and compared with the information supplied by the antibody manufacturer and literature. We have confirmed that subcellular distribution in immunohistochemistry and molecular weight in Western blotting are in concordance with published data of each protein.

RESULTS

1. Myosin double staining and separation of all fibers and fiber types (I)

All ATPase based fiber types were easily separated by the double immunostaining technique described in the publication. With this immunohistochemical method the fibers were identified as follows. Slow type 1 fibers stained deep brown with the peroxidase detection and fast type 2A/IIA fibers stained deep red using the alkaline phosphatase red detection system. Type 2C fibers as being hybrid fibers expressing both MyHC I and IIA isoforms stained red-brown showing the actual presence of both MyHC isoforms in these fibers. Moreover, this technique was able to also separate the ultrafast glycolytic MyHC IIX expressing type 2B fibers on the same slide as being immunonegative for the myosin antibodies used. Capillaries or other structures complicating the evaluation of small fibers when using the ATPase staining method were not labelled by the antibodies (figure 9).

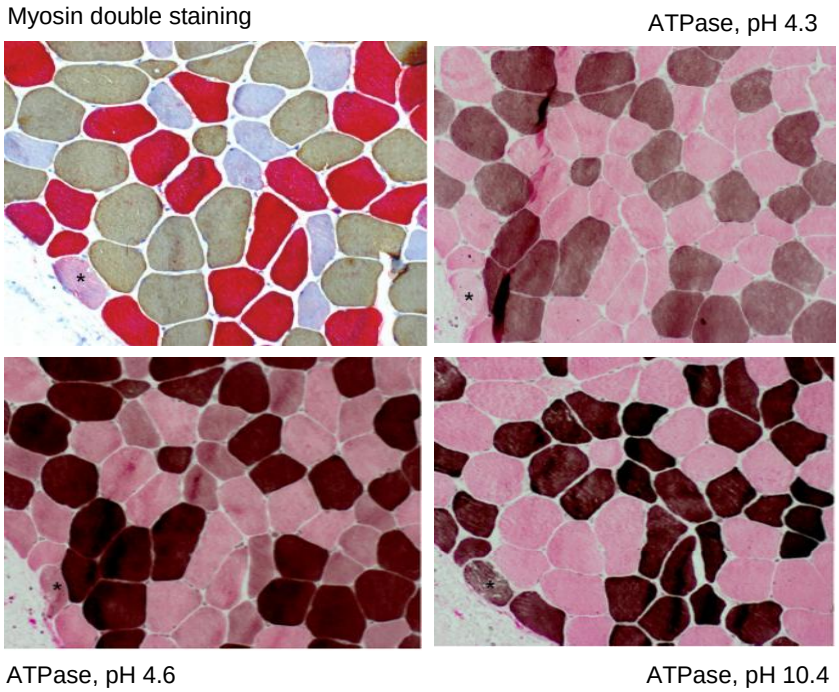


Figure 9. *Comparison of MyHC double staining with conventional ATPase staining, showing all fiber types on one single slide by immunohistochemistry. Type 1 fibers observed as brown, 2A/IIA fibers as red, 2B/IIX fibers in blue and one type IIA/X hybrid fiber as light pink in the MyHC double staining. Clinical implications of the presence of IIA/X hybrid fibers are currently not known. However, these fibers cannot be identified at all by the ATPase method.*

In muscle biopsies from patients with neurogenic muscular atrophies, fiber type grouping was comparably identified as with ATPase technique, but numerous highly atrophic fibers were more easily identified with the immunohistochemical double staining (figure 10), and the separation of different fiber types in these populations of highly atrophic fibers was definitely easier. Similarly the different fiber types were also easily distinguished in muscle samples from patients with other diseases.

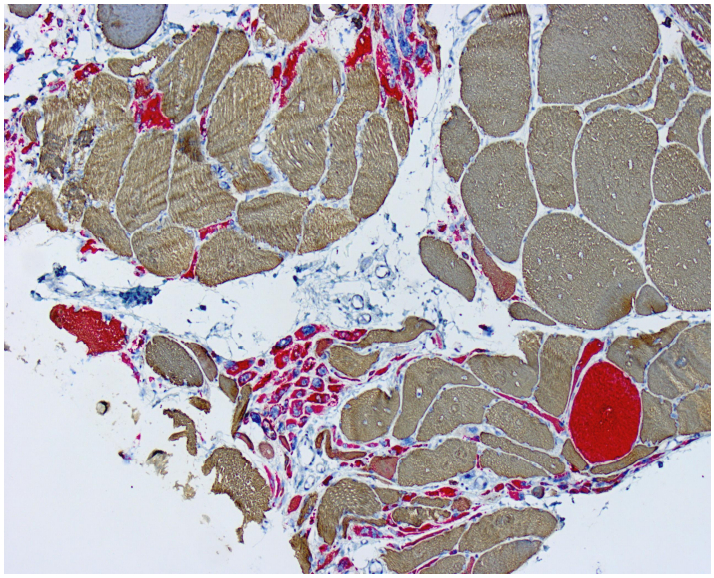


FIgure 10*. MyHC double staining showing fiber type grouping of atrophic type IIA and larger type I fibers in a neurogenic muscular atrophy sample.*

2. Identification of a novel disease: *MYH2* defects causing total lack of fast IIA myosin (II)

In five patients with ophthalmoplegia and generalized muscle weakness, muscle biopsies showed absence of MyHC IIA fibers (figure11), which was never observed in other neuromuscular diseases or normal controls. The finding was surprising and directly indicated a genetic defect in the corresponding gene *MYH2*. Collaborative research efforts proved that these patients harbour compound heterozygous truncating mutations (c.904+1G>A, c.2347C>T, c.1975-2A>G and c.2405T>A) in the *MYH2* gene. The expression of MyHC isoforms by SDS-PAGE confirmed the absence of MyHC IIA protein and consequently no fast 2/IIA fibers are present in the muscle biopsies of the patients.

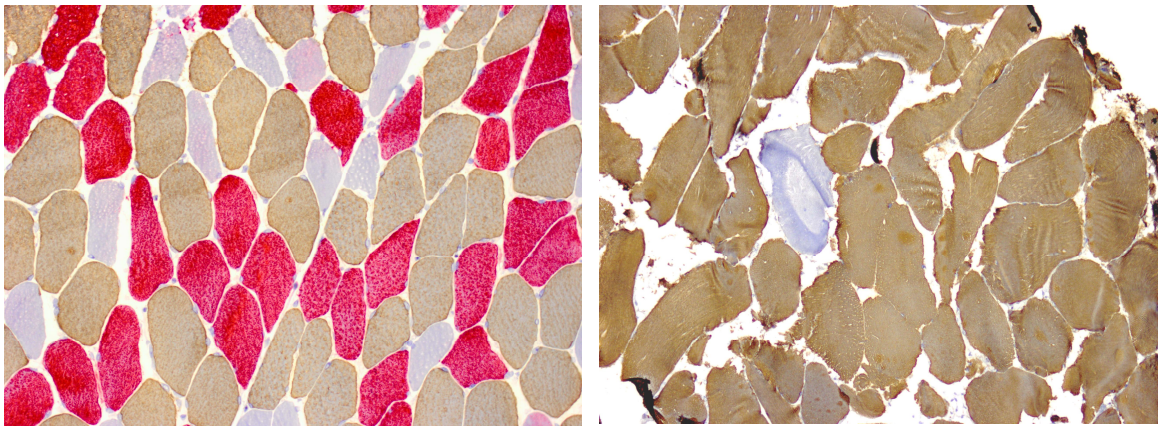


Figure 11. *Immunohistochemical MyHC double staining. Normal distribution of fiber types in the normal control (left) and absence of type IIA fibers in the muscle biopsy of one of the patients with compound heterozygous truncating MYH2 mutations (right).*

3. Identification of type 2 atrophic fibers in DM2 using the myosin double staining method (I)

In DM2 patients the MyHC double staining provided additional important information not easily obtained by ATPase enzyme histochemistry. All nuclear clump fibers and other highly atrophic fibers were readily detected as fast type IIA (ATPase type 2A) fibers in red (figure 12). This finding was re-confirmed by immunoreactivity for neonatal myosin heavy chain isoform as all these highly atrophic type IIA (ATPase type 2A) fibers also express neonatal MyHC as part of the reprogramming in the affected myofibers during the disease process. Fiber type grouping as a result of chronic neurogenic change was absent indicating that the presence of nuclear clump fibers is due to a different mechanism in DM2.

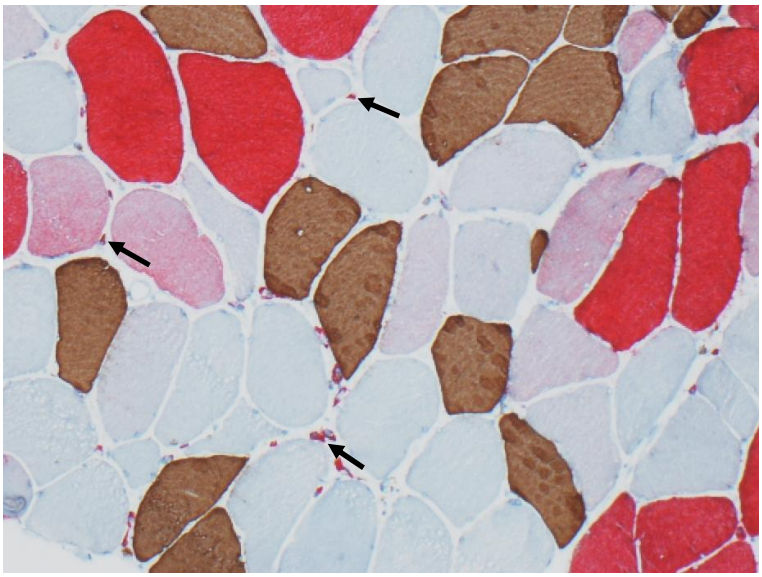


Figure 12. *Immunohistochemical MyHC double staining of the muscle biopsy sample of a DM2 patient showing numerous characteristic highly atrophic type IIA fibers (arrows).*

4. The expression of ZNF9 in DM2 (III)

4.1 ZNF9 mRNA transcript expression is reduced in DM2

Global microarray gene expression profiling of DM patient biopsies and normal controls, indicated that *ZNF9* mRNA levels were consistently lower than those of DM1 and normal individuals (figure 13). Further validation experiments by quantitative real-time RT-PCR using a *ZNF9*-specific TaqMan probe showed that the expression was reduced by approximately half in DM2 patients relative to DM1 and normal control individuals (figure 13), while no decrease was observed in DM1 patients and normal controls.

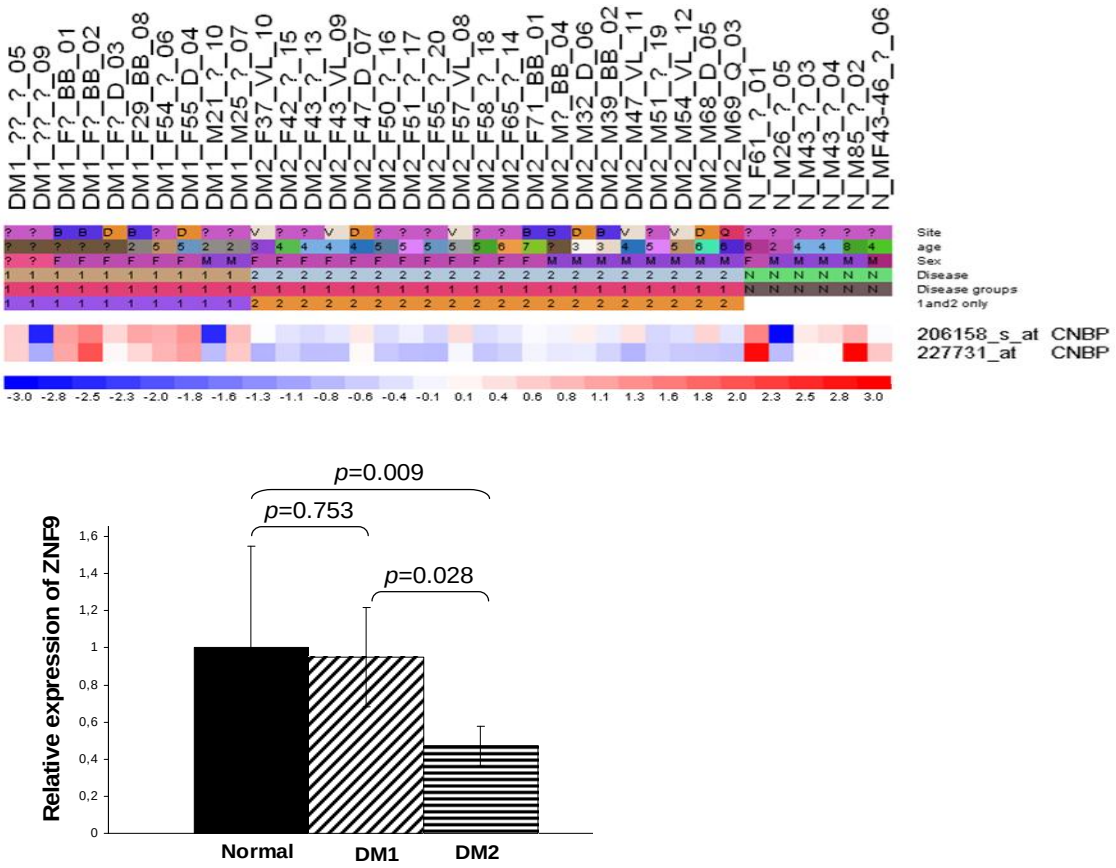


***Figure 13**. (Top) microarray expression profiling of mRNA from skeletal muscle biopsies for ZNF9 showing lower expression in DM2 (blue) when compared to DM1 and normal control samples (red). (Bottom) bar graphs showing results of real-time RT-PCR analysis of total ZNF9 mRNA with significant reduction of ZNF9 mRNA in DM2 samples.*

4.2 ZNF9 protein expression and aberrant subcellular localization in DM2 patients (III)

Consistent with the mRNA expression levels, total ZNF9 protein levels in DM2 muscle samples were reduced on western blots compared to DM1 and normal control samples. The reductions in mean protein levels were approximately 15% to 50%, depending on the antibody used, were observed (figure 2 of original publication III).

On longitudinal muscle biopsy sections, cytoplasmic ZNF9 was organized in sarcomeric striations at the Z-disc, in controls as well as DM1 and DM2 patient samples. Moreover, ZNF9 expression levels were higher in slow type 1 fibers and no localization in the nuclei was observed. Immunofluorescence analysis of ZNF9 in skeletal muscle tissue transverse sections showed somewhat less cytoplasmic and more sarcolemmal membrane-bound protein in DM2 patients compared to normal controls and DM1 disease controls. However, in the subpopulation of highly atrophic type 2/IIA fibers, characteristic for the muscle pathology in DM2, the expression was intense in the remaining cytoplasm. No cell subtype showed increased expression in DM1 or control samples (figure 1 of original publication III).

4.3 Expression of ZNF9 during muscle in vitro differentiation (III)

In contrast to the subcellular cytoplasmic and sarcomeric localization observed in the mature skeletal muscle, the expression of ZNF9 in early myoblasts (day 0) was markedly perinuclear in addition to the abundant nuclear localization. By day 3, the perinuclear staining was diminished, while nuclear staining was increased. Some cytoplasmic expression was observed towards the end of differentiation on day 7. However, in myoblast-myotube cell cultures no distinct differences in ZNF9 expression were observed between DM2 and control cells (figure 3 of original publication III).

4.4 Aberrant splicing of *ZNF9* in DM2 (III)

The observed reduction of ZNF9 mRNA and protein levels suggested a direct effect of the DM2 mutation on gene expression and prompted us to investigate *ZNF9* transcripts for possible alterations. To this end, we performed qualitative RT-PCR analysis with primers in various combinations in overlapping amplicons. Two

different amplicons covering exons 3 to 5 consistently detected a novel fragment in DM2 patients, while no other region of the transcript showed evidence of abnormal splicing. The observed size of the variant product and the intron-exon structure of *ZNF9* suggested retention of intron 3. Sequence analysis of cloned amplicons revealed that the novel fragment was the result of intron 3 retention. Retention of intron 3 gives rise to a novel open reading frame (ORF) extending 22 codons into intron 3 before terminating with a premature termination codon (PTC).

We cloned the entire RT-PCR reaction product from different DM patients and normal control individuals to estimate the abundance of intron 3 retaining transcripts. Analysis of at least 100 individual colonies from each sample established that variant products were present at low frequency of about 10% in DM2 and $\leq$ 3% in DM1 and not present in normal control samples.

4.5 Differential processing of mutant and wild type pre-mRNA *ZNF9* transcripts in DM2

Mutant ZNF9 pre-mRNA transcripts are abnormally processed and do not result in proper mRNA leading to the reduced amount of functional mRNA. A possible abnormal processing may occur as follows: as exons 1 and 2 are brought into close proximity in preparation for splicing, there is steric interference due to the expanded (CCUG)n repeat. As a result, splicing is retarded or may fail entirely for the majority of transcripts, accounting for the increased steady-state levels of pre-mRNA from the mutant allele, while the overall processed mRNA message is reduced. Most of intron 1 may be degraded normally, but the region close to the splice junction may be protected by its proximity to the expansion during splicing and may or may not end up in the foci. The presence of a small percentage of transcripts that retain intron 3 is consistent with retarded mRNA processing overall.

5. Chloride channel protein expression in DM1 and DM2 (IV)

The abnormal splicing of *CLCN1* in DM1 and DM2 was supposed to cause reduced levels of functional CLC-1 in the muscle tissue of the patients. However, sarcolemmal ClC-1 staining in DM1 and DM2 samples was very variable, ranging from severe reduction to more or less normal staining when compared to normal controls. This variability was to some degree expected given the variable nature of these diseases, but the consistent more severe myotonia in adult onset DM1 could not be correlated to consistently much lower ClC-1 protein expression in DM1 compared to DM2. One specific aim was to assess whether the co-segregation of a recessive *CLCN1* mutation with DM2 causes a significantly higher reduction of ClC-1 protein, based on the fact that the co-segregation causes a more severe myotonia phenotype in DM2 patients. However, only two muscle biopsy samples of DM2 patients with co-segregating recessive *CLCN1* mutations were available for the experiment. One with a co-segregating heterozygous R894X mutation showed total loss of ClC-1 protein while the other DM2 patient with a co-segregating heterozygous F413C mutation showed subtotal loss of the protein in both immunohistochemistry and Western blotting. These reductions were in the range of reduced ClC-1 expressions observed in DM1 and DM2 patients without known *CLCN1* mutations based on screening for the common mutations.

6. Chloride channel expression in patients with non-dystrophic myotonia and identification of new pathogenic mutations (IV)

We used the newly developed ClC-1 immunohistochemical double staining method to have a direct assessment of ClC-1 protein in frozen skeletal muscle tissue sections to aid the diagnostics of patients with non-dystrophic myotonia and the screening for *CLCN1* and *SCN4A* gene defects. Myotonia congenita patients with

homozygous R894X mutation show total loss of sarcolemmal ClC-1 protein. 25 patients with clinical and EMG myotonia, but just one heterozygous or none of the common mutations identified, showed a clear loss of sarcolemmal ClC-1 protein. Figure 14 shows immunohistochemical ClC-1 staining of patients with myotonia congenita. Sequencing the whole gene revealed a novel c. 264G>A (V88V) mutation in 2 of the patients with homozygosity and in 6 patients in compound heterozygosity with one of the common mutations. 9 of the patients were found to have a W118G mutation in compound heterozygosity with one of the common R894X and F413C mutations The W118G change has earlier been reported as a polymorphism (*Lehmann-Horn et al. 1995)*. However, when occurring in compound heterozygosity with the common R894X and F413C mutations, a clearly more severe reduction of ClC-1 protein is present compared to the protein amount observed in healthy heterozygous carriers with an R894X or F413C mutation alone.

The c.264G>A mutation is a silent mutation (V88V) with no amino acid change. However, cDNA sequencing of patients homozygous for c.264G>A revealed that the mRNA transcript lacked exon 2. Exon 2 was also lacking on one allele in patients heterozygous for the c.264G>A change.

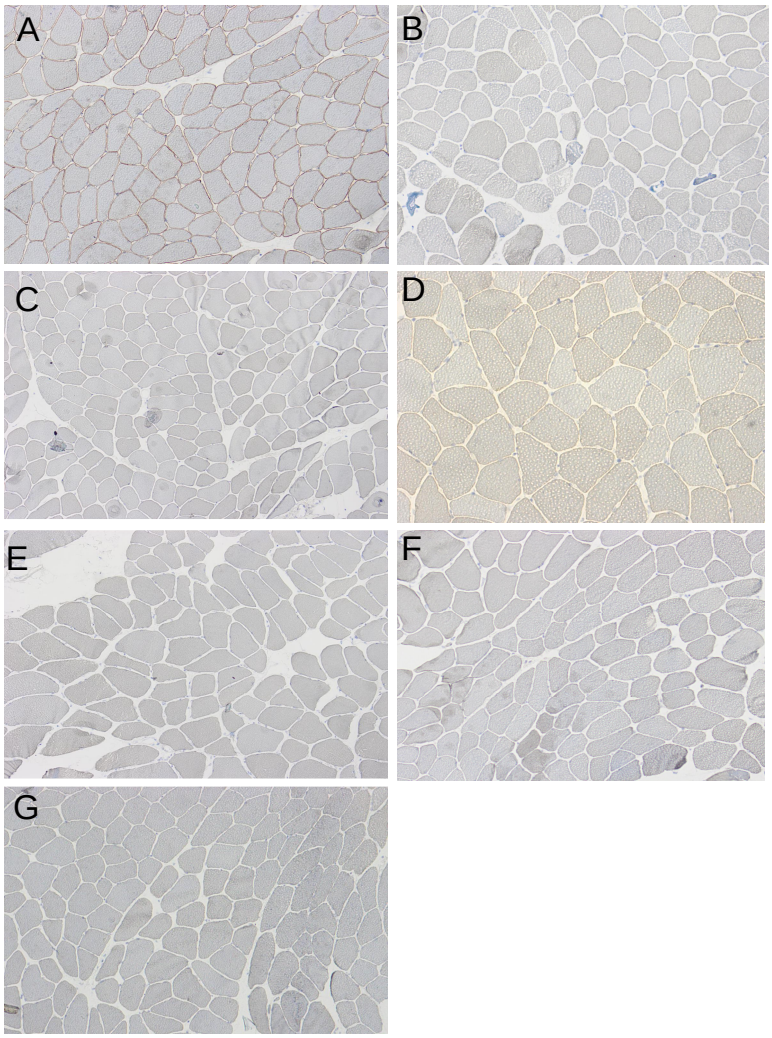


Figure 14*. Immunohistochemical staining of sarcolemmal ClC-1 in a normal biopsy (A). Total loss of sarcolemmal ClC-1 protein in patients with R894X homozygosity (B) Subtotal loss of sarcolemmal ClC-1 in compound heterozygosity R894 + W118G and (C) compound heterozygosity with F413C + W118G patient samples (D). In a patient muscle sample with compound heterozygous R984X + c.264G>A (E), compound heterozygous F413C + c.264G>A (F) and homozygous c.264G>A mutations showing total loss of sarcolemmal CLC-1 protein.*

In Western blots, there is a clear reduction ClC-1 protein in patient biopsies with homozygous R894X mutation. Heterozygous c.264G>A mutation combined with either R894X or F413C mutations also showed a clearly reduced amount of ClC-1 when compared to muscle biopsies from normal controls. Patients with combined heterozygous R894X and W118G showed almost normal total ClC-1 protein

expression in Western blots in contrast to the very clear reduction of sarcolemmal expression observed with immunohistochemistry (figure 15).

One patient with a dominant F307S mutation showed, as expected, normal sarcolemmal ClC-1 protein expression. In another myotonia patient with normal sarcolemmal ClC-1 protein and just one heterozygous R894 mutation identified despite sequencing the whole *CLCN1* gene the normal immunohistochemistry result prompted for an other explanation of the myotonia in the patient and subsequent sequencing of the *SCN4A* gene identified and known pathogenic mutation A1156T.

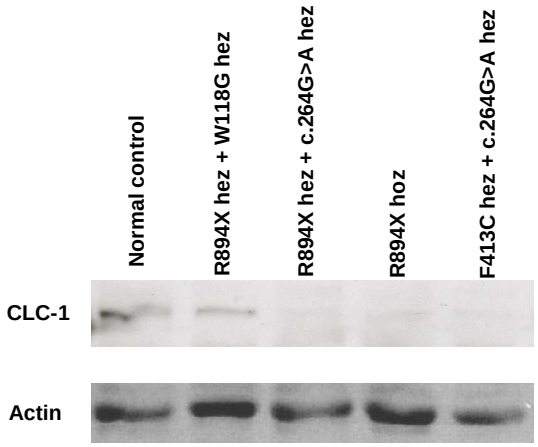


Figure 15. *Western blot showing total amount of CLC-1 protein in muscle tissue. Total protein amount in compound heterozygote R894X and W118G muscle is almost normal. The protein amount in compound heterozygous c.264G>A combined with R894X or F413C, and homozygous R894X is clearly reduced/almost absent. Ponceau stained actin as a loading control.*

6.1 Expression and localization of W118G mutated chloride channel in rat muscle fibers (IV)

First transfections of the chimeric GFP-CLC1 WT and W118G mutant into the living rat muscle fibers by means of electroporation seemed to show a more cytoplasmic endoplasmic reticulum abundance compared to the sarcolemmal

localization, but repeated experiments did not reveal consistent dramatic differences in the localization patterns. Both protein constructs were detected in the endoplasmic reticulum as well as on the plasma membrane (figure 16).

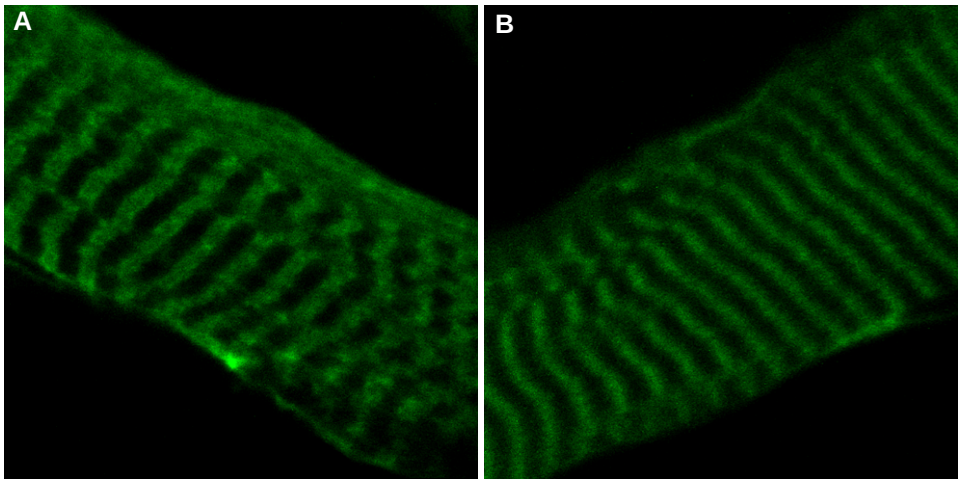
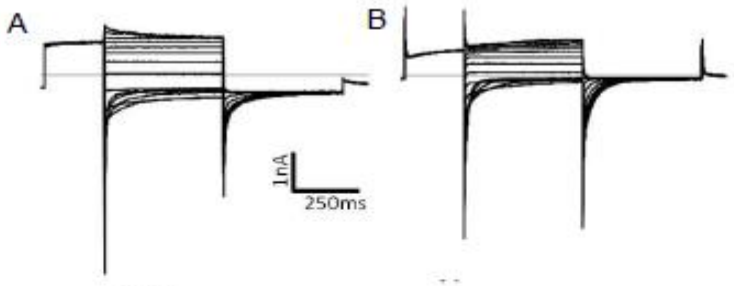


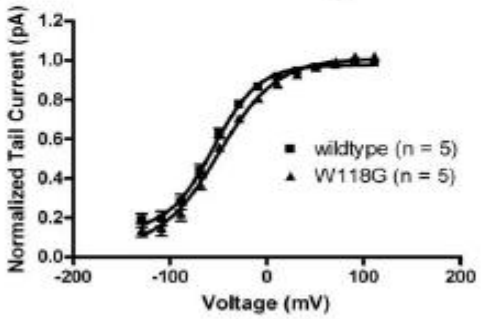
Figure 16. *Electroporation studies of WT GFP-ClC1 (A) and W118G mutant (B) on rat FDB muscle, both showing more cytoplasmic and less sarcolemmal ClC-1 expression.*

6.2 Chloride currents in cells with W118G mutation (IV)

Both W118G and wild-type CLC-1 channels produce robust chloride currents in transfected HEK cells. In contrast to currents produced by many dominantly inherited ClC-1 mutants, there was no obvious difference in current amplitudes between the wild-type and mutant clones. In addition, there is no significant difference in voltage dependence between the W118G-W118G homodimeric ClC-1 mutant and the wild-type (figure 20).



C



D

	wildtype	W118G
V_{50} (mV)	-55.0 (-60.2 to -49.8)	-49.4 (-56.1 to -42.7)
Slope (mV)	23.4 (19.0 to 27.8)	30.0 (24.2 to 35.8)

Figure 20. *Functional expression of wild-type ClC-1 and the W118G mutation by whole cell patch clamp of transfected HEK293T dells. Representative wild-type recording (A). Representative mutant recording (B). Boltzmann fits of the normalized tail current from 6 wild-type (squares) and 4 mutant (triangles) recordings to show the similar voltage dependence of activation (C). Error bars are obscured by symbol. V50 and slope (with 95 % confidence intervals) from the Boltzmann fits (D).*

6.3 W118G mutation frequencies in patients with myotonia (IV)

The W118G mutation occurred with an unexpectedly high frequency among myotonia patients from Finland and the UK. Nine out of 17 Finnish myotonia patients with inconclusive results by screening for the two common Finnish myotonia mutations harboured the W118G mutation. In 261 UK myotonia patients 31 patients were found to have the W118G mutation (10 of whom were homozygotes for this mutation) corresponding to a frequency of 12 %. This highly significant over representation ($p < 0.001$) in both patient cohorts compared to the

mutation frequency in the respective populations did not occur by chance and suggests a functional defect.

6.4 Population screening of chloride channel mutations (IV)

In the cohort of 100 population samples from Larsmo archipelago on the west coast of Finland, we found three heterozygous F413C mutations corresponding to a carrier frequency of 3 % in that specific population. In a cohort of 65 individuals from the same population the W118G mutation was found in a carrier frequency of 7,7 %, whereas no carriers of the c.264G>A mutations were found. In a cohort of 100 unselected population controls from central Finland the W118G mutation was found in a frequency of 3 % and in a cohort of 64 controls from the UK with a frequency of 4.7 %.

DISCUSSION

1. The role of MyHC double staining in the diagnostic routine

The immunohistochemical MyHC double staining is very efficient for the detection and separation of different muscle fiber types and their subtypes. In comparison with the conventional ATPase technique this immunohistochemical method provides advantages in the diagnostic routine: 1) The immunohistochemical MyHC double staining is faster and less labour intense. 2) Vanishing of the ATPase staining over time is not a problem with immunohistochemical labelling. 3) Frequent minor technical problems with ATPase due to improper reagents is not an issue with immunohistochemistry. 4). Crucial information about fiber type distribution was not compromised using this novel method. In fact, even more reliable information could be obtained with immunohistochemical double staining, i.e. regarding the occurrence of highly atrophic fibers because capillaries were not stained as with ATPase histochemistry. 5) The identification of 2C fibers being hybrids of MyHC isoforms I and IIA was easier and very reliable. Type IIX fibers, type 2B with ATPase, were readily separated as showing counterstaining with haematoxylin but no immunoreactivity. Moreover, the detection of hybrids expressing both IIA (ATPase type 2A) and IIX (ATPase type 2B) MyHC was possible, which is not the case with ATPase, although the biological or pathological significance of these specific hybrids is not determined. 6) The explicit advantage of this method is that all fiber type information is available on one and same slide.

The immunohistochemical method primarily developed for the screening detection of fast type IIA (ATPase type 2A) nuclear clump or other highly atrophic

fibers characteristic for DM2 disease, proved to be a very robust and reliable method for routine diagnostic purposes. Since the method provides more reliable and easier accessed information of the different fiber types than conventional ATPase histochemistry, we believe that MyHC isoform double staining immunohistochemistry can be used as a very good, if not superior, alternative in the routine diagnostics.

1.1 A new disease identified by using the new fiber typing method

With the method applied to our routine diagnostic procedure form muscle biopsies, we were able to easily identify the first patients with the total absence of type IIA fibers in muscle biopsies. In the further research collaboration and studies these patients were clarified to have a novel previously unknown disease, caused by recessive truncating mutations in the *MYH2* gene. The patients are born without fast IIA myosin but did not show evident muscle symptoms at birth, probably due to large usage of developmental MyHC isoforms during the first months of life (Butler-Browne et al. 1990). In early childhood symptoms manifested as generalized weakness with ophthalmoplegia and ptosis in some. The later evolution of the disease is rather stable with normal life expectancy but with incapacity for major muscle efforts.

2. Role of ZNF9 in DM2

DM2 disease has been considered to be caused primarily by RNA toxicity and that the mutation harbouring gene, *ZNF9* itself is of no significance for the disease pathogenesis. (Botta et al 2006; Margolis, et al. 2006). However, we have shown that ZNF9 expression is altered at multiple levels, including total mRNA and protein expression as well as subcellular localization to some extent. By exploring the mechanism of reduced ZNF9 expression we detected evidence for improper

processing of the pre-mRNA of the mutant allele, accounting for the overall decrease of mRNA transcript and protein.

ZNF9 has been reported to function as a DNA- and RNA-binding protein with alternatively spliced isoforms modulating β-myosin heavy chain gene expression (Rajavashisth et al.. 1989; Warden et al. 1994; Yasuda et al. 1995; Flink et al. 1995; Pellizzoni et al. 1997). However, the localization of ZNF9, changing from nuclear in undifferentiated myoblasts to cytoplasmic during differentiation to myotubes, and the cytoplasmic localization of ZNF9 in the Z-disc of the sarcomeres in mature human muscle fibers is unexpected for a transcription factor and suggests other functions in mature muscle fibers.

In addition to reduced *ZNF9* mRNA and protein levels, there is a minor difference in subcellular localization with less cytoplasmic and more membrane-bound protein in DM2 muscle. These localization changes apparently take place only after maturation of muscle tissue.

Clinical differences between DM1 and DM2 could in part be explained by different temporal and spatial expression patterns of *DMPK* and *ZNF9*, the genes harbouring the repeat mutations, leading to different amounts of toxic mRNA at different stages of development and in different tissues in DM1 and DM2. Moreover, effects of the resident genes may also account for certain aspects of the overall phenotype. This has been demonstrated by the use of knockout mouse models for *DMPK* and *ZNF9* (Chen et al.. 2007; Reddy et al. 1996) both of which show some phenotypic aspects of DM disease. There is a clear difference in ZNF9 expression between DM1 and DM2 patients. The observed clinical manifestations in DM1 and DM2 patients may thus be due to a combination of shared (nuclear pathology due to toxic RNA) and separate (cytoplasmic pathology due to, at least in part, *ZNF9*) pathomechanisms accounting for overlapping as well as distinct features.

3. Immunohistochemichal diagnostic method for myotonia

We have developed an immunohistochemical staining method for detecting ClC-1 in muscle fibers using two different protein specific antibodies. This proved to be a reliable method for the assessment of sarcolemmal ClC-1 protein on muscle sections. In our total cohort of 74 patients with sporadic/recessive non-dystrophic myotonia, 23 % had remained without a final genetic diagnosis after screening for the two common *CLCN1* mutations in Finland, R894X and F413C. Using this method we were able to establish the diagnosis in all these patients and moreover identified new *CLCN1* mutations that can cause or exacerbate decreased chloride conductance and myotonia by reduced amount of channel protein on the sarcolemma. We were able to identify a previously unreported c.264G>A mutation, as well as clarifying the W118G as a mutation with moderate harmful effect, although this was previously described as a polymorphism (Lehmann-Horn et al. 1995). Both mutations showed a clear loss of sarcolemmal ClC-1 protein in muscle biopsy sections. In addition to these findings, the robust expression of sarcolemmal ClC-1 in a patient harbouring a known dominant F307S mutation was verified consistent with the theoretical model of a dominant mutation. The usefulness of the method for diagnostics was also shown in a myotonia patient with only one R894 mutation despite whole gene *CLCN1* sequencing. The normal sarcolemmal ClC-1 protein on muscle biopsy directly indicated another cause for myotonia and the patient was later found to have a known pathogenic *SCN4A* mutation.

The total loss of sarcolemmal ClC-1 protein in homozygous R894X patients was expected since the mutation is a known truncating mutation and the protein is unstable (Furman et al. 1978).

The marked variability of ClC-1 expression detected by our assay in muscle biopsies from DM1 and DM2 patients is to some extent consistent with the highly variable phenotype of these diseases. Another purpose for developing this assessment technique was to be able to show on the molecular level the exacerbation

of the myotonia phenotype in DM2 patients with co-segregating recessive *CLCN1* mutations. (Suominen et al 2008). However, in the end we had access to only two muscle biopsies of DM2 patients with a co-segregating recessive *CLCN1* mutation and were thus not able to reliably distinguish the effect at the level of sarcolemmal ClC-1 expression in terms of significant decrease compared to DM2 patients without *CLCN1* mutation. Moreover, this approach, in order to be fully reliable, would have needed whole gene sequencing of all DM2 patients included in the study to make sure no uncommon *CLCN1* mutations were present in the cohort.

The combination of immunohistochemistry and gene sequencing is a powerful approach to achieve a final diagnosis in patients with non-dystrophic myotonia. Because the frequency of recessive mutation carriers in the general population can be relatively high, even in the range of 3-5 %, the distinction of asymptomatic carriers from a disease related heterozygous mutational finding is of high clinical relevance.

3.1 The c.264G>A mutation and ClC-1 expression

The previously unreported c.264G>A mutation was found in four different combinations: in compound heterozygosity in patients with R894X, F413C and V536I and as a homozygous mutation. Even though c.264G>A is silent, encoding no amino acid change, it leads to skipping of exon 2 on mRNA with subsequent frame shift and a premature truncation, which is a clear explanation for the loss of ClC-1 protein seen in all patients harbouring the mutation with immunohistochemistry as well as Western blotting.

3.2 The W118G mutation, function and ClC-1 expression

The W118G mutation has been reported as a polymorphism because of the relatively high frequency in the normal population (Lehmann-Horn et al. 1995). However, this mutation occurred with an unexpectedly high frequency among myotonia patients from Finland and The UK. Nine out of 17 Finnish myotonia

patients with inconclusive results by screening for the two common Finnish myotonia mutations harboured W118G. In the UK, where the population frequency of this mutation is calculated at 4.7%, 12% of patients with non-dystrophic myotonia congenita harboured the mutation. This highly significant over representation ($p< 0.001$) in both patient cohorts suggested a functional defect. In our Finnish patients W118G was found in two different combinations: compound heterozygosity with R894X in and with F413C.

On immunohistochemistry the combination W118G/R894X or W118G/F413C causes subtotal loss of sarcolemmal ClC-1 protein clearly distinct from the expression of heterozygous R894X or F413C alone. However, by Western blotting the total amount of ClC-1 protein in patients with a compound heterozygous W118G mutation is less abnormal. This discrepancy between sarcolemmal staining and blotting the total protein suggests the mutant W118G is not correctly transported and integrated into the sarcolemma. Our efforts to show this trafficking problem by electroporation transfection studies showed that a significant fraction of both wild-type and W118G was retained in the SR in rat muscle. Membrane extraction for the Western blot samples consists of all membrane components in the cells indicating that a large part of the denaturated mutant W118G protein detected is localized in cytoplasmic membrane components such as SR. Some studies have reported a high Cl^- conductance and the presence of YFP-ClC1:s in other intracellular compartments such as T tubules,(DiFranco et al. 2010; Lamb et al. 2011) but the antibodies used in the present study did not recognize any protein in T-tubules in patient or control samples. It should however be noted that in conduction studies using HEK cells and in electroporation studies using rat muscle fibers, the W118G mutation could not be combined with the other mutations in the compound heterozygous patients with subtotal loss of protein in immunohistochemisrty. Also, different handling of the W118G protein in non-human cells cannot be excluded. Low chloride conductance myotonia occurs when the summated loss of function of the two *CLCN1* alleles is greater than 60% (Peter et al. 2011; Kweicinski et al 1988; Colding-Jørgensen et al. 2005), owing to mutation of both alleles (Becker's disease), or a dominant negative interaction between a single mutant allele and the normal one (Thomsen's disease). Based on the high frequency in normal population controls and the absence of symptomatic homozygous W118G patients, the W118G apparently causes a mild loss of function

due to reduced abundance on the sarcolemma (for example 20%), which is insufficient to cause myotonia in a homozygote, but sufficient to cause myotonia when the other allele has lost its function, as with the R894X mutation.

SUMMARY AND CONCLUSIONS

The exact diagnosis of myotonic disorders can be very challenging for clinicians. In addition to clinical examination, laboratory tests including electrophysiology and muscle histopathology, diagnostics is increasingly based on molecular genetic DNA-testing. In all genetic disorders the causative gene mutation verification is the gold standard for final diagnosis. However, genetic DNA testing is rarely the first line of laboratory tests, but needs guidance from other examinations to be targeted to one or a few candidate genes. Even when correctly applied in the diagnostic procedure the DNA-test results do not always provide full clarification of the clinical disease.

The clinical presentation in DM2 is very variable and as a result patients are often misdiagnosed. In the past, previous diagnosis of DM2 patients have ranged from polymyositis, unexplained hepatopathy, chest pain, fibromyalgia, to psychiatric anxiety and other conditions. These incorrect diagnoses led to unnecessary and wrong treatment strategies. Also, many DM2 patients may have very late onset of symptoms, after the age of 60 or 70 when symptoms such as myalgia and / or mild proximal muscle weakness are frequently taken for normal aging and do not necessarily lead to neuromuscular examinations. There is a clear need for enhanced and improved diagnostic accuracy. Some DM2 patients may present severe cardiac conduction defects similar to the cardiac problems in DM1, with a risk of sudden cardiac death. Thus, correct diagnosis is needed and cardiac monitoring is recommended as part of the ENMC consensus guidelines of management in DM2.

1. One clue for considering DM2 diagnosis are the highly atrophic type IIA fibers including nuclear clump fibers on a routine muscle biopsy. The immunohistochemical MyHC double staining method described in this thesis provides the accurate tool for observing this finding, and directs the focus towards DM2 genetic testing.

2. A similar advantage is with the ClC-1 immunohistochemical staining method described in this thesis. This method provided means to identify one new *CLCN1* mutation, c.264G>A that causes the skipping of exon 2, to reclassify the W118G *CLCN1* change as a moderately pathogenic mutation, and to clarify recessive Becker myotonia patients in whom only one recessive mutation had been identified by genetic testing. The assay is most useful when screening for common *CLCN1* mutations fails to establish a genetic diagnosis in a patient with sporadic or recessive myotonia. Absence of protein in the muscle fibers directly indicates the presence of a second *CLCN1* mutation.

3. We were also able to show that the expression of the mutation carrying gene *ZNF9* in DM2 patients is definitely altered at several levels in contrast to previous reports. The exact importance for the disease pathomechanisms of this finding needs further research efforts but the decrease expression of ZNF9 could partly explain the phenotypic differences between DM2 and DM1.

4. We developed two new muscle biopsy based methods to aid the diagnostic evaluation of myotonic disorders. In addition to the above mentioned results, we were able to identify a completely novel previously unknown muscle disease caused by *MYH2* mutations, entirely based on findings acquired with the new MyHC double staining immunohistochemical technique. A few years of experience with this method in routine diagnostics has proved it to be extremely reliable and it has shown clear advantages over previous techniques. Our method has already been adopted in some diagnostic pathology laboratories in Finland as well as in some other countries.

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METHODS PAPER

Novel myosin heavy chain immunohistochemical double staining developed for the routine diagnostic separation of I, IIA and IIX fibers

Olayinka Raheem · Sanna Huovinen · Tiina Suominen · Hannu Haapasalo · Bjarne Udd

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Abstract The different histochemical ATPase properties of myosins separating the muscle fiber types have been utilized in diagnostic muscle biopsy routine for more than four decades. The ATPase staining method is rather laborious and has several disadvantages, such as weakening of staining over time and non-specific staining of capillaries, making the distinction of extremely atrophic muscle fibers difficult. We have developed a reliable and advanced immunohistochemical myosin double staining method for the identification of fiber types, including highly atrophic fibers in routine diagnostics. With this double staining method, we are able to distinguish among type I (ATPase type 1), IIA (ATPase type 2A), IIX (ATPase type 2B) and remodeled ATPase type 2C fibers expressing both fast and slow myosins using a one slide technique. Immunohistochemical double staining of myosin heavy chain isoforms can be used as an alternative for the conventional ATPase staining method in routine histopathology. The method provides even more detailed information of fast fiber subtypes and highly atrophic fibers on one single slide.

Keywords Myosin heavy chain · ATPase · Immunohistochemistry · Myotonic dystrophy · Muscle fiber

O. Raheem (✉)
Department of Neurology, Neuromuscular Molecular Pathology, University of Tampere, Biokatu 10, Finn-Medi 3, 33520 Tampere, Finland
e-mail: olayinka.raheem@uta.fi

S. Huovinen · H. Haapasalo
Department of Pathology, Pirkanmaa Hospital District Center for Laboratory Medicine, Biokatu 4, Finn-Medi 4, 33520 Tampere, Finland

T. Suominen
Department of Neurology, Neurogenetics, University of Tampere, Biokatu 10, Finn-Medi 3, 33520 Tampere, Finland

B. Udd
Department of Medical Genetics, Folkhälsan Institute of Genetics, University of Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland

B. Udd
Department of Neurology, Vaasa Central Hospital, Hietalahdenkatu 2-4, 65130 Vaasa, Finland

B. Udd
Department of Neurology, Tampere University Hospital, University of Tampere, Biokatu 10, Finn-Medi 3, 33520 Tampere, Finland

Introduction

Actomyosin filaments represent the principal structural contractile components of muscle cell sarcomeres [13]. Myosin is a molecular motor that converts chemical energy into movement. This is established by sliding of actin and myosin filaments over each other while hydrolysis of adenosine triphosphate (ATP) provides energy for this process [14]. The thick myosin filament consists of hexameric myosin molecules formed by two heavy chains (MyHC) and four associated light chains. Each myosin heavy chain contains two heads that are the site of myosin adenosine triphosphatase (ATPase), an enzyme that hydrolyzes ATP required for the actin and myosin cross bridge formation. These heads interact with a binding site on actin [14]. Myosin heavy chains are encoded by a multigene family and exist in several isoforms, which are expressed in a tissue-specific and developmentally regulated manner. More than one MyHC gene is expressed in

each muscle and developmental stage, but in the single mature and healthy muscle cell only one isoform is expressed [4].

In adult human skeletal muscle fibers, the major MyHC isoform in slow type I (ATPase type 1) fibers is encoded by the *MYH7* gene on chromosome 14, which is also the main isoform of the cardiac muscle. In the fast type 2A fibers, the corresponding MyHC isoform IIA is encoded by the *MYH2* gene on chromosome 17 [21]. Mutations in *MYH7* gene have been reported to cause both skeletal and cardiac or combined myopathies [2, 9, 11, 15, 16, 18], whereas mutations in *MYH2* were reported in rare families with skeletal myopathy [8, 10]. In the ultrafast glycolytic type 2B fibers, the corresponding MyHC IIX is expressed by the *MYH1* gene on chromosome 17 [21], but so far no human condition has been associated with mutations in this gene.

Hybrid fibers expressing both fast and slow myosin heavy chains are usually regenerated or remodeled fibers that often occur in an excess number in pathological stages as a result of reprogramming in altered muscle fibers. Such secondary changes in the expression of MyHC genes are also useful for the diagnostic assessment of muscle biopsies and reflect compensatory plasticity of muscle tissue [5, 7]. Although the exclusive expression of one MyHC gene per fiber is preprogrammed, various exogenic influences can modulate the expression, such as thyroid hormone, and innervation can also influence and induce isoform transitions [7, 12].

The myosin ATPase properties of different MyHC isoforms is widely used as the main diagnostic method for fiber-type separation. The histochemical method is based on the release of phosphate, the capture of phosphate by calcium resulting in calcium phosphate and substitution of calcium by cobalt. Phosphate is replaced by sulfide and the end product is a black precipitate of cobalt sulfide. The reaction is carried out at non-physiological pH of 9.4 and preincubation at different pHs of 4.3, 4.6 and 10.4 [1]. MyHC isoforms, their corresponding ATPase types, ATPase histochemical stains and immunohistochemical double staining patterns are shown in Table 1. The ATPase histochemical staining method is very laborious and there are disadvantages such as weakening of staining over time and the non-specific staining of capillaries, making a distinction of highly atrophic muscle fibers difficult.

Myotonic dystrophy (dystrophia myotonica, DM) is the most commonly inherited muscular dystrophy in adults. Two different types of myotonic dystrophy have been identified with similarities in their clinical features. Both myotonic dystrophy type 1 [DM1, Steinert's disease (OMIM #160900)] and type 2 [DM2, PROMM (OMIM #602668)] are dominantly inherited disorders with an estimated DM1 prevalence of 1/8,000 in European populations [3], while in DM2 the prevalence has not been established, but is proposed to be as common as in DM1 [17]. DM2 is caused by a tetranucleotide (CCTG)n expansion in the first intron of zinc finger protein 9 (*ZNF9*) gene on chromosome 3q21 [6]. The major symptoms of DM2 include proximal muscle weakness, muscle stiffness and/or pain, cataracts, myotonia, tremors, cardiac conduction defects and endocrinological abnormalities [18]. Clinical symptoms are however more inconsistent and diverse in DM2 than in DM1, which makes the clinical diagnosis a real challenge [18, 19]. We have previously reported the identification of a subpopulation of extremely small atrophic type 2 fibers, most of which are very difficult to detect by conventional ATPase staining, as a characteristic finding in DM2 [20]. Since fiber types by ATPase are paralleled by differences in MyHC isoforms, the full assessment of fiber-type distribution can also reliably be achieved by immunohistochemical staining.

Our MyHC immunohistochemical double staining method was primarily developed as an advanced screening method to identify the highly atrophic type 2 muscle fibers characteristic of DM2. However, this method proved a very informative and powerful tool for general diagnostic purposes and may substitute the ATPase stainings in the diagnostic routine. The MyHC double staining method can be performed on one slide only and our experience shows that this staining method is more reliable for the detection and separation of different fiber types and their subtypes, particularly regarding the highly atrophic fibers and hybrid fibers expressing more than one MyHC isoform.

Materials and methods

Frozen sections from ten normal cases, five DM2, five DM1, five muscle biopsies with neurogenic pathology and single biopsies with polymyositis, a genetically verified *FKRP*-mutated LGMD2I and a genetically verified *MYH7*-mutated early-onset Laing myopathy were used for MyHC double staining performed on a BenchMark (Ventana Medical Systems Inc., Tucson, AZ 85755, USA) immunostainer, using slow myosin monoclonal antibody against slow type I (ATPase type 1) fibers (clone WB-MHCs, Leica Microsystems, VisionBbiosystems, Newcastle upon Tyne, NE12 8EW, UK) at a dilution of 1:200 and myosin A4.74, a monoclonal antibody against fast type IIA (ATPase type 2A) fibers, at a dilution of 1:100. The myosin A4.74 antibody developed by Helen M. Blau was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, USA. The immunohistochemical stainings were performed using the official protocol of the BenchMark immunostainer with incubation of primary antibodies for

Table 1 Muscle fiber types based on ATPase staining (with van Gieson counterstain) related to their corresponding MyHC isoform content, the corresponding genes for the MyHC isoforms, the physiological and metabolic properties, and their staining patterns by ATPase histochemical stains and immunohistochemical double staining

ATPase fiber type	1	2A	2B	2C
Fiber type	Slow	Fast aerobic	Ultrafast glycolytic	Mixed
MyHC isoform	I	IIA	IIX	Hybrid I and IIA
MyHC gene	*MYH7*	*MYH2*	*MYH1*	Mixed
ATPase staining pH 10.4	Light brown	Brown	Dark brown	Dark brown
ATPase staining pH 4.6	Dark brown	No reaction (pale)	Light brown	Dark brown
ATPase staining pH 4.3	Dark brown	No reaction (pale)	No reaction (pale)	Light brown
MyHC double staining	Brown	Red	No reaction (light blue)	Red-brown

30 min at 37°C. Slow myosin was visualized with a peroxidase-based detection kit (Universal DAB detection kit, Ventana Medical Systems Inc., Tucson, AZ 85755, USA) and myosin A4.74 with alkaline phosphatase red detection system (ultraView^{TM} Universal Alkaline Phosphatase Red Detection kit, Ventana Medical Systems Inc., Tucson, AZ 85755, USA). Histochemical ATPase staining with pH 4.3, 4.6 and 10.4 was performed in parallel as described before [1]. Briefly, each of 10-µm sections were pre-incubated in sodium barbiturate and calcium chloride solution of pH 10.4, and barium acetate and hydrochloric acid solution of pH 4.3 and 4.6. The sections were then incubated in a solution of sodium barbiturate of pH 9.4, containing adenosine triphosphate for 30 min at +37°C. After incubation in 1% calcium chloride and 2% cobalt chloride, respectively, the sections were dipped into a solution of 0.01 M sodium barbiturate. The sections were washed and then dipped into a solution of 0.2% ammonium sulfide to form a black precipitate of cobalt sulfide. Fibers without reaction and connective tissue were stained using Van Gieson solution. The Van Gieson staining also changes the appearance of the black precipitate of cobalt sulfide into shades of brown. Nuclei were stained using Weigert's hematoxylin. The ATPase staining was done on serial sections of the same samples to compare staining methods and fiber-type distribution of muscle biopsies.

Results

All ATPase-based fiber types were easily separated by the double immunostaining technique (Fig. 1). Slow type 1 fibers stained deep brown with slow myosin MyHC I antibody with the peroxidase detection, and fast type 2A fibers stained deep pink-red with fast myosin MyHC IIA A4.74 antibody using the alkaline phosphatase red detection system. Type 2C fibers being MyHC I and IIA isoform hybrid fibers stained red-brown showing the presence of both slow and fast MyHC isoforms in these fibers. Moreover, this technique was also able to separate the ultrafast myosin MyHC IIX expressing type 2B fibers on the same slide that were immunonegative for the myosin A4.74 antibody. Capillaries or other structures were not labeled (Fig. 1).

In the disease samples, myosin immunohistochemistry provided additional information not easily obtained by ATPase enzyme histochemistry. In DM 2 samples, all nuclear clump fibers and other highly atrophic fibers were readily detected as fast type IIA (ATPase type 2A) in red (Fig. 2). This finding was re-confirmed by immunoreactivity for neonatal myosin heavy chain isoform, as all these highly atrophic type IIA (ATPase type 2A) fibers also express neonatal MyHC as part of the disease process (data not shown). Fiber-type grouping as a result of chronic neurogenic change was absent in both DM1 and DM2, whereas the number of hybrid fibers was increased in both. In the muscle biopsies of patients with neurogenic disease, fiber-type grouping was comparably identified as with ATPase technique, but highly atrophic fibers in severe neurogenic end-stage samples were more easily identified with the immunohistochemical double staining (Fig. 3). Different fiber types were also easily distinguished in muscle samples of patients with LGMD2I, myositis and a mutation in the *MYH7* gene (Fig. 4).

Discussion

In this study, we have analyzed the utility of immunohistochemistry of MyHC isoforms as an alternative to conventional ATPase histochemistry for fiber-type separation and identification. The immunohistochemical method primarily developed for the screening of fast type IIA (ATPase type 2A), nuclear clump or other highly atrophic fibers characteristic of DM2 disease proved to be a very robust and reliable method for routine diagnostic purposes. The immunohistochemical MyHC double staining was very efficient for the detection and separation of different muscle fiber types and their subtypes. In all

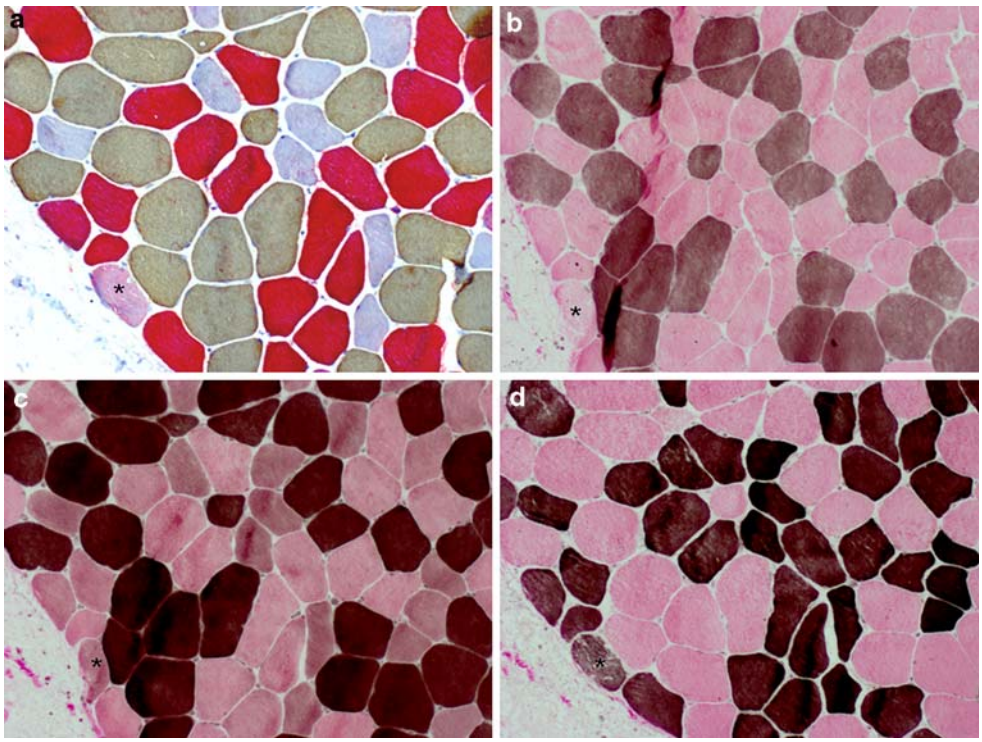

Fig. 1 Immunohistochemical MyHC double staining with *red* fibers expressing IIA, *blue* fibers showing IIX, and *brown* fibers showing I MyHC isoforms **a**. Corresponding ATPase histochemistry at pH 4.3 **b**, pH 4.6 **c**, pH 10.4 **d** distinguishing different fiber types. *A *pink* hybrid fiber expressing both IIA and IIX MyHC

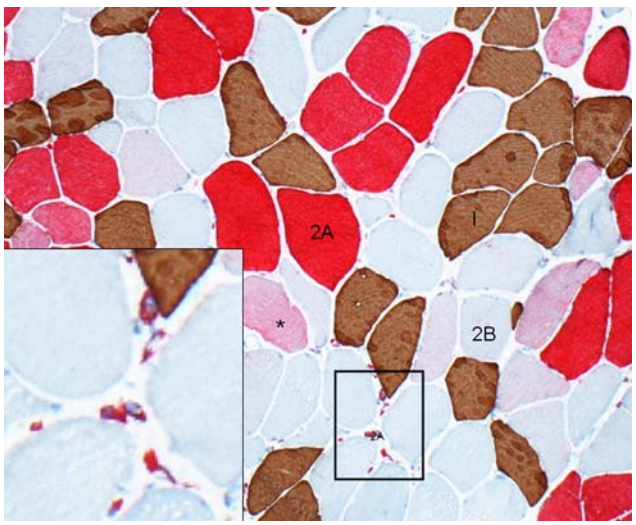

Fig. 2 Immunohistochemical MyHC double staining showing characteristic highly atrophic type IIA fibers in DM2 muscle biopsy. *Red* fibers expressing IIA, *blue* fibers IIX and *brown* fibers I MyHC isoforms. **Pink* hybrid fibers expressing both IIA and IIX MyHC

DM2 patient biopsies, the subpopulation of highly atrophic fast type IIA (ATPase type 2A) and nuclear clump fibers was clearly identified. In comparison with the conventional ATPase technique, this immunohistochemical method provides a few advantages in the diagnostic routine: (1) The immunohistochemical MyHC double staining is faster and less labor intense. (2) Vanishing of ATPase enzyme histochemistry staining over time is not a problem with immunohistochemical labeling. (3) Frequent minor technical problems with ATPase due to improper reagents is not an issue with immunohistochemistry. (4) Crucial information about fiber-type distribution was not compromised using this novel method. In fact, even more reliable information could be obtained with immunohistochemical double staining, i.e., regarding the occurrence of highly atrophic fibers because capillaries were not stained as with ATPase histochemistry. (5) The identification of 2C fibers as being hybrids of MyHC isoforms I and IIA was easier and very reliable. Type IIX fibers, type 2B with ATPase, were readily separated and showed counterstaining with hematoxylin, but no immunoreactivity. Moreover, the detection of hybrids expressing both IIA (ATPase type 2A) and IIX (ATPase type 2B) MyHC was possible (Fig. 1), which was not the case with ATPase, although the biological or pathological significance of these specific hybrids was not determined. (6) The explicit advantage of this method is that all fiber-type information is available on one and the same slide.

The primary purpose of finding a reliable method for the screening of DM2 disease by the identification of the highly atrophic type IIA (ATPase type 2A) and nuclear clump type IIA(ATPase type 2A) fibers in muscle biopsies was very successful (Fig. 2). However, in our 4 years of experience with this technique, it has proven to be a primary tool of much larger advantage in the routine

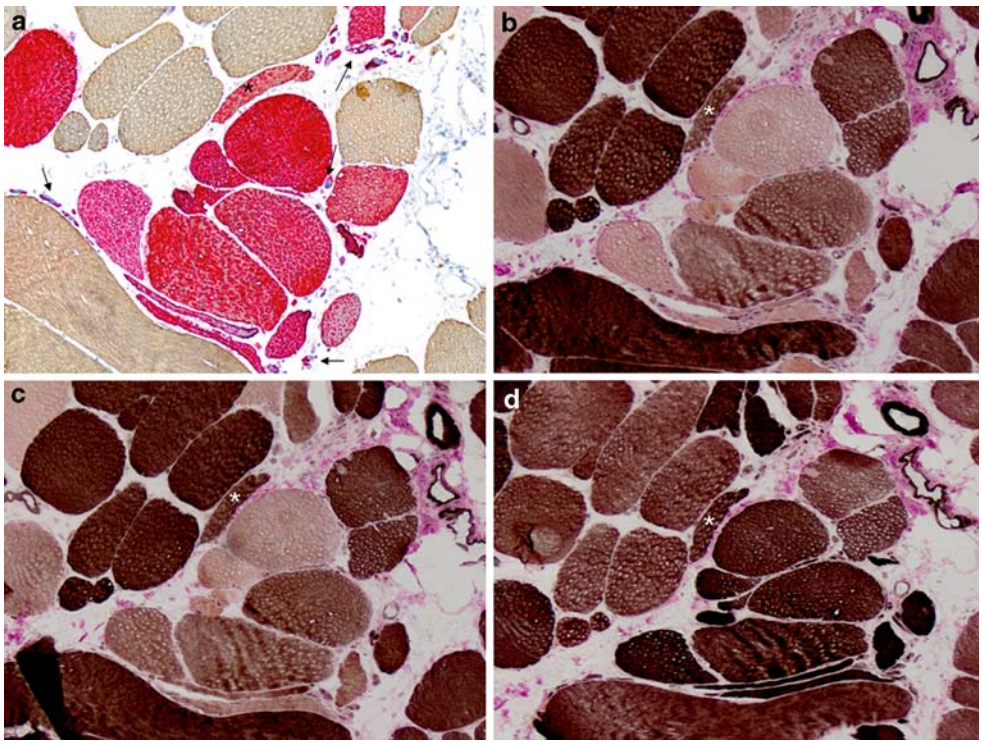

Fig. 3 Immunohistochemical MyHC double staining with *red* fibers expressing IIA, *blue* fibers IIX and *brown* fibers I MyHC isoforms **a** and ATPase stainings at pH 4.3 **b**, pH 4.6 **c** and pH 10.4 **d**. Highly atrophic fibers in severe neurogenic end-stage samples are more easily identified and typed with the MyHC double staining. *Arrows* in **a** show highly atrophic fibers. *The same *red-brown* hybrid fiber co-expressing MyHC isoforms I and IIA (ATPase type 2C)

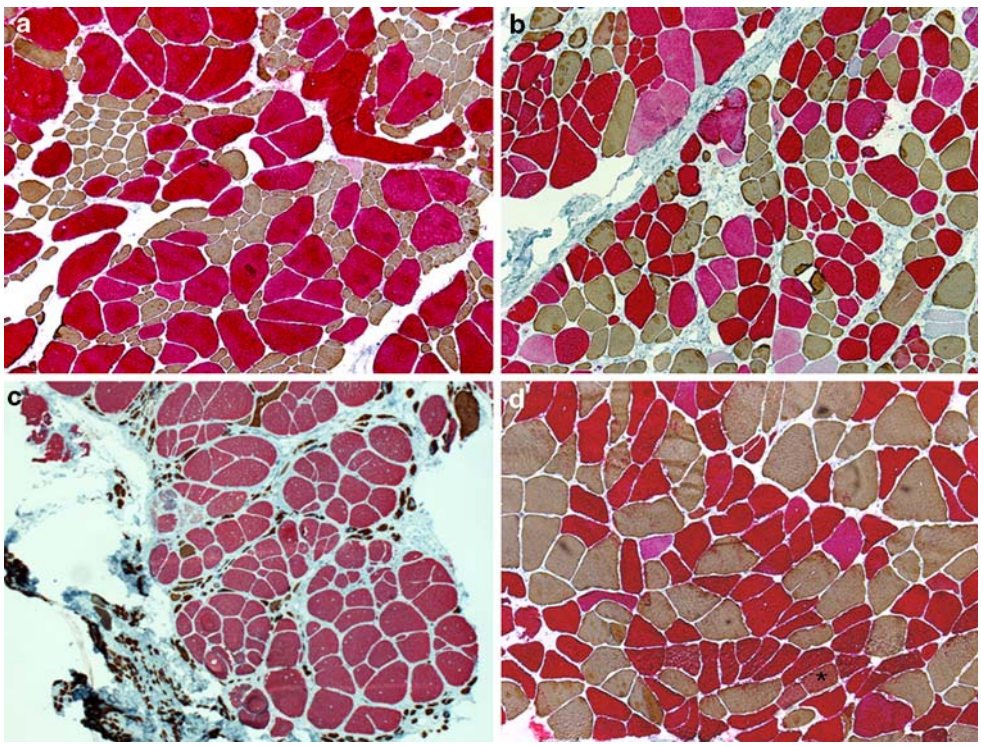

Fig. 4 Immunohistochemical MyHC double stainings of muscle sections with various pathologies. **a** Patient with genetically verified early-onset Laing myopathy showing fiber-type disproportion with smaller type I fibers. **b** Genetically verified LGMD2I. **c** Neurogenic atrophy showing marked fiber-type grouping and **d** polymyositis *asterisks* showing *red-brown* fiber co-expressing MyHC isoforms I and IIA (ATPase type 2C). *Red* fibers expressing IIA, *blue* fibers IIX and *brown* fibers I MyHC isoforms

diagnostic procedure. Since the method provides more reliable and easier access to information of the different fiber types than conventional ATPase histochemistry, we believe that MyHC isoform double staining immunohistochemistry can be used as a very good, if not superior, alternative in routine diagnostics.

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Conflict of interest statement None declared.

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Human disease caused by loss of fast IIa myosin heavy chain due to recessive *MYH2* mutations

Homa Tajsharghi,[1] David Hilton-Jones,[2] Olayinka Raheem,[3] Anna Maija Saukkonen,[4] Anders Oldfors[1] and Bjarne Udd[3,5,6]

1 Department of Pathology, Institute of Biomedicine, University of Gothenburg, Sahlgrenska Hospital, Gothenburg 413 45, Sweden
2 Department of Neurology, West Wing, John Racliffe Hospital, Oxford OX3 9DU, UK
3 Neuromuscular Centre, Tampere University and Hospital, Tampere 33520, Finland
4 Department of Neurology, Central Hospital of Northern Karelia, Joensuu 80210, Finland
5 Department of Neurology, Vasa Central Hospital, Vasa 65130, Finland
6 Folkhälsan Genetic Institute, Department of Medical Genetics, Helsinki University, Helsinki 00290, Finland

Correspondence to: Anders Oldfors,
Department of Pathology,
Institute of Biomedicine,
University of Gothenburg,
Sahlgrenska University Hospital,
413 45 Gothenburg, Sweden
E-mail: anders.oldfors@gu.se

Striated muscle myosin heavy chain is a molecular motor protein that converts chemical energy into mechanical force. It is a major determinant of the physiological properties of each of the three muscle fibre types that make up the skeletal muscles. Heterozygous dominant missense mutations in myosin heavy chain genes cause various types of cardiomyopathy and skeletal myopathy, but the effects of myosin heavy chain null mutations in humans have not previously been reported. We have identified the first patients lacking fast type 2A muscle fibres, caused by total absence of fast myosin heavy chain IIa protein due to truncating mutations of the corresponding gene *MYH2*. Five adult patients, two males and three females, from three unrelated families in UK and Finland were clinically assessed and muscle biopsy was performed in one patient from each family. *MYH2* was sequenced and the expression of the corresponding transcripts and protein was analysed in muscle tissue. The patients had early-onset symptoms characterized by mild generalized muscle weakness, extraocular muscle involvement and relatively favourable prognosis. Muscle biopsy revealed myopathic changes including variability of fibre size, internalized nuclei, and increased interstitial connective and adipose tissue. No muscle fibres expressing type IIa myosin heavy chain were identified and the *MYH2* transcripts were markedly reduced. All patients were compound heterozygous for truncating mutations in *MYH2*. The parents were unaffected, consistent with recessive mutations. Our findings show that null mutations in the fast myosin heavy chain IIa gene cause early onset myopathy and demonstrate that this isoform is necessary for normal muscle development and function. The relatively mild phenotype is interesting in relation to the more severe phenotypes generally seen in relation to recessive null mutations in sarcomeric proteins.

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Abbreviations: MyHC = myosin heavy chain; PCR = polymerase chain reaction

Introduction

Myosin is one of the most abundant proteins in the body and is indispensable for body movement and heart contractility. Three major myosin heavy chain (MyHC) isoforms are present in adult human limb skeletal muscle: MyHC I, also called slow/β-cardiac MyHC, is the gene product of *MYH7* and is expressed in slow, type 1 muscle fibres as well as in the ventricles of the heart; MyHC IIa (*MYH2*) is expressed in fast, type 2A muscle fibres; and MyHC IIx (*MYH1*) is expressed in fast, type 2B muscle fibres. The three different muscle fibre types display distinct physiological properties and have unique roles in the function of skeletal muscle (Larsson and Moss, 1993). We describe the clinical and morphological characteristics of patients from three unrelated families lacking the production of MyHC IIa due to non-sense and truncating mutations in *MYH2*.

Materials and methods

Patients

Five patients were clinically assessed (Table 1). Three of the patients had mild to moderate generalized muscle weakness from early childhood, with minor progression. Two were subjectively asymptomatic. All had facial muscle weakness and marked external ophthalmoplegia and two had ptosis.

Muscle morphology

Muscle biopsy specimens were obtained from one patient of each family. In Patient II:1 (Family A) muscle biopsy specimens were obtained from the vastus lateralis of the quadriceps femoris muscle, at age 38, and from the deltoid muscle at age 40. In Patient II:2 (Family B) and Patient II:1 (Family C) muscle biopsies were obtained from the vastus lateralis of the quadriceps femoris muscle at age 55 and 58, respectively. Enzyme and immunohistochemical analyses, including MyHC isoforms, of freshly frozen muscle biopsy specimens were performed as previously described (Tajsharghi *et al.*, 2002). In Patients II:2 (Family B) and II:1 (Family C) a new double immunostaining method for MyHC isoforms was performed that shows the expression of different MyHC isoforms in different muscle fibres in a single section (Raheem *et al*., 2010).

DNA analysis

Genomic DNA was extracted from frozen skeletal muscle or peripheral blood using DNA Extraction Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) analysis was performed in a master mixture (ReddyMix PCR Master Mix; Abgene, Epsom, UK) after addition of 20 pmol of each primer and genomic DNA. PCR amplifications were performed as previously described (Tajsharghi *et al.*, 2005). Nucleotide sequence determination was performed by cycle sequencing using a BigDye Terminator DNA sequencing kit (Applied Biosystems, Hercules, CA).

RNA analysis

The complementary DNA of MyHC isoforms, including the three adult skeletal isoforms, are highly homologous. In order to solely amplify fragments of *MYH2* by PCR, we performed alignment of *MYH2*, *MYH1*, *MYH7*, *MYH4*, *MYH3* and *MYH8* complementary DNA (http://bio.lundberg.gu.se/edu/msf.html) to design *MYH2* specific primers. Total RNA was extracted from muscle tissue of the patients using the Total RNA Isolation System (Promega, Madison, WI). Synthesis of first-strand complementary DNA was performed using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions using 1 μg total RNA.

To analyse the splicing of exon 8 of *MYH2* in Patient II:1 (family A), PCR was performed on complementary DNA with forward primer AGTGACGGTGAAGACTGAGGGA (corresponding to nucleotide 177–198 of human MyHC IIa complementary DNA sequence) combined with a backward primer ATCTGTGGCCATCAGTTCTTCCT (corresponding to nucleotide 986–1008 of human MyHC IIa complementary DNA). The resulting PCR products were analysed by sequencing after separation on 2% agarose gel and purification using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). In addition, PCR was performed on complementary DNA with forward primer AGGGAGCTGGTGGAGGGGCC (corresponding to nucleotide 1898–1917 of human MyHC IIa complementary DNA sequence) combined with a backward primer CTTGACATTCATGAAGGATCT (corresponding to nucleotide 2473–2493 of human MyHC IIa complementary DNA sequence) covering exon 15 through 20 to analyse the p.R783X mutation in Patient II:1 (Family A). This primer pair was also used to analyse the p.L802X mutation and the splicing of exon 16 in Patient II:2 (Family B) and Patient II:1 (Family C). The PCR amplifications consisted of an initial preheating step for 5 min at 94°C, followed by a touchdown PCR with denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 1 min with a 1°C temperature decrement per cycle during the first 10 cycles. The subsequent cycles (40 cycles) each consisted of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min.

To analyse the proportion of transcripts of the three major MyHC isoforms, PCR was performed on complementary DNA extracted from skeletal muscle and fragment analysis was performed as previously described (Tajsharghi *et al.*, 2002).

Protein analysis

To analyse the expression of the MyHC isoforms, proteins extracted from muscle biopsy specimens were separated by 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described (Tajsharghi *et al.*, 2002).

Haplotype analysis

Haplotype analysis was performed with micro-satellite markers.

Table 1 Clinical data

Patient	II:1 (Family A) UK	II:2 (Family A) UK	II:3 (Family A) UK	II:2 (Family B) Finland	II:1 (Family C) Finland
Sex (F/M)	F	F	M	M	M
Age (years)	41	42	44	58	59
Age at onset of muscle weakness	Early childhood. Little or no progression	Asymptomatic, except for 'lazy eye' noted in childhood. No subsequent ocular symptoms	Asymptomatic	General muscle weakness from early childhood	Ptosis, ophthalmoplegia and mild general weakness since early childhood
Ophthalmoplegia	Pronounced	Pronounced	Pronounced	Pronounced	Pronounced
Ptosis	Yes	No	No	No	Yes
Distribution of muscle weakness	Facial muscle weakness	Facial muscle weakness	Facial muscle weakness	Facial muscle weakness	Facial muscle weakness
				Upper limbs MRC grade 4–5	Upper limbs MRC grade 4–5
				Abdominal muscle weakness MRC grade 3	Abdominal muscle weakness MRC grade 3
				Mild proximal weakness in lower limbs	Mild proximal weakness in lower limbs
	Neck flexion weakness Diffuse limb muscle slimness–mild weakness most marked proximally	Neck flexion weakness Elbow flexion and ankle dorsiflexion	Neck flexion weakness Elbow flexion and ankle dorsiflexion		
Other signs or symptoms	Symptomatic joint hypermobility	Asymptomatic joint hypermobility	Asymptomatic joint hypermobility	Congenital pectus carinatum surgically corrected. No improvement on strength training	
EMG	Myopathic—more marked in proximal muscles	Not investigated	Not investigated	Mild myopathic	Myopathic
s-CK	Normal	Not investigated	Not investigated	Normal	Normal
Muscle imaging	Not investigated	Not investigated	Not investigated	Moderate diffuse fatty degenerative change in thigh and in medial gastrocnemius	Moderate diffuse fatty degenerative change in thigh and in medial gastrocnemius

MRC = Medical Research Council scale for grading of muscle strength (*Aids to the Examination of the Peripheral Nervous System*. Elsevier, 2000); s-CK = Creatine kinase in serum.

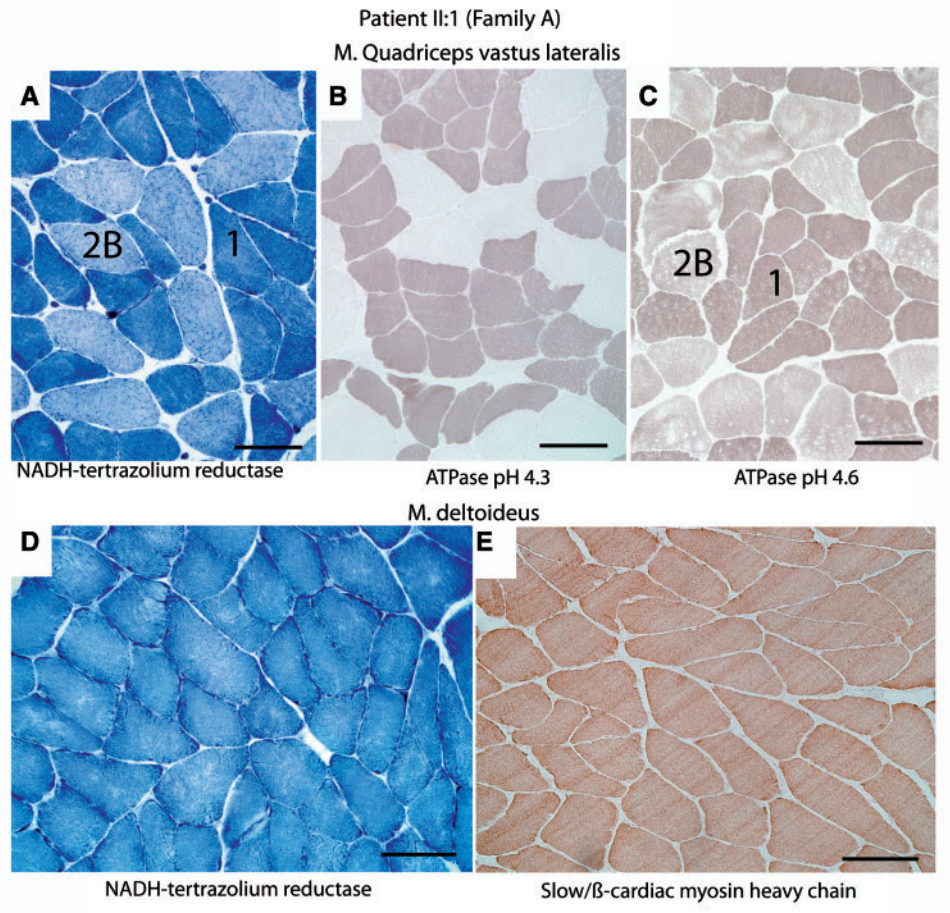

Figure 1 Muscle biopsy from quadriceps and deltoid muscles of Patient II:1 (Family A). (**A–C**) The quadriceps muscle include type 1 and type 2B fibres. (**D–E**) The deltoid muscle specimen shows type 1 fibre uniformity with expression of only slow/β cardiac myosin heavy chain. Bar corresponds to 100 μm.

Results

Laboratory investigations

Morphological analysis of biopsy specimens from the quadriceps femoris and deltoid muscles of Patient II:1 (Family A) demonstrated type 1 fibre uniformity in the deltoid muscle and absence of type 2A fibres in both muscles (Fig. 1). A biopsy specimen from vastus lateralis of the quadriceps femoris muscle of Patient II:2 (Family B) demonstrated absence of MyHC IIa and myopathic features including increased variability of fibre size and internalized nuclei (Fig. 2). In Patient II:1 (Family C) a muscle biopsy of the vastus lateralis of the quadriceps muscle showed absence of muscle fibres expressing type IIa MyHC, as well as myopathic changes that included marked variability in fibre size, internalized muscle fibre nuclei, increased interstitial fat and connective tissue and type 1 fibre uniformity (Fig. 3A–C).

MRI or CT of skeletal muscle in two of the patients showed diffuse fatty infiltration with an unusual pattern of predominant involvement of medial gastrocnemius in the lower legs, combined with predominant involvement of the semitendinosus, gracilis and vastus lateralis muscles in the thigh. The tibialis anterior muscle, which mainly consists of slow muscle fibres, showed normal appearance (Fig. 3D–G).

Molecular genetics

The incentive to consider mutated *MYH2* as a plausible cause of the disease was the ophthalmoplegia in the patients of Family A since in skeletal myopathy associated with a dominant missense mutation, p.E706K in *MYH2*, all patients had ophthalmoplegia and abnormal type 2A muscle fibres (Martinsson *et al.*, 2000). In Families B and C it was the total absence of fast IIa fibres with the new double immunostaining technique (Raheem *et al.*, 2010) in proximal muscle biopsy specimens that indicated a *MYH2* defect.

Mutation analysis of *MYH2* was performed in six individuals. In Patient II:1 (Family A), we identified two sequence variants. First, a heterozygous G to A change affecting a highly conserved nucleotide of the 5′ splice junction of intron 8 (c.904+1G>A). PCR analysis of complementary DNA in a region covering exons 2–10 of *MYH2* revealed two different fragments: one fragment of normal size and a shorter fragment. Sequence analysis of the short fragment demonstrated skipping of exon 8, shifting of

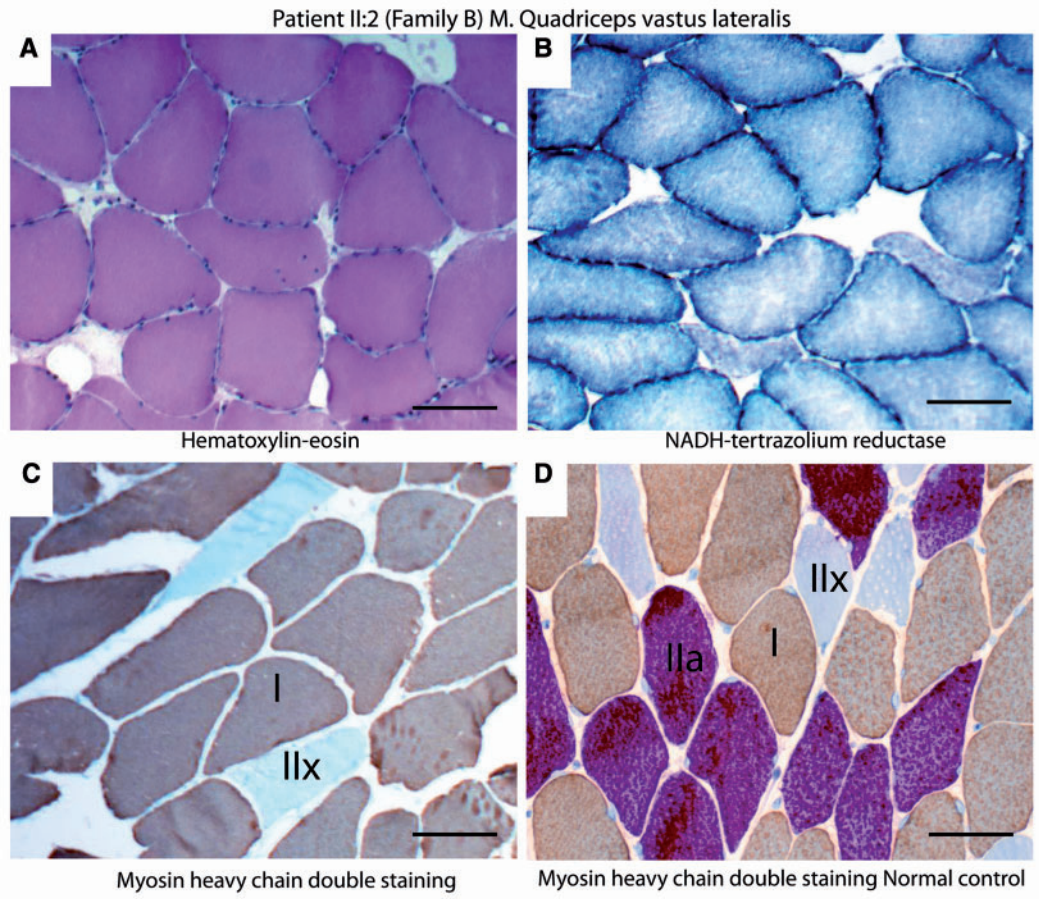

Figure 2 Quadriceps muscle biopsy sections of Patient II:2 (Family B). (**A–B**) There is increased variability of muscle fibre size with atrophic and hypertrophic fibres and occasional fibres with internalized nuclei and lack of type 2A muscle fibres. (**C**) Immunohistochemical staining demonstrates muscle fibres with expression of either of myosin heavy chain I and IIx. No fibres expressing IIa MyHC are present. (**D**) Immunohistochemical of control muscle demonstrating muscle fibres expressing IIa myosin heavy chain (red fibres). Bars correspond to 50 μm.

the reading frame and a premature stop codon (p. Tyr269-Glu302delfsX) (Fig. 4B). The second variant was a heterozygous nonsense mutation, c.2347C>T, changing Arginine at position 883 to a stop codon (p.Arg783X) in exon 19 (Fig. 4C). The same two mutations were also identified in siblings II:2 and II:3 (Family A). The unaffected father (I:1, Family A) had only the heterozygous 5′ splice site mutation of intron 8 indicating that the c.2347C>T mutation was inherited from the mother.

In Families B and C, we identified in each of two patients (Patient II:2 of Family B and II:1 of Family C) two variants with truncating effects in *MYH2*. The two different variants were identical in both families. The first was a heterozygous A to G change affecting the highly conserved second nucleotide of the 3′ splice site of intron 15 (c.1975-2A>G) which resulted in skipping of exon 16 and shifting of the reading frame (p. Glu659-Gly687delfsX11) (Fig. 4E). The second variant was a heterozygous non-sense mutation, c2405T>A, changing leucine at position 802 to a stop codon (p.Leu802X) in exon 19 (Fig. 4F). Sequence analysis of complementary DNA demonstrated that the patients were compound heterozygous for the two truncating mutations. PCR amplification of complementary DNA of Patient II:2 (Family B) and Patient II:1 (Family C) in the region covering exon 15 through exon 20 of *MYH2* generated two products: a large fragment derived from normal splicing and a small fragment with skipping of exon 16. Sequence analysis of the large fragment revealed normal splicing of exon 16 in combination with the c.2405T>A mutation in exon 19. Sequence analysis of the small PCR fragment revealed skipping of exon 16 and creation of a stop codon combined with wild-type c.2405T in exon 19.

Analysis of *MYH2* transcripts

To determine the effect of the mutations on *MYH2* gene expression, analysis of the relative level of expression of different isoforms of MyHC mRNA was performed by PCR on complementary DNA and fragment analysis. These results demonstrate that the three patients express very low levels of *MYH2* transcripts (Fig. 5A).

Protein analysis

The expression of MyHC isoforms by SDS–PAGE analysis of the deltoid muscle of Patient II:1 (Family A) and the quadriceps muscle

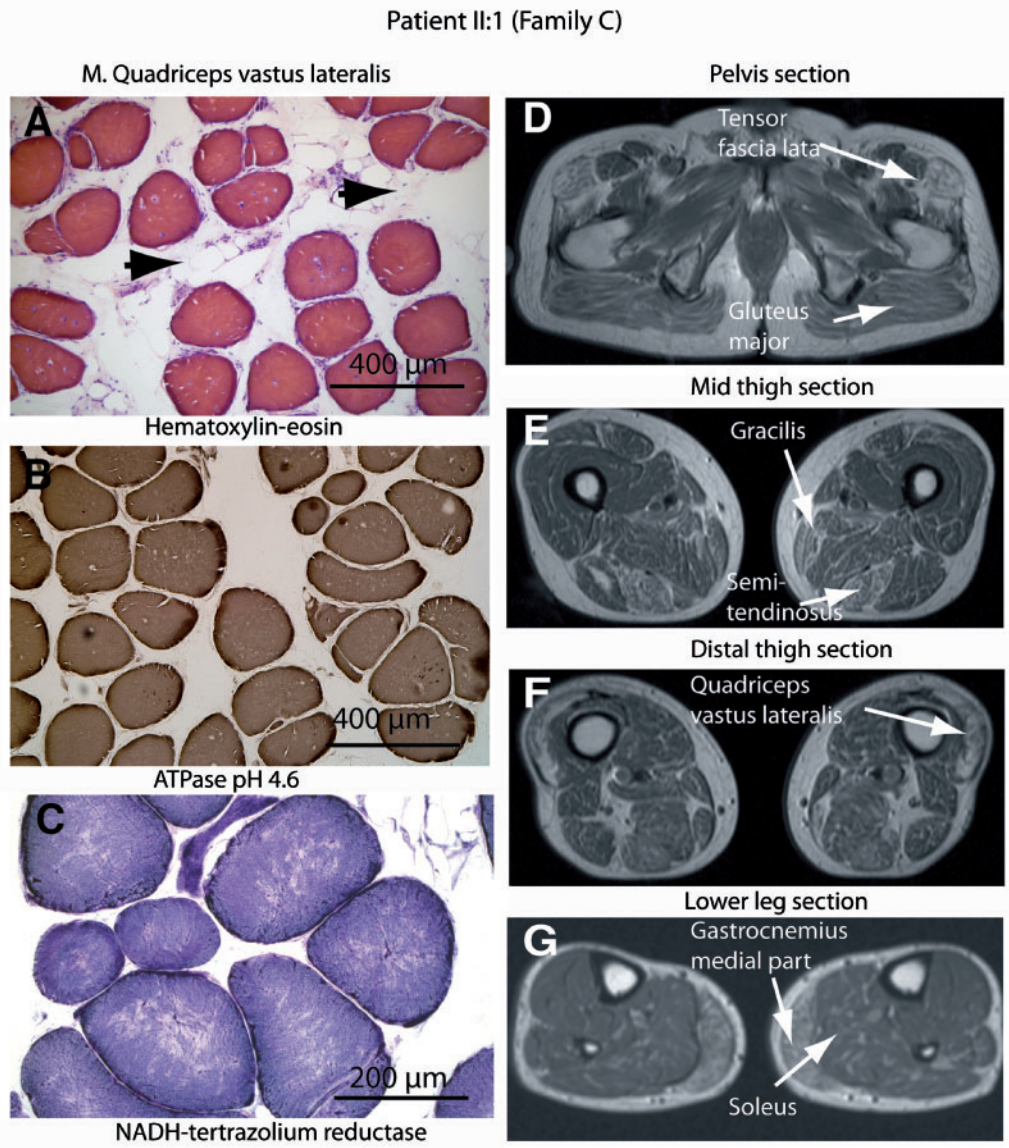

Figure 3 Muscle histopathology and MRI of Patient II:1 (Family C). (**A–C**) Sections of a muscle biopsy specimen from vastus lateralis of the quadriceps femoris muscle demonstrating fatty infiltration (arrow heads), hypertrophic and atrophic muscle fibres with internalized nuclei, type 1 fibre predominance, as well as slight disorganization of the intermyofibrillar network as revealed by NADH-tetrazolium reductase. (**D–G**) MRI of pelvis and legs at age 58 years demonstrating fatty infiltration in semitendinous, gracilis, vastus lateralis of the quadriceps femoris and medial gastrocnemius muscles.

of Patient II:1 (Family C) confirmed the absence of MyHC IIa protein (Fig. 5B). There was a predominant expression of slow/β-cardiac MyHC (MyHC I) in these two muscle biopsy specimens.

Haplotype analysis

Haplotype analysis of Patient II:2 (Family B) and Patient II:1 (Family C) revealed that the two patients carried the identical haplotype over a distance ~3.3 Mb on one chromosome with the c.1975-2A>G mutation, whereas sharing of a shorter segment (0.7–1.5 Mb) on the other chromosome indicates that the c.2405T>A mutation was more ancient.

Discussion

We have identified the first patients with loss of a MyHC isoform, MyHC IIa and complete loss of one of the major muscle fibre types, type 2A. Our patients were compound heterozygous for truncating mutations in *MYH2* resulting in loss of expression of MyHC IIa mRNA as well as any functional protein. Whether the reduced transcript expression was the result of non-sense mediated mRNA decay could not be established, but the functional consequences would be similar to complete inactivation of *MYH2*. The parents in all three families had no symptoms or signs of muscle dysfunction implying that all four mutations are

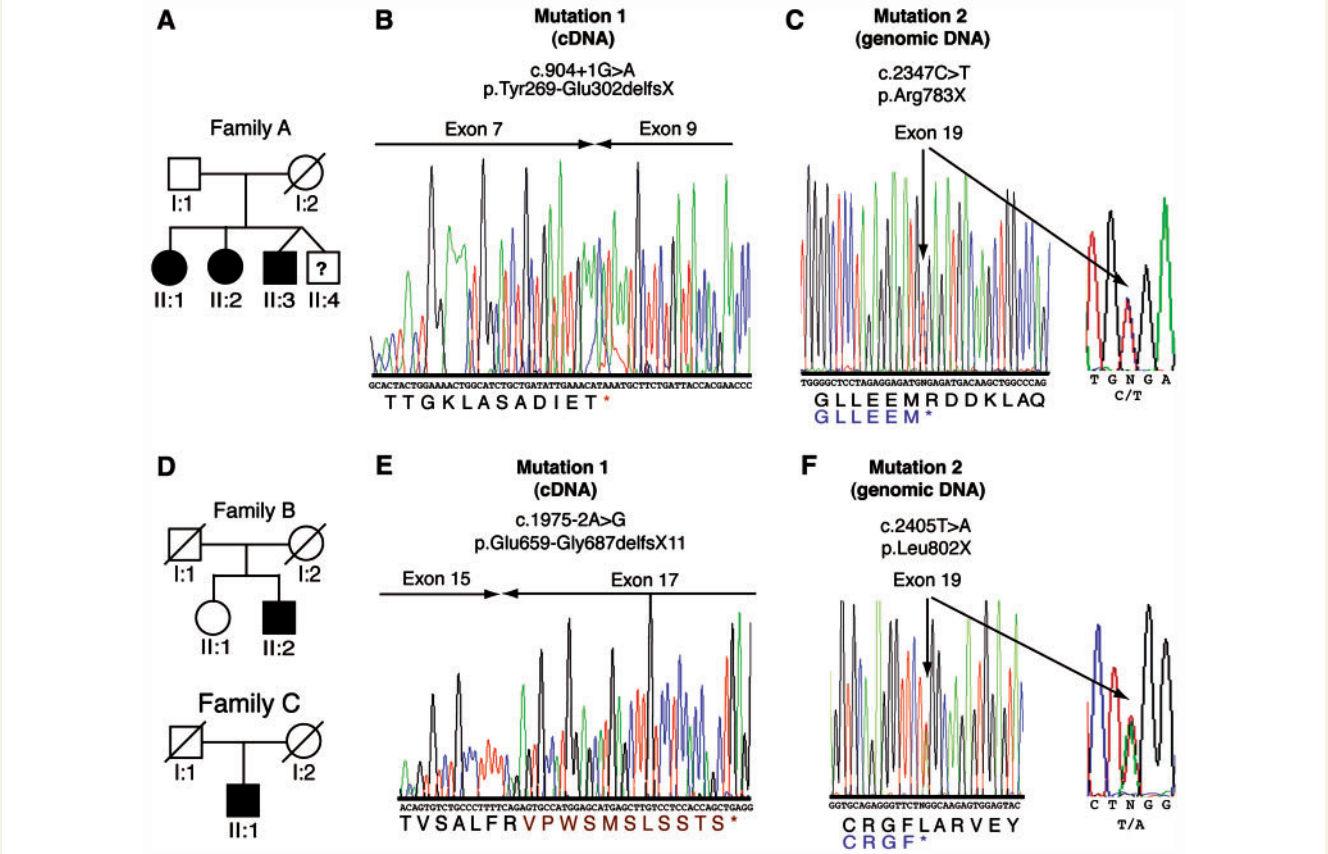

Figure 4 Pedigrees and DNA sequencing chromatograms of *MYH2* in the patients. (**A**) Pedigree of Family A. (**B**) Complementary DNA (cDNA) sequence chromatogram of exons 7 and 9 demonstrating skipping of exon 8 in Patient II:1 (Family A). The normal sequence is illustrated in Supplementary Fig. 1. (**C**) Genomic DNA sequence chromatogram of exon 19 of Patient II:1 (Family A) carrying the heterozygous c.2347C>T mutation, changing the arginine at position 783 to a stop codon. (**D**) Pedigrees of Families B and C. (**E**) Complementary DNA sequence chromatogram of exons 15 and 17 showing skipping of exon 16 in Patient II:2 (Family B). The same results were obtained in Patient II:1 (Family C). (**F**) Genomic DNA sequence chromatogram of exon 19 of Patient II:2 (Family B) carrying the heterozygous c.2405T>A mutation, changing the leucine at position 802 to a stop codon. The same results were seen in Patient II:1 (Family C). Amino acid sequences in black indicate the normal sequences; sequences in red indicate the amino acid changes due to the mutations; and sequences in blue indicate the mutant allele. Filled symbols in the pedigrees show the individuals that are clinically and genetically affected.

recessive and that hemizygous loss of MyHC IIa expression does not lead to haploinsufficiency and disease.

In human limb muscle there are two fast MyHC isoforms: MyHC IIa (corresponding to MyHC IIa in the mouse) and MyHC IIx (corresponding to MyHC IId/x in the mouse). Mice also express a third fast MyHC isoform in limb skeletal muscle: MyHC IIb. Results from studies on MyHC IId/x and MyHC IIb null mice demonstrate that these genes are required for the normal muscle development and function of adult skeletal muscle in the mouse and that the different fast MyHC isoforms are functionally unique and cannot substitute for one another (Acakpo-Satchivi *et al.*, 1997; Sartorius *et al.*, 1998; Allen *et al.*, 2001; Allen and Leinwand, 2001). MyHC IIa null mice have been reported but not characterized in detail (Geurts *et al.*, 2006).

Our patients with loss of fast MyHC IIa expression exhibited muscle weakness and myopathic changes with predominant involvement of semitendinous, gracilis, vastus lateralis and medial gastrocnemius muscles in the lower limbs. The reason for preferential involvement of these muscles remains to be demonstrated but may reflect the relative proportion of MyHC IIa in these muscles, since the tibial anterior muscle, which is predominantly composed of slow fibres, showed normal appearance on imaging. In MyHC IIb knockout mice, two factors appeared to determine the extent to which a muscle was affected: the level of MyHC IIb and the amount of muscle activity (Allen *et al.*, 2000). In the MyHC IId/x null mice there was no such correlation suggesting that other factors may also be of importance (Allen *et al.*, 2000).

The expression of MyHC IIa can be detected from around 24 weeks gestational age to adulthood in humans and it is one of the major MyHC isoforms expressed in human skeletal muscle (Butler-Browne *et al.*, 1990; Cho *et al.*, 1994; Smerdu *et al.*, 1994). This implies that our patients had disturbed development and maturation of skeletal muscle from around 24 weeks of gestational age, which is consistent with the early onset of symptoms. However, none of the patients were identified at birth as having a

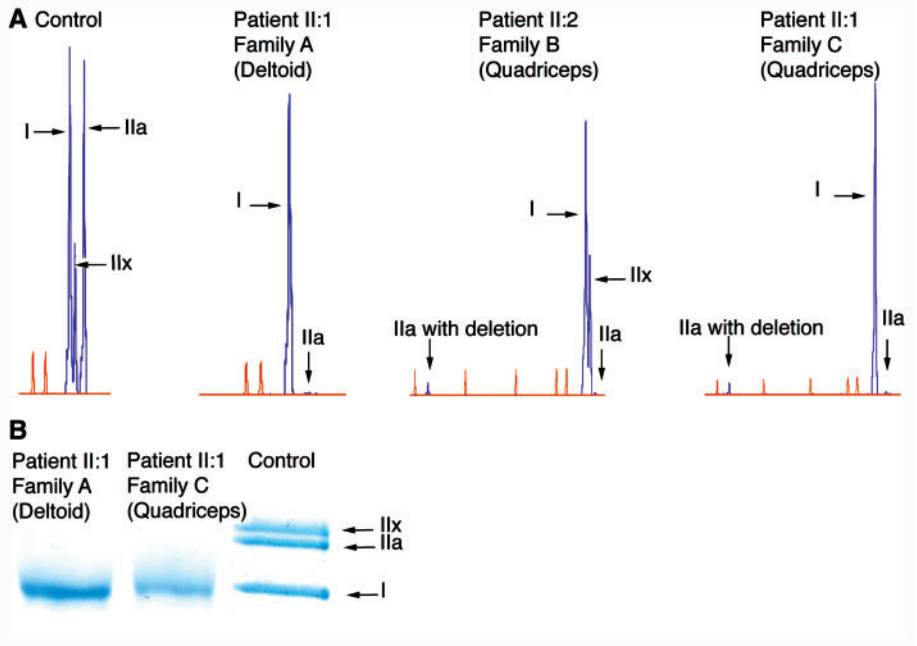

Figure 5 Expression of myosin heavy chain isoforms. (**A**) Quantitative analysis of relative expression of MyHC I, MyHC IIa and MyHC IIx messenger RNA based on reverse transcription PCR analysis. The complementary DNA fragment with skipping of exon 16 differs from the wild-type cDNA fragment by 106 nucleotides. Small amounts of the transcripts of MyHC IIa with skipping of exon 16 in Patient II:2 (Family B) and Patient II:1 (Family C) are present. In the deltoid muscle of Patient II:1 (Family A) and the quadriceps muscle of Patient II:1 (Family C) MyHC IIx transcripts are undetectable whereas in the quadriceps muscle of Patient II:2 (Family B) MyHC IIx is expressed at levels comparable to those of the normal control. (**B**) Expression of MyHC isoforms by SDS–PAGE in muscle homogenate of Patient II:1 (Family A) (deltoid muscle) and Patient II:1 (Family C) (quadriceps femoris muscle) showing MyHC I predominance in both samples. A control muscle sample from a biceps muscle of an individual, without evidence of muscle disease, demonstrates the normal occurrence of three major MyHC isoforms.

congenital myopathy. Analogous with the MyHC IIb and IId/x null mice, our patients showed slow progression of muscle wasting with increasing age (Acakpo-Satchivi *et al.*, 1997; Allen *et al.*, 2001; Allen and Leinwand, 2001). As in mice, the progression may be related to ongoing degeneration and regeneration as indicated by the pathological changes in muscle biopsy specimens. Why degenerative changes in the type 1 and type 2B fibres occur when one MyHC isoform and fibre type is lacking is not clear. A possible explanation could be that proper maintenance of muscle tissue requires all myosin isoforms and fibre types, and that muscle fibre degeneration is a consequence of less capability to sustain mechanical load during normal activity if one fibre type is lost.

The explanation of the apparent type 1 fibre uniformity and predominant expression of slow myosin in two of the investigated muscles (deltoid muscle of Patient II:1 in Family A and quadriceps muscle of Patient II:1 in Family C) is not clear. It is well known that in many congenital or early onset myopathies, such as nemaline myopathy and central core disease, there is predominance and sometimes uniformity of type 1 fibres. However, in our patients, type 1 fibre predominance was not a consistent finding in all muscles since Patient II:1 (Family A) had a normal amount of type 2B fibres in the quadriceps muscle and Patient II:2 (Family B) expressed *MYH1* gene transcript at a nearly normal level in the quadriceps muscle, and up to 15% of fibres expressed MyHC IIx on immunohistochemistry. In the quadriceps muscle the normal proportion of type 1 fibres is between 44 and 57% (Lexell *et al.*, 1983).

The clinical phenotype of our patients with compound heterozygous null mutations of *MYH2* was rather mild. This was unexpected since several other myopathies caused by recessive null mutations of sarcomeric proteins that exist in different isoforms show a much more severe clinical phenotype. Absence of α-tropomyosin slow (*TPM3*) (Tan *et al.*, 1999) or muscle troponin T slow (*TNNT1*) (Jin *et al.*, 2003) is associated with severe forms of nemaline myopathy. Complete loss of β-tropomyosin (*TPM2*) is associated with Escobar syndrome with nemaline myopathy (Monnier *et al.*, 2009) and absence of α-skeletal muscle actin (*ACTA1*) is associated with persistent expression of developmental actin and severe or intermediate nemaline myopathy (Nowak *et al.*, 2006). In addition, the various isoforms of skeletal muscle MyHC genes and proteins show a high degree of conservation in genomic structure and amino acid sequences. The orthologous isoforms of MyHC in different species have a greater extent of conservation than different isoforms within a species (Weiss *et al.*, 1999) suggesting an important functional diversity within the MyHC gene family.

The fact that MyHC IIa is expressed in extraocular muscle can explain the ophthalmoplegia observed in all of our patients (Pette and Staron, 1997; Pedrosa-Domellöf *et al.*, 2000). In patients with

autosomal dominant myopathy associated with the heterozygous *MYH2* p.E706K missense mutation, there was a clear correlation between pathology and expression of MyHC IIa indicating a dominant negative effect of this missense mutation (Tajsharghi *et al.*, 2002). In the patients with no fast IIa MyHC due to compound heterozygous truncating *MYH2* mutations, the situation is different and illustrates the importance of expression of MyHC IIa, even if hemizygous loss is well tolerated. Total absence of MyHC IIa cannot be substituted for by an increased expression of another MyHC isoform but the consequence of the total loss is a surprisingly mild phenotype.

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Supplementary material

Supplementary material is available at *Brain* online.

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New immunohistochemical method for improved myotonia and chloride channel mutation diagnostics

[1]Olayinka Raheem, [1]Sini Penttilä, [1]Tiina Suominen, [2]Mika Kaakinen, [3]James Burge, [3]Andrea Haworth, [4]Richa, Sud, [3]Stephanie Schorge, [5]Hannu Haapasalo, [1,6]SatuSandell, [2]Kalervo Metsikkö, [3]Michael Hanna, [1,7,8]Bjarne Udd

Raheem O,MSc, (1) *Neuromuscular Research Unit, University of Tampere and Tampere University Hospital, Tampere, Finland; address: University of Tampere, Neuromuscular Molecular Pathology, Finn-Medi 3, 1-122, Biokatu 10, 33520 Tampere, Finland*; telephone number +358 443480651, fax number +358 3 3551 8430, email address: olayinka.raheem@uta.fi
Penttilä S, MSc, (1), e-mail address: sini.penttila@uta.fi
Suominen T, MSc, (1), e-mail address: tiina.suominen@uta.fi
Kaakinen M, PhD, (2) *Institute of Biomedicine, Department of Anatomy and Cell Biology, University of Oulu, Oulu, Finland* e-mail address:mika.kaakinen@oulu.fi
Burge J, MRCP (UK), (3)*Medical Research Council Centre for Neuromuscular Diseases, National Hospital for Neurology and Neurosurgery, University College London, Queen Square, London, UK,* e-mail address: j.burge@ucl.ac.uk
Haworth A, PhD,*(4)Neurogenetics Unit, Institute of Neurology, NationalHospital for Neurology and Neurosurgery, Queen Square, London, UK,* e.mail address: andrea.haworth@uclh.nhs.uk
Sud R, PhD,*(4),* e-mail address: richa.sud@uchl.nhs.uk
Schorge S, PhD, (3), e-mail address: s.schorge@ucl.ac.uk
Haapasalo H, MD, PhD, (5)*Department of Pathology, Fimlab Laboratories Ltd, Tampere, Finland,* e-mail address: hannu.haapasalo@fimlab.fi
Sandell S, MD, (1), (6)*Department of Neurology, SeinäjokiCentralHospital, Seinäjoki, Finland,* e-mail address: satu.sandell@epshp.fi
Metsikkö K, PhD, (2), e-mail address: kalervo.metsikko@oulu.fi
Hanna M, FCRP (UK), (3), e-mail address: m.hanna@ucl.ac.uk
Udd B, MD, PhD, (1), (7)*Folkhälsan Institute of Genetics, Department of Medical Genetics and Haartman Institute, University of Helsinki, Helsinki, Finland,* (8)*Department of Neurology, Vaasa Central Hospital, Vaasa, Finland,* e-mail address: bjarne.udd@netikka.fi

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SEARCH TERMS: Cohort studies [54], All genetics [91], Ion channel gene defects [97], All neuromuscular disease [176], Muscle disease [185]

ABSTRACT

Objective: The objective of this study was to validate the immunohistochemical assay for the diagnosis of non-dystrophic myotonia and to provide full clarification of clinical disease to patients with who basic genetic testing has failed to do so.

Methods: An immunohistochemicalassay of sarcolemmal chloride channel abundance using two different ClC1-specific antibodies.

Results: This method lead to the identification of new mutations, to the reclassification of W118G in *CLCN1* as a moderately pathogenic mutation, and to confirmation of recessive (Becker)MyotoniaCongenita in cases when only one recessive*CLCN1* mutation had been identified by genetic testing.

Conclusions: We have developed a robust immunohistochemical assay that can detect loss of sarcolemmal ClC-1 protein on muscle sections. This in combination with gene sequencing is a powerful approach to achieving a final diagnosis of non-dystrophic myotonia.

INTRODUCTION

We have previously reported an increased frequency of co-existing recessive *CLCN1* mutations in the currently diagnosed myotonic dystrophy type 2 (DM2) patients (1), because DM2 patients heterozygous for a recessive *CLCN1* mutation have more pronounced myotonia (1). With the aim of showing this modifying effect of the co-segregating *CLCN1* mutations on the protein level, we developed an immunohistochemical assay for ClC-1 protein expression. The method proved to be efficient in the molecular diagnostic clarification of non-dystrophic myotonias caused by mutations in *CLCN1* and *SCN4A* genes.

Autosomal recessive Becker (OMIM #255700) and dominant Thomsen (OMIM #160800) congenital myotonia are non-dystrophic myotonias caused by mutations in *CLCN1* on chromosome 7q35 (2). More than 100 different *CLCN1* mutations have been identified (3). Some *CLCN1* mutations are clearly more common than others. R894X (c.2680C>T) has an estimated carrier frequency of about 1 % in the European population. In the Finnish population the mutation F413C(c.1238T>G) is almost as frequent at least in the northern Finland (4). However, these two mutations explain only about half of the congenital myotonias in the studied population, and many myotonia patients remain with just one mutation identified when screening for these two common mutations.

In this study we focused on the validation of an immunohistochemical assay for the diagnosis of non-dystrophic myotonia. With this method combined with molecular genetics we were able to clarify all undetermined myotonia patients, identify new recessive mutations and verify normal protein expression with dominant *CLCN1* mutations.

METHODS

Standard protocol approvals, registration and patient consents

All blood and tissue samples were obtained with written informed consent according to the Helsinki declaration and the study was approved by the local ethical board.

Patients and controls

The study included patients of whom muscle biopsy was available: 29 patients with non-dystrophic myotonia (NDM), 15 males and 14 females with an average age of 49 years ranging from 20-78 years, eight DM1 patients,10 DM2 patients, five asymptomatic carriers of recessive *CLCN1* mutations, and six non-related normal controls.

Twenty five NDM patients had clear clinical/subclinical myotonia. Twenty four were from sporadic/recessive pedigrees. Five of the 24 were homozygotes for R894X, one was a compound heterozygote R894X and F413C, and in the remaining 18 screening for R894X and F143C had failed to establish a final genetic diagnosis (single mutation or no mutation detected). One patient, in whom screening for the two common *CLCN1* mutations had been negative, was from an autosomal dominant pedigree. In the patients without a final genetic diagnosis the whole *CLCN1*exome and/or cDNA was sequenced so that the efficacy of our assay for ClC-1 expression to detect a second mutation could be determined.

Four NDM myalgic patients had myotonia detectable by EMG but not by clinical examination.

All DM1 and DM2 patients had been genetically diagnosed. Two of the DM2 patients had a co-segregating *CLCN1* mutation. The remaining 8 DM2 patients and two DM1 patients that had been screened were negative for R894X and F413C. None of the patients were on antimyotonic drugs.

Patients are summarized in table e-1.

Population controls

We screened 100 Finnish population controls for the W118G mutation. Additionally, a cohort of 65 Finnish population samples from a genetically isolated Larsmo island region was screened for both W118G and c.264G>A changes and 100 from the same population were also screened for F413C mutations.

The W118G change had been previously screened for in 261 unrelated myotonia patients and in 64 unrelated population control samples from the UK as a part of the clinical genetics service of the National Hospital for Neurology & Neurosurgery, Queen Square, London (unpublished data).

ClC-1 immunohistochemistry on human skeletal muscle

Frozen sections of muscle tissue were used for immunohistochemical double staining of ClC-1 protein using two different antibodies pooled together, a commercial ClC-1 antibody against an extracellular domain close to the C-terminal (Alpha diagnostic international, TX, USA) and a ClC-1 antibody generated against the 15 C-terminal amino acids (5). The double immunohistochemical staining was performed on the BenchMark (Roche Tissue Diagnostics / Ventana Medical Systems Inc.) immuno-stainer, visualized with a peroxidase based detection kit and the signal amplified (Roche Tissue Diagnostics / Ventana Medical Systems Inc.). The stainings were analyzed and compared to normal controls. Samples used for immunohistochemistry are listed in table e-1.

Genomic DNA and cDNA sequencing of CLCN1 gene

Genomic DNA was extracted from peripheral blood leucocytes. Primer sequences to the 23 *CLCN1* exons are available upon request. All 23 exons were amplified by polymerase chain reaction and sequenced using bidirectional fluorescent sequencing on an ABI3130xl automatic DNA sequencer system (Applied Biosystems, CA, USA), with Big-Dye Version 3.1 chemistry. For cDNA analysis,

RNA was extracted from muscle biopsies using Trizol according to the manufacturer´s suggestions (Invitrogen, CA, USA) and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *CLCN1* gene transcript was sequenced using five overlapping primer pairs. All sequences were analyzed with Sequencer software (Gene Codes Corporation, Ann Arbor, MI, USA).

SDS-PAGE and Western Blotting

Membrane proteins were extracted from muscle biopsies: two normal controls, one co-segregating DM2 and heterozygous F413C mutation, one homozygous R894X, three heterozygous R894X and two heterozygous F413C (table e-1). The membrane protein phases were used for SDS-PAGE and Western blotting according to standard protocols. Nitrocellulose membranes with transferred proteins were immunolabeled with ClC-1 antibody (5).

In vivo electroporation and expression analysis of chimeric GFP ClC1 constructs

Mammalian expression plasmids encoding chimeric GFP ClC-1 and GFP ClC-1 W118G were prepared. In vivo electroporation of plasmids encoding GFP ClC1:s into the living rat flexor digitorumbrevis-(FDB) muscle was performed as described (6). After three to five days of the operation rats were sacrificed the transfected muscles were excised, frozen in liquid nitrogen- cooled isopenthane and cryosectioned.

Patch clamp analysis

The chloride currents supported by either homodimeric W118G mutant or wildtype channels were assessed by whole cell patch clamp. The W118G point mutation was introduced into the cDNA for human *CLCN1* in a mammalian expression vector (PCDH1, System Biosciences) using the QuickChange Site-directed Mutagenesis Kit (Agilent Technologies, Inc., CA, USA). HEK293T

(ATCC) cells were transfected with 0.5µg *CLCN1* DNA using Lipofectamine2000 (Invitrogen) and studied by patch clamp 24 to 48 hours after transfection.

To obtain the voltage dependence of activation, the instantaneous current on stepping to -100 mV (tail current) was measured after pre-pulses to variable voltages from -140mV to +120mV. The full voltage protocol started from a holding potential of -40mV after which the voltage was first stepped to +60mV (which fully activates wildtype channels) before applying the variable pre-pulse voltage and then the step to -100mV. The normalized tail current, I, was plotted against pre-pulse voltage was fitted with a Boltzmann function ($y = I_{min} + [I_{max} – I_{min}]/[1 + \exp((V_{50}– V_{prepulse})/slope)])$ to estimate the voltage of half maximal activation (V_{50}) and slope factor.

RESULTS

Immunohistochemistry and sequencing

Patients with clinical and EMG myotonia

All five patients with homozygous R894X mutations showed total loss of sarcolemmalClC-1 expression. Ten patients with clinical myotonia but just one heterozygous R894X mutation after first screening had total / subtotal loss of sarcolemmal ClC-1 protein (figure 1).Sequencing of the whole gene revealed that out of these 10 patients, six harbored an additional heterozygous W118G (c.352T>G) change located in exon 3 and four had an additional heterozygous synonymous change, c.264G>A located in exon 2.

Of the four patients with clinical myotonia and heterozygous F413C mutation (table e-1, patients P12-P14, P20), one showed total loss of protein and was found to be compound heterozygous with a c.264G>A change. The three other patient biopsies showed subtotal loss of sarcolemmal ClC-1 protein and sequencing the whole gene identified compound heterozygosity with W118G.

One patient without common mutations and subtotal loss of sarcolemmal ClC-1 was compound heterozygous for two previously unknown mutations, V536I (c.1606G>A) and the c.264G>A. In two other patients without common mutations and total loss of sarcolemmal ClC-1, sequencing the whole *CLCN1* gene revealed a homozygous c.264G>A change only.

The c.264G>A mutation is silent mutation with no amino acid change (p.V88V). However, cDNA sequencing of patients homozygous for c.264G>A revealed that all mRNA transcripts lacked exon 2. Exon 2 was also lacking in one allele in patients heterozygous for the c.264G>A change (Figure 2). According to Human Splicing Finder (7) c.264G>A breaks several potential exonic splicing enhancer sites. Nucleotides c.263-270 are markedly conserved in mammals (Figure 2). To ensure that c.264G>A is the cause of exon skipping and not just linked to it, we sequenced introns 1 and 2 (apart from base pairs c.180+938_180+1167) in patients P22 and P16, and no variants were found..

One patient with dominant familial myotonia had a dominant F307S (c.920T>C) mutation located in exon 8 in compound heterozygosity with c.2284+5C>T that has been suggested to be a splice mutation (8) but is known to occur in 1 % of normal population (1000 genes database). There was no loss of ClC-1 protein on the sarcolemma. One patient with clinical myotonia and heterozygous R894X mutation had more or less normal amount of sarcolemmal ClC-1. Sequencing of the whole gene and mRNA in this patient did not disclose other *CLCN1* mutations. The normal ClC-1 immunohistochemistry directly suggested a different cause and a A1156T mutation in the sodium channel *SCN4A* gene was subsequently identified.

<u>Asymptomatic first degree carrier relatives of patients with Becker myotonia</u>

In the case of the homozygous c.264G>A brothers F1:II-1 and F1:II-2, we were able to study the asymptomatic mother F1:I-1 who logically was a carrier of the mutation. Each asymptomatic parent (F2:I-1 and F2:I-2) of patient F2:II-1 compound heterozygous for c.264G>A and R894X, was found to be a carrier for one of the mutations each. Furthermore, the asymptomatic mother (F3:I-2) of the

compound heterozygous patient F3:II-1 harboring c.264G>A and F413C carried the F413C mutation only. On immunohistochemistry, carriership of the F413C mutation in the mother was not associated with any clear loss of sarcolemmal protein. Thus, the segregation of c.264G>A is compatible with a recessive pathogenic mutation.

Heterozygosity for R894X in the asymptomatic father F4:I-2 of patient P23 produced an irregular minor reduction of sarcolemmal ClC-1 (table e-1). Altogether, these results suggest that the compound heterozygous mutations in patients were on separate alleles.

Patients with EMG myotonia and myalgia but without clinical myotonia

In patients (P26-P29) with only EMG myotonia, sarcolemmal ClC-1 protein was close to normal, slightly irregular or just moderately reduced. These patients were found to have heterozygous R894X and F413C mutations only, even after sequencing the whole *CLCN1* gene.

DM1 and DM2 patients

The sarcolemmal ClC-1 staining in DM1 and DM2 samples was variable from severe reduction to normal staining when compared to normal controls. Muscle biopsy samples were available only of two DM2 patients with co-segregating recessive *CLCN1* mutations. One with a co-segregating heterozygous R894X mutation showed loss of ClC-1 protein while the other DM2 patient with a co-segregating heterozygous F413C mutation showed subtotal loss of the protein in both immunohistochemistry and Western blotting. These reductions were in the range of ClC-1 expression seen in DM1 and DM2 patients. ClC-1 immunohistochemical results and results from *CLCN1* sequencing are summarized in table e-1.

Western blotting

In Western blots we observed 80-90 % reduction ClC-1 protein in biopsies with a homozygous R894X mutation (Figure 3). Heterozygous c.264G>A mutations combined with both R894X and F413C mutation also showed 80-90 % reduction of ClC-1 when compared to muscle biopsies from normal controls (Figure 3). These results correlated well with the results in ClC-1 immunohistochemistry. Patients with combined heterozygous R894X and W118G showed less clear reduction of total ClC-1 protein expression in Western blots (Figure 3) in contrast to the very clear reduction of sarcolemmal expression observed with immunohistochemistry. The method should however be refined if further standardized quantification is needed.

Results from functional analysis of W118G

Both W118G and wildtype ClC-1 channels produced robust chloride currents in HEK cells. In contrast to currents produced by many dominantly inherited ClC-1 mutants, there was no obvious difference in current amplitudes between the wildtype and mutant clones. In addition there was no significant difference in voltage dependence between the W118G-W118G homodimeric ClC-1 mutant and the wildtype (figure 4). Wt ClC-1 has been shown to localize in the sarcolemma and T-tubules of wild type rat myofibers (5, 9, 10, 11, 12). Transfections of the chimeric GFP-ClC1 WT and W118G mutant into the living rat muscle fibers by means of electroporation did not reveal clear differences in the localization patterns between wildtype and mutant (data not shown).

Population screening

In the cohort of 100 samples from Larsmo population, we found three heterozygous F413C mutations corresponding to a carrier frequency of 3 %. In 65 individuals from the same population the W118G mutation was found in a carrier frequency of 7,7 %, whereas no carriers of the c.264G>A mutation were found. In a cohort of 100 controls from Central Finland the W118G

mutation was found in a frequency of 3 % and in a cohort of 64 controls from the UK with a frequency of 4.7%.

Of 261 UK myotonia patients 31 patients were found to have the W118G mutation (10 of whom were homozygotes for this mutation) corresponding to a frequency of 12 %.

DISCUSSION

We developed an immunohistochemical assay for ClC-1 in muscle fibers using two different antibodies that proved to be a robust method for the detection of presence or absence of sarcolemmal ClC-1 protein on muscle sections. In our total cohort of 74 patients with sporadic/recessive non-dystrophic myotonia 23% had remained without genetic diagnosis after screening for the two common *CLCN1* mutations in Finland, R894X and F413C. Using this method we were able to establish diagnosis in all and identified new *CLCN1* mutations that can cause or exacerbate low chloride conductance myotonia.

The previously unreported c.264G>A mutation was found in four different combinations. The silent c.264G>A is the apparent cause of exon 2 skipping on mRNA subsequently leading to frame shift and expected to cause nonsense mediated mRNA decay, which is supported by the absent protein in c.264C>A homozygotes.

The W118G mutation has been considered a polymorphism (13). However, it occurred with an unexpectedly high frequency among myotonia patients from Finland and The UK. Nine of 19 Finnish myotonia patients with inconclusive results by screening for the two common Finnish myotonia mutations harbored W118G, which corresponds to 12 % of the total myotonia patient cohort. Also in The UK, 12% of patients with confirmed or suspected myotonia congenita harbored the mutation, compared to 5% in the general population. This highly significant over representation ($p < 0.001$, Fisher´s test) in both patient cohorts suggests a functional defect of muscle chloride

conductance. When expressed in HEK cells, homodimeric W118G mutant channels yielded robust chloride currents with the same voltage-dependence as wildtype channels, thus the defect is not at the level of ClC-1 protein function. However, on muscle immunohistochemistry the combination W118G/R894X or W118G/F413C causes subtotal loss of sarcolemmal ClC-1 protein clearly distinct from the expression of heterozygous R894X or F413C alone. By Western blotting, which measures the combined ClC-1 content of intracellular and surface plasma membranes, the total amount of ClC-1 protein in patients with a combined heterozygous W118G mutation is less abnormal. This discrepancy between sarcolemmal staining and blotting the total protein suggests mutant W118G is not correctly transported and integrated into the sarcolemma, which is in accordance with recent results reported for *CLCN1* mutations Q43R, Y137D and Q160H (14). The W118G mutation has been reported to occur in healthy controls with a frequency of 2.9% - 3,5 % (13, dbSNP). However, it affects a highly conserved amino acid in the first transmembrane region of the protein. Our population studies comparing the isolated Larsmo population to a cohort of Central Finland show that the frequency of a certain mutation may be highly variable even within a population considered to be genetically homogeneous. The carrier frequency of the pathogenic F413C mutation varied in these geographical cohorts from 0,6 % to 3 % and the W118G showed frequencies of 3 % and 7,7 % respectively.

Low chloride conductance myotonia occurs when the summated loss of function of the two ClC-1 alleles is greater than 60% (15, 16, 17) owing to mutation of both alleles (Becker's disease), a dominant negative interaction between a single mutant allele and the normal one (Thompsen's disease), or a wider mRNA spliceopathy affecting both alleles (DM1 and DM2). Based on the high frequency in normal population controls and the absence of symptomatic homozygous W118G patients in our cohort, one explanation is that the W118G causes a moderate loss of function (for example 40-50% in a homozygote) that is insufficient to cause myotonia by itself, but sufficient to cause myotonia when the other allele shows loss of function.

Expression of ClC-1 is stimulated by action potentials (18) in the muscle cell; robust ClC-1 expression in the patient harboring the known dominant F307S mutation (19) is consistent with the notion of positive feedback between myotonia and expression of the dominant allele. Furthermore this is the first confirmation in muscle from a patient with a dominant mutation that the dominant negative interaction must occur at the level of channel function and not by disrupted expression.

The marked variability of ClC-1 expression detected by our assay in muscle from DM1 and DM2 patients is consistent with the highly variable phenotype of these diseases. While the exacerbation of the DM2 phenotype by co-segregating recessive *CLCN1* mutations is detectable as a selection bias in a large population (1), in our two DM2 patients we were not able to reliably distinguish the effect of a co-segregating *CLCN1* mutation from inherent variability in the DM2 phenotype at the level of ClC-1 sarcolemmal expression.

A fully normal ClC-1 protein expression in a myotonia patient may suggest Thomsen´s disease or a different genetic background such as sodium channel myotonia as was the case in some of our patients. The assay is most useful when screening for common *CLCN1* mutations fails to establish a genetic diagnosis in patients with sporadic or recessive myotonia; absence of protein indicates the presence of a second *CLCN1* mutation, and results also assist in the classification of novel sequence variants as pathogenic or benign.

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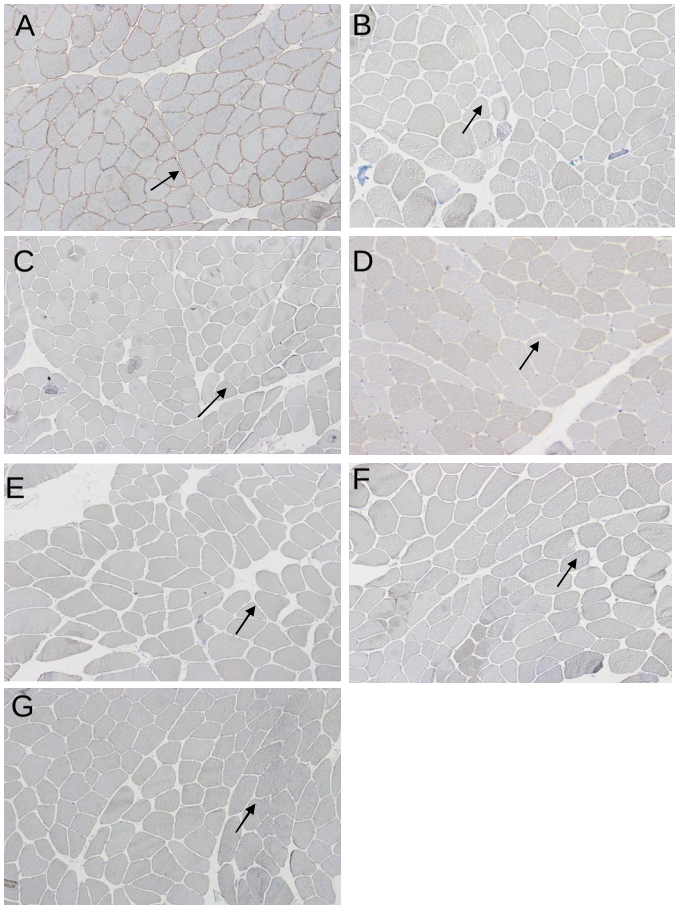


Figure 1. CLC-1 immunohistochemical staining of muscle biopsies.

Sarcolemmal staining of ClC-1 in a normal biopsy (A). Total loss of sarcolemmal ClC-1 protein in R894X homozygous (B) and in compound heterozygous (R894X and W118G) patient samples (C). Subtotal loss of sarcolemmal ClC-1 protein in a compound heterozygous (F413C and W118G) patient (D). Patient with compound heterozygous R984X and c.264G>A (E), compound heterozygous F413C and c.264G>A (F) and homozygous c.264G>A mutations showing total loss of sarcolemmal ClC-1 protein.

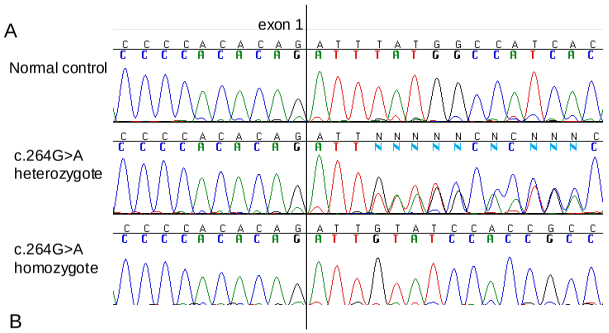


Figure 2.The effect of c.264G>A on *CLCN1*cDNA.

A) *CLCN1* cDNA sequences of c.264G>A carriers compared to normal control. Sequence of a patient homozygous for c.264G>A shows that exon 2 has been skipped totally so that exon 1 is followed by exon 3. Patient heterozygous for c.264G>A has two alleles: one normal and one lacking exon 2. B) The nucleotide alignment of *CLCN1* cDNA shows marked conservation for c.264G (in red).

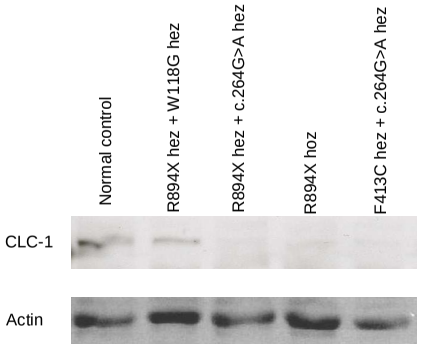
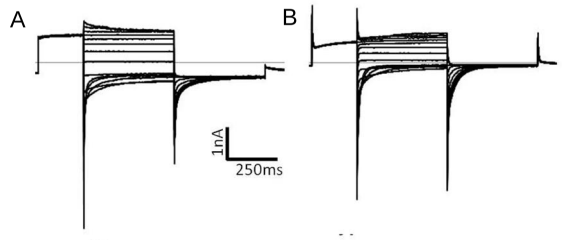


Figure 3. CLC-1 western blot on patient biopsies:

Western blot showing total amount of ClC-1 protein in muscle tissue. Total protein amount in a patient compound heterozygous for R894X and W118G is almost normal. The protein amount in heterozygous c.264G>A combined with R894X or F413C, as well as in homozygous R894X is clearly reduced/almost absent. Ponceau stained actin as a loading control. Hez = heterozygous, hoz = homozygous.



D

	wildtype	W118G
V_{50} (mV)	-55.0 (-60.2 to -49.8)	-49.4 (-56.1 to -42.7)
Slope (mV)	23.4 (19.0 to 27.8)	30.0 (24.2 to 35.8)

Figure 4. Functional expression of wildtype CLC-1 and W118G in cells.

Functional expression of wildtype ClC-1 and the W118G mutation by whole cell patch clamp of transfected HEK293T cells. Representative wildtype recording (A). Representative mutant recording (B). Boltzmann fits of the normalized tail current from 6 wildtype (squares) and 4 mutant (triangles) recordings to show the similar voltage dependence of activation (C). Error bars are obscured by symbol. V50 and slope (with 95 % confidence intervals) from the Boltzmann fits (D).

Table e-1. All patients and individuals studied, sarcolemmal ClC-1 protein amounts with immunohistochemistry and respective *CLCN1* mutations by gDNA and cDNA sequencing. Hez = heterozygous; hoz = homozygous; na = not assessed.

Patient No.	**Family**	**Result after screening for R894X and F413C**	**ClC-1 protein in immunohistochemsitry**	***ClC-1 protein in Western blot***	***CLCN1* mutations after sequencing the whole gene**	***CLCN1* mutations after cDNA sequencing**
Patients with clinical/subclinical and EMG myotonia						
P1		R894X hoz	Total loss	Reduced	na	na
P2		R894X hoz	Total loss	na	na	na
P3		R894X hoz	Total loss	na	na	na
P4		R894X hoz	Total loss	na	na	na
P5		R894X hoz	Total loss	na	na	na
P6		R894X hez	Subtotal loss	Normal	R894X hez + W118G hez	R894X hez + W118G hez
P7		R894X hez	Subtotal loss	Normal	R894X hez + W118G hez	R894X hez + W118G hez

P8		R894X hez	Subtotal loss	na	R894X hez + W118G hez	R894X hez + W118G hez
P9		R894X hez	Subtotal loss	na	R894X hez + W118G hez	R894X hez + W118G hez
P10		R894X hez	Subtotal loss	na	R894X hez + W118G hez	R894X hez + W118G hez
P11		R894X hez	Subtotal loss	na	R894X hez + W118G hez	R894X hez + W118G hez
P12		F413C hez	Subtotal loss	na	F413C hez + W118G hez	na
P13		F413C hez	Subtotal loss	na	F413C hez + W118G hez	na
P14		F413C hez	Subtotal loss	na	F413C hez + W118G hez	na
P15		R894X hez	Total loss	na	R894X hez + c.264G>A hez	R894X hez + skip exon 2 hez
P16		R894X hez	Total loss	na	R894X hez + c.264G>A hez	R894X hez + skip exon 2 hez
P17	F2:II-1	R894X hez	Total loss	na	R894X hez + c.264G>A hez	R894X hez + skip exon 2 hez
P18		R894X hez	Total loss	na	R894X hez + c.264G>A hez	R894X hez + skip exon 2 hez
P19		R894X hez	Slight reduction/ normal	na	R894X hez + A1156T in *SCN4A*	na
P20	F3:II-1	F413C hez	Total loss	Reduced	F413C hez + c.264G>A hez	na
P21		None	Subtotal loss	na	V536I hez + c.264G>A hez	V536I hez + skip exon 2 hez
P22	F1:II-1	None	Total loss	na	c.264G>A hoz	Skip exon 2 hoz
P23	F1:II-2	None	Total loss	na	c.264G>A hoz	Skip exon 2 hoz
P24		None	Normal	na	F307S hez + c.2284+5C>T	na

P25	F4:II-1	R894X hez + F413C hez	na	na	na	na

Patients with EMG myotonia and myalgia but without clinical myotonia

P26		R894X hez	Irregular minor reduction	na	R894X hez	R894X hez
P27		R894X hez	Irregular minor reduction	na	R894X hez	R894X hez
P28		F413C hez	Irregular minor reduction	Normal	na	na
P29		R894X hez	Moderately reduced	Normal	R894X hez	na

Asymptomatic first degree family members carriers of *CLCN1* mutations

A1	F2:I-2	R894X hez	na	na	na	na
A2	F2:I-1	na	na	na	c.264G>A hez	na
A3	F1:I-1	na	na	na	c.264G>A hez	na
A4	F3:I-1	F413C hez	Normal	na	na	na
A5	F4 I-2	R894X hez	Irregular minor reduction	na	na	na

DM2 patients

P30		R894X hez (+ DM2)	Total loss	na	na	na

P31		F413C hez (+ DM2)	Subtotal loss	Slightly reduced	na	na
P32		None	Reduced	na	na	na
P33		None	Reduced	na	na	na
P34		None	Reduced	na	na	na
P35		None	Reduced	na	na	na
P36		None	Reduced	na	na	na
P37		None	Reduced	na	na	na
P38		None	Slight reduction/ normal	na	na	na
P39		None	Slight reduction/ normal	na	na	na

DM1 patients

P40		None	Reduced	na	na	na
P41		None	Reduced	na	na	na
P42		na	Slight reduction/ normal	na	na	na
P43		na	Slight reduction/ normal	na	na	na
P44		na	Slight reduction/ normal	na	na	na
P45		na	Slight reduction/ normal	na	na	na

P46		na	Slight reduction/ normal	na	na	na
P47		na	Slight reduction/ normal	na	na	na

Normal controls

N1		na	Normal	na	na	na
N2		na	Normal	na	na	na
N3		na	Normal	na	na	na
N4		na	Normal	Normal	na	na
N5		na	Normal	na	na	na
N6		na	Normal	Normal	na	na