TIINA SUOMINEN

Dystrophia Myotonica Type 2 (DM2) in Finland

A mutation with extensive clinical implications

ACADEMIC DISSERTATION
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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the text by Roman numerals I-IV. The articles are reprinted with the permission of their copyright holders.

I. **Suominen T**, Schoser B, Raheem O, Auvinen S, Walter M, Krahe R, Lochmüller H, Kress W, Udd B. High frequency of co-segregating *CLCN1* mutations among myotonic dystrophy type 2 patients from Finland and Germany. J Neurol. 2008 Nov;255(11):1731-6

II. Auvinen S, **Suominen T**, Hannonen P, Bachinski LL, Krahe R, Udd B. Myotonic dystrophy type 2 found in two of sixty-three persons diagnosed as having fibromyalgia. Arthritis Rheum. 2008 Nov;58(11):3627-31

III. **Suominen T**, Bachinski LL, Auvinen S, Hackman P, Baggerly KA, Angelini C, Peltonen L, Krahe R, Udd B. Population frequency of myotonic dystrophy: Higher than expected frequency of myotonic dystrophy type 2 (DM2) mutation in Finland. Eur J Hum Genet. 2011 Jul;19(7):776-82.

IV. **Suominen T**, Deng Y, Bachinski LL, Raheem O, Haapasalo H, Kress W, Krahe R, Udd B. Proximal myalgic myopathy associated with short mutant $(CCTG)_{DM2}$ repeats. [Manuscript submitted]

ABBREVIATIONS

ATP	adenosine triphosphate
CISH	chromigenic *in situ* hybridization
CK	creatine kinase
DM1	myotonic dystrophy type 1
DM2	myotonic dystrophy type 2
DNA	deoxyribonucleic acid
EMG	electromyography
FISH	fluorescent *in situ* hydridization
FM	fibromyalgia
FSHD	facioscapulohumeral dystrophy
LD	linkage disequilibrium
MRI	magnetic resonance imaging
mRNA	messenger RNA
MyHC	myosin heavy chain
NMD	neuromuscular disease
PCR	polymerase chain reaction
RNA	ribonucleic acid
RP-PCR	repeat-primed PCR
SCA	spinocerebellar ataxia
SNP	single nucleotide polymorphism
SP-PCR	small-pool PCR, single genome equivalent amplification
SR	sarcoplasmic reticulum
UTR	untranslated region

ABSTRACT

Myotonic dystrophy type 2 (DM2) is a multisystemic genetic disease caused by a repeat expansion of four nucleotides (CCTG) in the first intron of *CNBP* (previously called *ZNF9*) gene on chromosome 3q21. The main clinical features include proximal muscle weakness, muscle pain (myalgia) and cataracts. Myotonia, although present in the disease name, is not so prominent and may be absent even when studied by electromyography.

DM2 is clinically variable and especially milder presentations may be easily misdiagnosed or get mingled with usual complaints of elderly persons. The phenotypic variability remains without conclusive explanations, but in some cases it has been suggested that mutations in other genes may affect the phenotype. Some explanations might also come from the pathomechanism of the disease, implicating secondary splicing abnormalities of a number of effector genes. Repeat numbers from 75 to as many as 11,000 have been reported from patients with DM2 disease. Because of the challenges in diagnosing DM2, the disease prevalence has been unknown. Nevertheless, DM2 has been considered much more infrequent than myotonic dystrophy type 1 (DM1), which is reported to have a prevalence of 1 in 8,000. This estimation of DM1 prevalence is based on clinical ascertainment of patients before the genetic discrimination of DM1 and DM2 was available. The repeat number varies widely from patient to patient, but does not correlate with the disease severity.

In this thesis the prevalence of DM2 was studied by investigating the frequency of DM2 mutation in the population. A surprisingly high mutation carrier frequency of 1 in 1,830 was obtained by analyzing over 5,500 Finnish samples. High prevalence was also suggested by the finding of a higher frequency of recessive *CLCN1* mutation carriers among currently diagnosed DM2 patients compared to healthy individuals or DM1 patients. Co-segregating heterozygous recessive *CLCN1* mutations in combination with DM2 mutation was found to have an aggravating effect on the symptoms and signs, i.e. myotonia. The DM2 phenotype is very

variable, and according to our results DM2 can also be misdiagnosed as fibromyalgia, a chronic pain disorder, because of the similarity of the myalgia symptoms in these two disorders. As a result of our ascertainment strategy for DM2 disease, we also identified a new disease type linked to the DM2 repeat expansion. In one family a very short $(CCTG)_{55-61}$ repeat expansion mutation was identified causing a late onset muscle disease without myotonia. Our preliminary studies on pathomechanism of this new proximal myalgic myopathy suggest that it is caused by a different mechanism compared to DM2 disease.

The results of this study have provided new and more exact information on DM2 disease and mutation prevalence, on the effects of modifying genes, on significant alternatives for differential diagnosis, and even a completely new disease type associated with short DM2 expansion. Clinicians will benefit from this new data by understanding the extent of the disease prevalence and the variability of the phenotype in order to get correct diagnosis and management for the patients. The results will also aid researchers to understand the pathomechanisms associated with specific multisystemic features in DM2.

TIIVISTELMÄ

Tyypin 2 myotoninen dystrofia (DM2) on dominantisti periytyvä sairaus, jonka oireet kohdistuvat moniin eri elimiin. Taudin pääoireet ovat lihasten heikkous ja lihasjäykkyys, lihaskipu sekä harmaakaihi. Vaikka taudin nimi viittaa myotoniaoireeseen eli lihaksen heikentyneeseen kykyyn rentoutua supistuksen jälkeen, suurella osalla DM2-tautia sairastavista potilaista myotoniaa ei nähdä edes lihaksen sähköisen toiminnan tutkimuksella (elektromyografia). DM2-taudin aiheuttava mutaatio on neljän emäksen, CCTG, toistojakson laajentuma *CNBP*-geenin (aiemmin *ZNF9*) ensimmäisessä intronissa. Geeni sijaitsee kromosomipaikassa 3q21.

DM2-taudin oireet ovat hyvin vaihtelevat, eikä oireiston raja-alueita vielä tarkkaan tunneta. Myös taudin esiintyvyys on laajalti tuntematon, vaikka arvioita on esitetty. Yleisesti sen ajatellaan olevan harvinaisempi kuin yleisin aikuisiän lihasdystrofia, tyypin 1 myotoninen dystrofia (DM1), jonka esiintyvyyden on arvioitu olevan 1/8000. Arvio perustuu kliinisiin diagnooseihin ennen DM2-taudin geneettistä erottamista omaksi taudikseen. DM2-taudin aiheuttava toistojakson laajentuma voi sisältää hyvin vaihtelevan määrän CCTG-nukleotidien toistumia. Kirjallisuudessa kuvatuilla potilailla toistojen määrä on vaihdellut 75:n ja 11000:n välillä. Vaikka vaihtelu on näin suurta, korrelaatiota toistojen määrän ja oireiden vakavuuden välillä ei ole voitu osoittaa. Oireiden vaihtelevuuden syitä voivat sen sijaan olla muiden geenien mutaatioiden tai polymorfismien vaikutus tai taudin syntymekanismiin liittyvät tekijät.

Tässä tutkimuksessa selvitettiin DM2-taudin yleisyyttä tutkimalla DM2-mutaation esiintyvyyttä normaaliväestössä yli 5500 suomalaisesta henkilöstä. DM2-mutaation esiintyvyys oli yllättävän suuri, 1/1830, joka on moninkertainen verrattuna aikaisempiin arvioihin taudin yleisyydestä. DM2-mutaation aiemmin luultua suurempaan yleisyyteen viittaavat myös tulokset *CLCN1*-mutaatioiden suuremmasta frekvenssistä diagnosoitujen DM2-potilaiden joukossa verrattuna normaaliin väestöön tai DM1-potilaisiin. Heterotsygoottisilla *CLCN1*-mutaatioilla

oli yhdessä DM2-mutaation kanssa myös vaikutus taudinkuvaan. Näillä potilailla oli selkeämpi myotoniaoire kuin tyypillisillä DM2-potilailla keskimäärin. Vaihtelevan taudinkuvan takia DM2-tauti saatetaan helposti diagnosoida väärin, kuten tuloksemme fibromyalgiapotilaiden osalta viittaavat. DM2-taudille tyypillistä lihasten kipua, myalgiaa, nähdään myös fibromyalgiassa, joka on krooninen kipuoireyhtymä. Tutkimuksessa kuvataan myös uudentyyppinen DM2-mutaatioon liittyvä erilainen taudinkuva, proksimaalinen myalginen myopatia, jonka aiheuttaa hyvin lyhyt CCTG-toistojakson laajentuma. Tutkimustulostemme perusteella taudin mekanismi näyttäisi olevan erilainen kuin DM2-tautia aiheuttavan mutaation patogeneettinen mekanismi.

Tutkimuksen tulokset ovat uusia ja niiden kautta saadaan uutta ymmärrystä DM2-taudin esiintyvyydestä, muiden geenien vaikutuksesta taudinkuvaan sekä käytännön erotusdiagnostiikkaan. Lisäksi tutkimuksessa on kuvattu uudentyyppinen lyhyeen DM2-mutaatioon liittyvä tauti. Tutkimuksessa saaduista tuloksista on hyötyä potilaille, jotka näiden tulosten valossa voivat saada oikean DM2-taudin diagnoosin aikaisempaa useammin. Tulokset ovat tärkeitä myös DM2-taudin patogeneettisten mekanismien tutkimukselle, jossa voidaan hyödyntää lyhyen toistojakson aiheuttaman uuden taudin selvittämistä DM2-taudin moninaisten oireiden syntymekanismien ymmärtämisessä.

1. INTRODUCTION

Myotonic dystrophies are muscular dystrophies with multiorgan involvement. They are genetically and clinically divided into two separate diseases: myotonic dystrophy type 1 (dystrophia myotonica 1, DM1, Steinert's disease, OMIM #160900) and myotonic dystrophy type 2 (dystrophia myotonica 2, DM2, PROMM, OMIM #602668). The clinical features include progressive muscle weakness, which starts in distal upper and lower limbs in DM1 and in proximal lower limbs in DM2. Other core features are cataracts and myotonia, which in DM2 may be very mild or even absent (Udd et al., 2006; Udd et al., 2011). One marked symptom characteristic of DM2 is muscle pain, myalgia with onset long before any muscle weakness or wasting is apparent.

DM1 and DM2, although caused by mutations in different genetic loci, are associated with expansions of microsatellite repeats located in transcribed but untranslated regions of their respective genes. In DM1 the mutation is an expansion of a trinucleotide $(CTG)_n$ repeat in the 3′ untranslated region (3′UTR) of *DMPK* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992), whereas DM2 is caused by an expansion of a tetranucleotide $(CCTG)_n$ repeat in the first intron of *CNBP* (*ZNF9*) gene (Liquori et al., 2001).

Diseases associated with repeat expansions may show anticipation, i.e. more severe phenotype and earlier disease onset in successive generations. In DM1 this phenomenon has been observed and accounts for the occurrence of four different phenotypes of the disease: 1) congenital DM1, 2) childhood-onset DM1, 3) adult-onset classic form of DM1, and 4) late-onset oligosymptomatic DM1. The genetic explanation of the anticipation is the positive correlation of the repeat expansion size with the disease severity, i.e. the expansion of the repeat length in subsequent generations. In DM2, on the other hand, anticipation is the exception and not the rule. Although the phenotype of DM2 is highly variable, the size of the repeat expansion has not been reported to correlate with the disease severity. This paradox

still lacks conclusive explanation. (Ashizawa and Sarkar, 2011; Udd and Krahe, 2012)

The pathomechamism in both DM1 and DM2 involves toxic RNA effects. The transcribed mutant RNA repeats are not normally processed and accumulate in the nuclei and affect among others the splicing of several effector genes, including *CLCN1*. (Klein et al., 2011; Schoser and Timchenko, 2010) The splicing pattern of the affected proteins is changed towards more fetal isoforms, which are not functional in the adult mature muscle tissue. It has been suggested that other pathomechanisms such as reduction of translational activity in muscle cells are involved in the pathogenesis of DM1 and DM2 (Huichalaf et al., 2009; Huichalaf et al., 2010). Differences in the molecular pathogenesis responsible for the different phenotypic characteristics of DM1 and DM2 have also been described, such as CNBP down-regulation and reduced cap-independent translation in DM2, and the effects of reduced DMPK levels and *DMPK* antisense transcription in DM1 (Udd and Krahe, 2012).

DM1 is considered the most common muscular dystrophy affecting adults. This view is based on prevalence studies on myotonic dystrophy disease before the identification of DM2 mutation. The prevalence may vary a lot in different populations, especially in genetic isolates, although the overall prevalence is estimated to be 1 in 8,000 (Harper, 2001). For DM2 the prevalence has not been established but it has been generally considered much more infrequent than DM1. However, preliminary data on DM2 prevalence or frequency supports the hypothesis that DM2 is at least as common as DM1 in many populations (Udd et al., 2006; Udd et al., 2011). Prevalence studies of DM2 based on clinical ascertainment are challenging because of the wide variability of the disease phenotype, for which the full spectrum of presentations is not yet defined. The frequency of DM2 mutation in the population is therefore of importance in order to have an objective rationale for assessment of the frequency of the disease.

The aspect of large phenotype variability can be approached by studying possible genetic factors affecting the disease phenotype. *CLCN1* gene is an interesting candidate as its mutations cause myotonia, a symptom also part of the DM2 disease. In addition, *CLCN1* is a target for aberrant splicing in DM2, which makes it an even more important candidate for studying phenotype variability. Another way to investigate this variability is to assess possible differential diagnostic alternatives for

DM2, i.e. whether DM2 could be misdiagnosed as fibromyalgia, a chronic pain disorder. Patients with fibromyalgia present with widespread pain including muscle pain, which is very common in DM2 patients (Suokas et al., 2012).

For the correct diagnosis of DM2 it is important to understand the borders of the clinical spectrum and the prevalence of the disease. Without correct diagnosis DM2 patients easily become frustrated with their unexplained symptoms. Many clinical or laboratory tests are performed unnecessarily and many patients have been incorrectly treated for other disorders or referred to the psychiatrist in the past. Even when curative treatment is not available, the correct diagnosis is very important and makes a difference to the patients and health care. A definitive diagnosis provides correct management of the disease in order to avoid possible complications of DM2 such as heart conduction defects. Moreover, when identified in one patient, usually many other close or even more distant family members can also get the correct explanation for their health problems.

2. REVIEW OF THE LITERATURE

2.1 Introduction to genetics

2.1.1 Genes and protein synthesis

Genes are molecular units of heredity and consist of DNA. Genes code for proteins or RNA molecules, which function in biological processes in human cells. Structurally genes can be divided into different sequence regions: promoter region, which regulates the expression of a gene, 5′ untranslated region (UTR), exons, introns and 3′UTR at the end of a gene (Figure 1).

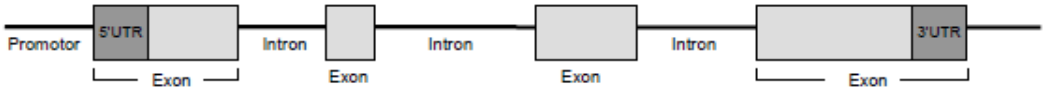

Figure 1. Schematic structure of a gene. (The figure is modified from Gemayel et al., 2010.)

To apply the genetic information of a gene to synthesize a protein, the information is first transcribed to messenger-RNA (mRNA). In this process called transcription, one strand of DNA acts as a template (antisense strand) to guide the mRNA formation. RNA polymerase enzymes build the mRNA by joining together ribonucleosides complementary to the template DNA strand, thus producing a pre-mRNA molecule similar to the sense strand of the DNA. After this transcription several modifications are made to the produced pre-mRNA to create a mature final mRNA. The most important modification is the splicing of introns. Introns are spliced out by a complex called spliceosome, which consists of specific proteins and different small-nuclear RNAs (snRNAs). In this process the splicing machinery is

also capable of including or excluding exons of the gene. For the multi-exon genes, which comprise 94 % of all human genes, exons can be reconnected in multiple ways to produce multiple different mRNA isoform variants, which in turn account for multiple protein isoform variants. This alternative splicing is very frequent and is estimated to apply to approx. 86 % of human genes (Wang et al., 2008). In addition to creating multiple mRNA isoform variants from one gene by alternative splicing, the use of alternative promotors is also quite common. The mechanisms of differential mRNA processing are regulated spatially and temporally in order to produce multiple protein isoforms with functional specifications for different cell types, differentiation stages or locations in the cell.

Proteins are composed of amino acids. To use the genetic information of mature mRNA to produce amino acids and proteins, mRNA is transported from the nucleus to the cytoplasm for translation. Ribosomes, large complexes containing proteins and ribosomal RNAs, catalyze the polymerization of amino acids according to the nucleotide sequence code of the mRNA. Each amino acid is decoded by one or several codons, a three-letter code of bases, which specifies all the 20 different amino acids available for constructing a protein. As with transcription, modifications can be made also after translation to build up a mature protein.

2.1.2 Microsatellites

In addition to genes coding for proteins or RNAs, the human genome contains a large number of repeated DNA sequences, which encompass as much as 46 % of the genome (Gemayel et al., 2010). Microsatellites are short tandem repeats (STRs, also called simple sequence repeats, SSRs) of 1-9 nucleotide motifs. Usually they are found in the non-coding regions of the genome, but especially trinucleotide repeats are often found in the coding regions as well. Because of the highly polymorphic nature of microsatellite repeats, they can be used as molecular markers for a particular unique chromosomal location in genotyping and linkage studies. Microsatellite repeats are more prone to errors in replication or recombination than the non-repetitive DNA sequences, and these errors change the number of tandem repeats. In other words, the number of repeats in some instances can increase or decrease in successive generations. Sometimes errors in the length of a

microsatellite repeat region can cause a human disease (more on this in section 2.1.3.1 Repeat mutations and human diseases).

2.1.3 Mutations

Mutations are changes in the DNA sequence. Such DNA changes can occur spontaneously without a known cause or by the effect of mutagenic agents, such as chemicals, viruses or radiation. Mutations occurring in the germ-line cells are passed on to future generations. When one nucleotide is changed for another, the mutation is called substitution. About two-thirds of reported human disease-causing mutations are base substitutions (The Human Gene Mutation Database, HGMD, http://www.hgmd.cf.ac.uk/ac/index.php, 19.3.2012). Small insertions and deletions are also quite common mutation classes, accounting for almost 25 % of human disease-causing mutations (HGMD 19.3.2012). Changes in the repeat numbers, for example in the microsatellite repeat regions, can also cause human diseases. The number of mutations in this class is very low, accounting for only 0.3 % of all human mutations (HGMD 19.3.2012). Other types of mutations include gross insertions, duplications or deletions and complex rearrangements.

The classification of mutations can be based on the effect they have on the gene. Base substitutions can be divided into missense (one amino acid is changed to another), nonsense (amino acid coding codon is changed to a stop codon), or silent (the mutation causes a change of codon without exchange of amino acid). Small insertions and deletions are classified in respect of the effect on genetic code. If the mutation preserves the reading frame of the genetic code with or without introducing new codons it is called an in-frame mutation. If the mutation causes a disruption of the reading frame in the mRNA, it is called a frame-shift mutation, which usually leads to a STOP codon in the later mRNA sequence. When a mutation disrupts the reading frame, the effect is dependent on the location and quality of the mutation.

2.1.3.1 Repeat mutations and human diseases

Several diseases, especially neurodegenerative disorders, are associated with the abnormal repeat number of microsatellites. The first genetically characterized diseases associated with repeat expansion mutation were fragile X syndrome (FRAXA) (Verkerk et al., 1991) and spinal and bulbar muscular dystrophy (SBMA) (La Spada et al., 1991) in the year 1991. A noteworthy group of trinucleotide repeat-associated diseases are those caused by $(CAG)_n$ repeat expansions. One of the best-known is Huntington's disease (HD), which is an autosomal dominant neurodegenerative disorder associated with glutamine-coding repeat expansion of over 37 CAG repeats (Duyao et al., 1993). Also several types of spinocerebellar ataxias (SCA) are caused by either translated or untranslated CAG repeats: SCA1, SCA2, SCA3, SCA6, SCA7, SCA12 and SCA17. SCAs are rare progressive neurodegenerative diseases characterized by cerebellar ataxia and additional heterogeneous symptoms. A common pathomechanism for diseases caused by translated CAG repeats, called polyglutamine expansion diseases, has been suggested (Williams and Paulson, 2008). SCA diseases can also be caused by other repeat expansions aside from $(CAG)_n$. Expansion of untranslated CTG repeat in *ATXN8OS* gene causes SCA8. It has been suggested that the pathomechanism also involves a polyglutamine expansion of CAG repeat from *ATXN8* gene on the opposite DNA strand (Moseley et al., 2006).

Even though most of the repeat expansion associated diseases are inherited dominantly, one exception is Friedreich ataxia (FRDA), which is caused by GAA trinucleotide repeat expansion mutation in intron 1 of the frataxin (*FXN*) gene (Campuzano et al., 1996). Also point mutations have been reported, and patients can either be homozygous for the repeat expansion of approximately over 70 GAA repeats or compound heterozygous with a repeat expansion in one allele and a missense mutation in another (Filla et al., 1996; Patel and Isaya, 2001).

Besides trinucleotide repeat expansions, unstable tetranucleotide, in myotonic dystrophy type 2 (Liquori et al., 2001), and pentanucleotide, in spinocerebellar ataxia type 10 (Matsuura et al., 2000) and SCA31 (Sato et al., 2009) have also been reported. In addition, the recently identified mutation associated with amyotrophic lateral sclerosis (ALS) contains tandem repeats of a hexanucleotide (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

While the majority of repeat-associated diseases are caused by an expansion of the repeat region, facioscapulohumeral dystrophy (FSHD) is exceptionally associated with repeat contraction. An array of macrosatellite tandem repeats of 3.3 kb in size, called D4Z4, has been identified in chromosomal location 4q35. Normal individuals have 12 to over 100 copies of D4Z4, whereas patients with FSHD have only 1-10 copies. However, the complete absence of D4Z4 repeats is not associated with disease. (Griggs and Amato, 2011)

The actual pathological effect of the repeat expansions is very variable. If the repeat is located in the coding sequence it usually is translated into a corresponding amino-acid stretch with abnormal consequences for the protein product. If the repeat expansion is located in a transcribed but untranslated region it may cause an abnormal toxic RNA product with or without consequences for the protein product, or the pathogenic effect of the mutation is related to abnormal activation of an associated gene, as occurs with the D4Z4 contraction in FSHD (Lemmers et al., 2010).

2.1.4 Inheritance of human diseases

Many human diseases are caused by mutation(s) in a single gene. Over 10,000 human diseases are monogenic. The mode of inheritance is classified as autosomal or sex-linked depending on the chromosomal location of the disease-causing gene: if the gene is in an autosome, a disorder shows autosomal inheritance, whereas with a disorder caused by a gene in either sex chromosome the inheritance is sex-linked. Autosomal inheritance is termed dominant if a mutation on one allele causes a disease phenotype, and recessive if mutation(s) need to be present on both alleles to cause a disease. Sex-linked inheritance involves a gene mutation located in either X- or Y-chromosome. Y-chromosomal inheritance is very rare, but several diseases are X-linked, both dominant and recessive. In the case of sex-linked recessive inheritance, the carrier females transmit a mutated gene to their affected sons. Other patterns of inheritance include mitochondrial, polygenic and multifactorial inheritance. (Mueller and Young, 1998)

In some cases genetic disorders do not seem to follow any patterns of inheritance described above. Some diseases are originated from novel mutations in the germ-

line cells and these *de novo* mutations can be transmitted to offspring. In such cases the parents do not have the disease, but their children may. These mutations are also called sporadic. Another mechanism for producing an atypical inheritance pattern is incomplete penetrance. Penetrance is a definition for the proportion of individuals carrying disease-causing mutation(s) and also expressing an associated clinical trait. If some of the individuals carrying the mutation(s) are not presenting the disease phenotype, the penetrance of the mutation(s) is said to be incomplete. Incomplete penetrance may be due to several reasons: modifier genes, epigenetic mechanisms or environmental factors can influence the clinical outcome, or the disease phenotype could be very mild and therefore undetected. In late-onset diseases incomplete penetrance can be age-related.

In some autosomal dominant diseases, especially those caused by repeat expansion mutations, the disease may be more severe or symptoms begin at younger age in the offspring than in the parents. This phenomenon is called anticipation. In the case of repeat expansion diseases it is caused by expansion of unstable repeats in meiosis producing larger expansion and more severe disease in the offspring if the size of the repeat expansion correlates with the disease severity. Similarly, short expansions in previous generations may be asymptomatic causing incomplete penetrance.

2.1.5 Genetic diagnostics

The genetic diagnosis of a disease is a process involving several steps and requires first a clinical analysis of the phenotype. Currently genetic testing is usually performed with methods that can detect specific mutations in a candidate gene or all the mutations causing changes on the mRNA level of a gene. Genetic tests are available for several inherited diseases and the number is continuously growing.

The most widely used method for genetic diagnostics is sequencing (Sanger et al., 1977). As opposed to targeted mutation analysis focusing on a single specific mutation, sequencing covers all genetic variations in the investigated part of a gene. For the repeat expansion diseases sequencing is not a suitable diagnostic method, because the instability and large size of the repeat regions makes the sequencing very challenging or even impossible. PCR-based methods such as repeat-primed

PCR (also called triplet-primed or tetraplet-primed PCR, TP-PCR) have been developed for rapid detection of pathogenic expansions in repeat regions (Bachinski et al., 2003; Sermon et al., 2001; Warner et al., 1996).

With targeted mutation analysis a single candidate-gene mutation is analyzed. Several methods are used for targeted mutation analysis including methods applying restriction enzymes, minisequencing technique, and probe-based sequence detection methods (TaqMan 5′nuclease assay). These methods are quite fast and results more straightforward to analyze compared to conventional sequencing. Nevertheless, a disadvantage is that they require prior knowledge of the mutation.

The field of genetic diagnostics is changing due to recent advances in sequencing techniques. These rapid techniques, referred to as next-generation sequencing, enable simultaneous genetic analysis of several genes cost-effectively. It is especially advantageous in diagnosing diseases that are genetically heterogeneous as opposed to those caused by mutations in a single gene (Ku et al., 2012). For research these next-generation sequencing approaches will provide means to rapidly screen for all human exons or the whole genome and facilitate the discovery of new disease-associated mutations or even new diseases (Ku et al., 2012). With these new sequencing techniques, the analysis and interpretation of the data becomes a more significant step in the process. Finding the important variants from a vast amount of data is a consequential challenge for geneticists and bioinformaticians. However, the techniques are also moving into the diagnostics as they will be cheaper than Sanger sequencing at least for very large genes or sets of candidate genes.

2.2 Skeletal muscle

Understanding the structure and function of skeletal muscle is important for the understanding of myotonic dystrophies, which are the main objects of this study. Skeletal muscles are also called voluntary because some of their contraction activity is also controlled by the individual's own volition. Skeletal muscle comprises about 40 % of human body mass in men and 32 % in women. Other muscle types include smooth muscle and cardiac muscle, which are entirely controlled by the autonomous nervous system, i.e. non-voluntary mechanisms. (Sherwood, 2010)

2.2.1 Muscle structure

Muscle cells, also termed muscle fibers, are organized in bundles called fascicles, which are surrounded by a sheath of connective tissue called perimysium. Each muscle fiber is also surrounded by connective tissue, endomysium, which binds the fibers together. Fascicles are wrapped together by epimysium, which is a very dense layer of connective tissue and covers up the entire muscle, and ultimately coordinates the force generated by each muscle fiber and fascicle, via connective tendon tissue, into movements. Skeletal muscles are connected to the bones by tendons, which are extensions of the connective tissues in the muscle. Muscle structure is illustrated in Figure 2.

Skeletal muscles are connected to axons of motor neurons in complex molecular structures called neuromuscular junctions. Each motor neuron innervates one or more commonly a group of individual muscle fibers, but one muscle fiber can receive innervation from only one motor neuron. The combination of one motor neuron and the muscle fibers it innervates is called a motor unit. (Karpati et al., 2010)

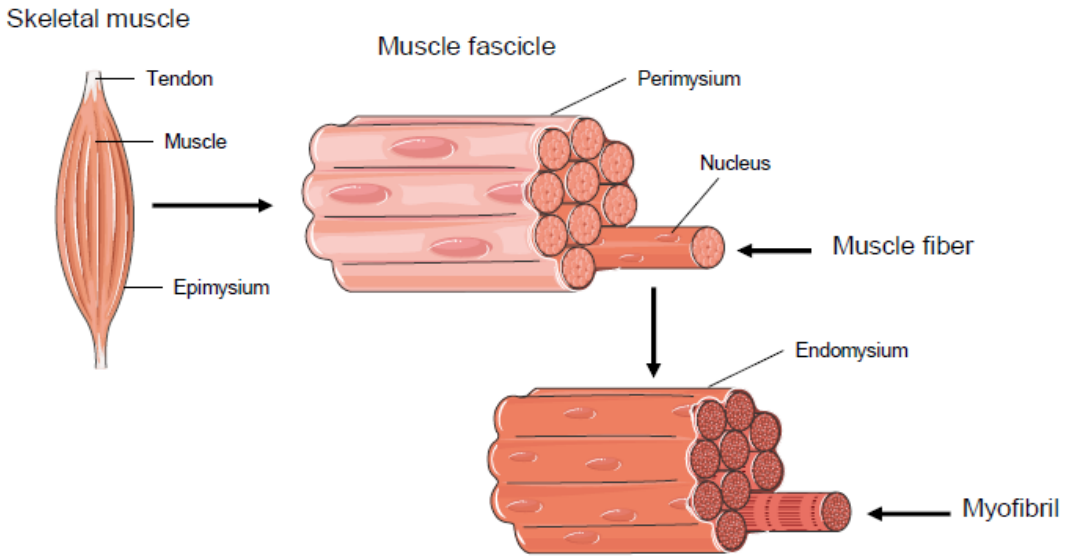

Figure 2. Structure of a skeletal muscle. (The figure was produced using Servier Medical Art at http://www.servier.com.)

Muscle fibers are composed of numerous myofibrils, which are long sequential repeats of the contracting basic units called sarcomeres. The sarcomere is structurally divided into several sections, which are illustrated in Figure 3. Sarcomeres are separated by the Z-disc and one sarcomere extends from one Z-disc to another. The Z-disc is composed of complexes of several proteins with the functional property of transducing force. The thin filaments are anchored in the Z-disc, whereas the thick filaments are connected to the M line. The major structural component of thin filaments is actin, which is organized in a filamentous helical form. Thin filaments also include regulatory proteins tropomyosin and troponin, which have a function in the exitation—contraction mechanism of the sarcomere. Thick filaments are formed of myosin protein. The third filament, structure and backbone of the sarcomere, is generated and composed of titin proteins, which extend from the Z-disc up to the M line with overlapping interactions of titin molecules at both ends.

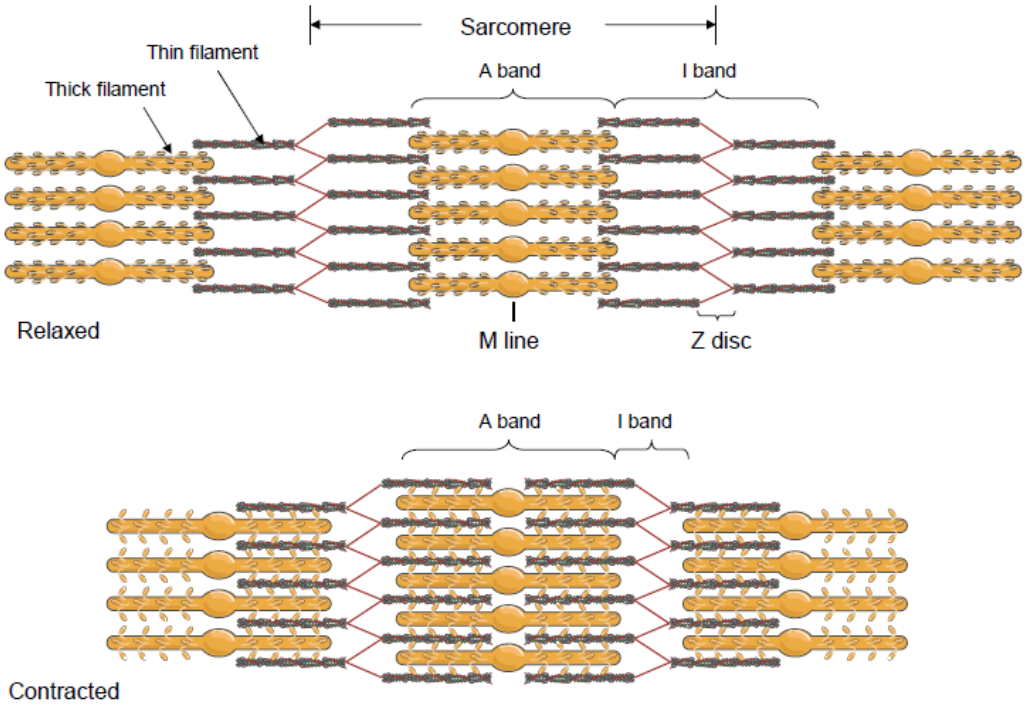

Figure 3. Schematic structure of a relaxed (above) and contracted (below) state of a sarcomere. (The figure was produced using Servier Medical Art at http://www.servier.com.)

2.2.2 Muscle cell

Skeletal muscle fibers are highly differentiated cells, which are formed by fusion of precursor cells, myoblasts, during embryonic development. As a result of this fusion process the mature muscle cells are multinucleated with nuclei located in the subsarcolemmal parts of the fibers. Muscle fiber contains a high number of mitochondria, the organelles generating energy in the cell, necessitated by the high energy demand of muscle tissue. The internal structure of the muscle fiber is composed of numerous myofibrils, which are responsible for the capacity of the muscle to contract based on the contractile property of each serially connected sarcomere along the myofibril. The cell membrane of a muscle fiber is called sarcolemma and, correspondingly, endoplasm is termed sarcoplasm. (Sherwood, 2010)

2.2.3 Molecular differences of individual muscles: Fiber types

There are three types of muscle fibers classified by their biochemical capacities: slow-oxidative (type I) fibers, fast-oxidative (type IIA) fibers and fast-glycolytic (type 2B/IIX) fibers. Fast-glycolytic fibers have higher usage of glycolysis for energy recruitment and a higher capacity of using ATP (adenosine triphosphate), which is a major energy reservoir in cells, and consequently contract faster than slow-type fibers. On the other hand, fibers of the slow type are more resistant to fatigue because of their dependence on oxidative generation of ATP and are predominantly found in muscles that are needed to maintain activity for long periods of time, for instance muscles that support the body weight. Fast types of fibers are divided into either oxidative or glycolytic depending on the mechanism they synthesize ATP. Type IIA fibers have high oxidative phosphorylation capacity and type 2B/IIX fibers synthesize ATP primarily by anaerobic glycolysis requiring large glycogen storages. Fast fibers are recruited for strong and rapid contractions. (Sherwood, 2010; Spangenburg and Booth, 2003)

In each mature muscle fiber one single type of myosin heavy chain (MyHC) is expressed in the myosin thick filament. The type of MyHC protein determines the type of muscle fiber. In slow type I fibers myosin heavy chain protein is expressed by the *MYH7* gene. In fast type IIA fibers the MyHC protein isoform IIA is encoded

by the *MYH2* gene, and in fast type 2B/IIX fibers the corresponding MyHC isoform IIX is expressed by the *MYH1* gene. Muscles are a mixture of cells expressing different MyHC isoforms, but in a single motor unit all fibers are of the same fiber type. (Raheem et al., 2010a) However, these characteristics of slow and fast fiber types are just one feature of the differences between different muscles regarding their molecular setup in individual muscles.

2.2.4 Muscle function

The contractile unit of the muscle is the sarcomere. When the muscle contracts, thin filaments slide into the thick filaments as shown in Figure 3, and as a result the sarcomere will shorten. This process is powered by ATP hydrolysis. The contraction starts with an electrical action potential arriving from the motor nerve and consequently the neurotransmitter acetylcholine is released to the neuromuscular junction, which via the corresponding receptor ion channel activation triggers the action potential in the muscle fiber. The action potential, i.e. the opening of the Na-channels on the sarcolemma, is spread rapidly to the inner parts of muscle fiber through highly organized structures of transverse tubules (T-tubules), which are invaginations of the sarcolemma (Figure 4). The action potential causes a depolarization which activates specific voltage-gated transmembrane proteins on T-tubules. These proteins are calcium channels, called dihydropyridine receptors (DHPRs). Activation of DHPRs triggers the opening of the next calcium channel, the ryanodine receptors, which reside on the sarcoplasmic reticulum (SR). T-tubules and sarcoplasmic reticulum are very closely organized enabling the connection between DHPRs and ryanodine receptors. When ryanodine receptors on SR are opened, they release a large amount of stored Ca^{2+} ions to the cytosol which in turn initiates the contraction of myofibrils via calcium-sensitive proteins in the thin filaments. The signal to open the Ca^{2+}-releasing ryanodine channels is transmitted through T-tubules and SR within milliseconds, enabling every myofibril in the muscle fiber to contract simultaneously. (Sherwood, 2010; Sorrentino, 2011)

How then is the release of Ca^{2+} ions further transformed into muscle contraction? The key proteins are the accessory proteins of the thin filament, troponin and

tropomyosin. Troponin has three subunits, one of which binds up to four Ca^{2+} ions and acts as Ca^{2+} sensor. The binding of Ca^{2+} changes the conformation of another, inhibitory subunit, so that the troponin molecule is released from binding actin, the major component of thin filaments. Consequently, a conformational change in tropomyosin moves it from its resting-state position, thus revealing the myosin-binding sites of the actin molecule. Binding of myosin to actin is necessary for the muscle contraction. (Alberts et al., 1994)

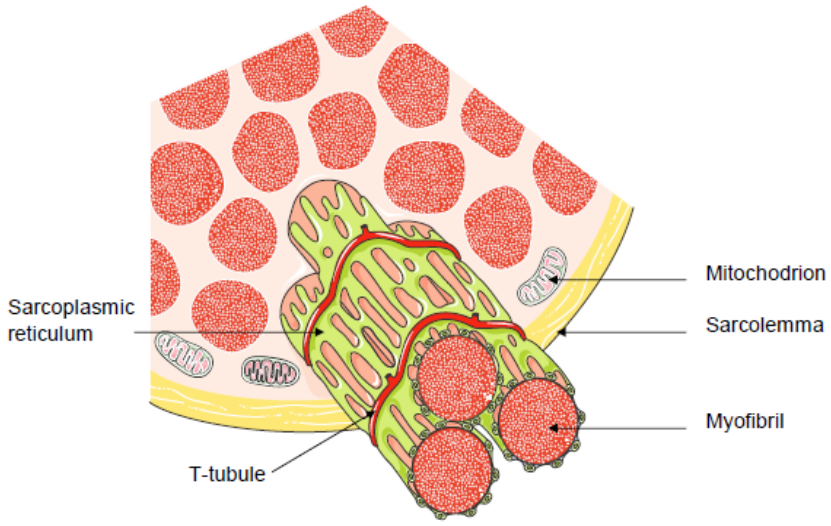

Figure 4. Sarcoplasmic reticulum and T-tubules. Myofibrils are surrounded by sarcoplasmic reticulum network, which is in close connection with T-tubules. (The figure was produced using Servier Medical Art at http://www.servier.com.)

The sliding of thin filaments into thick filaments is achieved by repeated attaching and releasing of the myosin molecule to and from the actin. This is powered by hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate (P_i). The hydrolysis of ATP is catalyzed by the ATPase activity of the myosin molecule. The release of ADP and P_i causes a conformational change and consequently the bending of the head of the myosin molecule. This bending of the myosin head attached to the actin results in actin sliding past myosin and the contraction of the sarcomere. (Alberts et al., 1994; Sherwood, 2010)

The relaxation of the muscle is achieved by active, energy-consuming, removal of Ca^{2+} ions from the cytosol back to the sarcoplasmic reticulum. The protein pump responsible for this is sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). Calcium-binding proteins in SR, notably calsequestrin, facilitate the uptake of Ca^{2+} ions. (Sorrentino, 2011)

The action potential in the muscle fiber is the initial event for the muscle contraction. The resting membrane potential of the sarcolemma relative to the extracellular space is -70 to -90 mV. This membrane potential is created by different permeability and active transport of potassium (K^+), sodium (Na^+) and chloride (Cl^-) ions. The action potential is generated by large voltage-dependent increase in the membrane conductance of Na^+, while the increase in membrane conductance of K^+ results in the returning of membrane potential to the resting state. The function of the chloride channel in the muscle fiber is to stabilize the resting membrane potential and prevent unnecessary action potentials leading to contractions. If chloride channels are deficient, K^+ accumulation leads to depolarization of the membrane, which initiates self-sustained action potential and protracted contraction. (Karpati et al., 2010)

2.2.5 Muscle biopsy in diagnostics

Muscle tissue samples obtained from a patient with neuromuscular disease are usually needed for making the right diagnosis. Despite the increasing knowledge of disease-causing genes and the availability of diagnostic genetic tests, muscle biopsy remains an important tool in diagnostics. There are several ways to study the biopsy, such as histological, histochemical, immunohistochemical, Western blot, *in situ* hybridization, electron microscopy and genetic based techniques. Selection of the muscle from which the biopsy should be taken is essential and the best location is usually case-specific. The results of the techniques used have to be interpreted in the light of clinical history and other laboratory findings. (Karpati et al., 2010)

2.3 Neuromuscular disorders

Neuromuscular disorders (NMD) are a heterogeneous group of diseases impairing the function of muscles. As a result the ability to perform voluntary movements is compromised. The vast majority of neuromuscular disorders is caused by genetic defect and are thus inherited. A simplified classification of neuromuscular diseases is shown in Table 1.

Neuromuscular disorders can be divided into three categories based on the site of dysfunction: 1) myopathies caused by primary defects in the muscle tissue, 2) myastenias or diseases of the neuromuscular junction and 3) neurogenic muscular atrophies caused by defects in the motor neuron of peripheral nervous system. More than 200 genes and similar numbers of additional genetic loci have been identified, associated with neuromuscular diseases, and many are still to be identified (Kaplan, 2011; Laing, 2012). Classification by genes alone is difficult as some diseases are caused by mutations in several different genes (e.g. ALS is caused by mutations in *C9orf72*, *SOD1*, *TARDBP*, *FUS, etc.*). Alternatively, mutations in one gene may cause several different diseases (e.g. mutations in *LMNA* gene have been associated with LGMD1B, EDMD, cardiomyopathy, Charcot-Marie-Tooth disease and lipodystrophy). Furthermore, even the same mutation in one gene can cause different phenotypes in different individuals, as is the case with a homozygote *DYSF* mutation associated with both Miyoshi distal myopathy and LGMD2B (Weiler et al., 1999). Despite the difficulties in classifying neuromuscular diseases, the precise final diagnosis can be made only after the genetic cause of the disease has been identified. Molecular diagnosis is therefore considered the gold standard for the diagnosis of neuromuscular disorders (Laing, 2012).

Table 1. Classification of neuromuscular diseases.

- Myopathies
  - **Muscular dystrophies**
    - Dystrophinopathies
      - Duchenne muscular dystrophy (DMD)
      - Becker muscular dystrophy (BMD)
    - Limb-girdle muscular dystrophies (LGMD)
    - Facioscapulohumeral muscular dystrophy (FSHD)
    - Distal muscular dystrophies
    - Oculopharyngeal muscular dystrophies (OPMD)
    - Emery-Dreifuss muscular dystrophy (EDMD)
    - Congenital muscular dystrophies (CMD)
    - Other and unclassified muscular dystrophies
    - **Myotonic dystrophies**
      - Myotonic dystrophy type 1 (DM1)
      - Myotonic dystrophy type 2 (DM2)
  - **Congenital myopathies**
  - **Myotonia and other channelopathies**
  - **Immune mediated inflammatory myopathies**
  - **Myopathies termed by histopathology criteria (MFM, etc.)**
  - **Myopathies termed by clinical presentation (HMERF, etc.)**
  - **Myopathies termed by genetic definition (FHL1-myopathy, etc)**
  - **Mitochondrial myopathies**
  - **Metabolic myopathies**
  - **Other and unclassified myopathies**
- Myasthenias
  - **Myasthenia gravis (MG)**
  - **Congenital myasthenic syndromes (CMS)**
  - **Lambert-Eaton myasthenic syndrome (LEMS)**
- Neurogenic disorders
  - **Motor neuron diseases**
    - Amyotrophic lateral sclerosis (ALS)
    - Spinal and bulbar muscular atrophy (SBMA)
    - Spinal muscular atrophies (SMA)
  - **Charcot-Marie-Tooth disease (CMT)**

2.3.1 Diagnostics of muscular dystrophies

Muscular dystrophy has two definitions. Its clinical definition is a genetic disease of the muscle cell causing progressive loss of muscle tissue. Its histopathologic definition is characterized by fiber necrosis and regeneration combined with replacement of muscle fibers by adipose or connective tissue. Muscular dystrophies are clinically very heterogeneous, but they all involve progressive muscle weakness and wasting. Defining the pattern of muscle weakness and wasting is instrumental in clinical evaluation and differential diagnostics. The onset of disease symptoms can vary from prenatal to late adulthood. (Griggs and Amato, 2011)

Diagnostics of dystrophies needs a combination of many tools. Clinical evaluation is the first and maybe the most important way to obtain an overall impression of the disease phenotype. Laboratory features, i.e. increased serum creatine kinase (CK) levels, specific changes in muscle and nerve electric properties studied by electromyography (EMG), and muscle imaging using magnetic resonance imaging (MRI) will further define the specific features involved in certain types of dystrophies. Extensive studies on muscle biopsy, including immunohistochemistry and immunoblotting, are essential in delineating the diagnostic options for further molecular genetic clarification of the background of a disease. Precise genetic diagnosis can be achieved by genetic testing. (Griggs and Amato, 2011)

2.3.2 Myotonic dystrophies type 1 and 2 (DM1 and DM2)

Myotonic dystrophies constitute the most common forms of muscular dystrophies in the adult population. Two different forms have been identified: myotonic dystrophies type 1 and type 2. The mutation underlying DM1 is an unstable expansion of a trinucleotide $(CTG)_n$ repeat in the 3′UTR of *DMPK* (dystrophia myotonica protein kinase) gene on chromosome 19q13.3. The mutation causing DM2 is similar, but not identical: an unstable expansion of a tetranucleotide $(CCTG)_n$ repeat in the first intron of *CNBP* gene (CCHC-type zinc finger, nucleic acid binding protein; previously called *ZNF9*, zinc finger protein 9) on chromosome 3q21.

CNBP gene contains five exons, of which exons 2-5 code for a protein product of 177 amino acids. The large size, over 14,000 bps, of *CNBP* gene is explained by the huge intron 1 containing approximately 12,000 bps. The protein contains seven CCHC-type zinc finger domains, which bind RNA or single-stranded DNA. These nucleic acid binding domains are necessary for CNBP to function as a transcription regulator. *CNBP* gene was identified in 1989 to code for a protein that binds certain DNA sequences, called sterol regulatory elements. The protein functions as a trans-acting factor involved in cellular sterol-mediated control of transcription (Rajavashisth et al., 1989).

DM1 was first described by the German neurologist Hans Steinert in 1909, and the disease is also called Steinert's disease. The mutation was found in 1992 by three separate groups (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). When the molecular cause of DM1 was clarified, clinicians observed that some patients having a disease clinically resembling DM1 did not carry the mutation and the possibility of another disease was considered. In the first reports of DM2 disease, before the genetic etiology was known, it was named proximal myotonic myopathy (PROMM) (Ricker et al., 1994; Thornton et al., 1994) or proximal myotonic dystrophy (PDM) (Udd et al., 1997) on account of its clinical presentation. Later, when the disease in one large Minnesota family (MN1) was linked to a new locus on 3q21, the locus and later the disease came to be known as DM2 (Day et al., 1999; Ranum et al., 1998). The mutation was characterized in 2001 (Liquori et al., 2001) and the names of the two myotonic dystrophies were uniformed to DM1 and DM2.

2.3.2.1 Prevalence

The prevalence of DM1 in European populations has been established to 1 in 8,000 (Harper, 2001). However, this estimate has been made before the genetic differentiation of DM1 and DM2 diseases was possible, and is thus likely to represent both DM1 and some DM2 patients. Based on this prevalence DM1 is considered the most frequent muscular dystrophy in adults. However, in different populations the prevalence of DM1 may vary widely. For example a very high

prevalence of 1 in 2,114 has been reported in an isolated population of Yemenite Jews (Segel et al., 2003).

For DM2 only a few estimates of its prevalence have been proposed. DM2 has generally been considered much rarer than DM1, but recent studies have suggested that DM2 may be as frequent as DM1 at least in Finland and some Central and Eastern European populations (Udd et al., 2011).

2.3.2.2 Clinical features

DM1 and DM2 are multi-systemic disorders causing other features besides muscle-related symptoms and signs. Clinical presentation of DM2 has similarities but also clear differences from that of DM1. DM2 is generally less severe and congenital or childhood-onset forms present in DM1 are not part of the spectrum of DM2. Muscle weakness and wasting predominantly affects distal limb and facial muscles in DM1, whereas in DM2 weakness presents later and in proximal lower limb muscles. The other core features, such as myotonia, cataracts, cardiac, gastrointestinal and endocrine abnormalities, are much more prominent in DM1 than DM2, whereas myalgic pain is prominent in DM2. (Ashizawa and Sarkar, 2011; Udd and Krahe, 2012)

Core features of DM1. DM1 disease phenotype correlates to some degree in severity and age of onset with the number of $(CTG)_n$ repeats of the expanded mutant allele. The clinical outcome thus consists of a continuum of disease severities rather than separate phenotypic forms (Karpati et al., 2010). However, to help the clinical diagnosis and classification of patients, different phenotypic groups have been established: congenital, childhood-onset and adult-onset. In addition to classic adult-onset form there is also a category of late-onset, oligosymptomatic very mild phenotypes, caused by 50-100 CTG repeats. Classic adult-onset form is caused by expanded alleles containing 50 to 1000 CTG repeats (Turner and Hilton-Jones, 2010). The most constant symptoms of adult-onset DM1 are muscle weakness, myotonia and cataracts. Weakness presents in muscles of distal upper and lower limbs and also facial and neck muscles are severely affected causing the typical facial features of a DM1 patient. Myotonia, which is clinically detected as a delayed relaxation of muscles after contraction, can be seen in many muscles, but is most

readily identified as grip activation myotonia in the hands. Myotonia is invariably present on EMG, which is a very sensitive method for detecting myotonia as the typical changes may be seen even in the absence of clinical myotonia. Early cataracts (before the age of 50 years) are a common feature of adult-onset DM1. In addition to the core clinical features, heart conduction abnormalities are very marked in DM1 and may even cause sudden death. Several other symptoms are part of the adult-onset DM1 phenotype, including gastrointestinal problems, testicular atrophy, diabetes, frontal balding in males, neoplasias and reduced levels of gammaglobulin. (Ashizawa and Sarkar, 2011)

Symptoms in the congenital form of DM1 differ from the adult-onset form. Myotonia and other muscle symptoms are not present at birth, but occur later in life. Instead, affected neonates are hypotonic and have respiratory and feeding difficulties and will develop a mental retardation. Similarly to congenital form, childhood-onset DM1 usually does not present with muscle symptoms. Instead, the patients have moderate problems at school, although on examination their muscle bulk is small and facial weakness may exist. (Udd and Krahe, 2012)

Symptoms and signs in congenital and childhood-onset form of DM1 are caused by developmental abnormalities, and the degenerative changes characterizing the adult onset DM1 will develop later in life causing severe disability and reduced life span. (Ashizawa and Sarkar, 2011; Udd and Krahe, 2012)

Symptoms in DM2. DM2 in general is much less severe, the onset is later and the progression is slower than in adult-onset DM1. Except for severe cardiac conduction abnormalities in certain DM2 families, the life span of a DM2 patient is normal. The phenotype is very variable and any of the common symptoms may be absent in any individual patient. Also the disease onset is variable, ranging from early twenties to around the age of 70-75 years (Udd et al., 2006). Core features are muscle weakness, myalgic muscle pain, muscle stiffness, cataracts and myotonia. Muscle weakness first involves proximal lower limb muscles and later abdominal, axial and neck flexor muscles. Facial muscles are usually spared and no DM1 facial appearance is present, but mild ptosis may occur in a minority of patients. Muscle pain, which is a common problem in DM2, and calf muscle hypertrophy are features clearly distinguishing DM2 from DM1 (Udd and Krahe, 2012). Of DM2 patients 76 % have been reported to experience pain, which is usually widespread and can resemble the pain seen with fibromyalgia patients (Suokas et al., 2012). Patients also

complain of muscle stiffness, commonly located on hip and thigh muscles (Karpati et al., 2010). Myotonia in DM2 is not as prominent as in DM1. Many DM2 patients do not show clinical myotonia at all and in a considerable portion of patients myotonia is absent even on EMG (Udd et al., 2003). Myotonia may also be variable over time. Other symptoms of DM2 include cataracts, tremor, cramps, endocrinological abnormalities and cardiac conduction defects. Cataracts are similar to those seen in DM1 patients and usually develop very late in life, but are sometimes present also in younger patients around their twenties as their first symptom (Day et al., 2003). Insulin resistance is a frequent sign, but clinically manifesting diabetes is found in only 23 % of DM2 patients (Day et al., 2003). Other endocrinological problems include hypogonadism. Cardiac conduction defects are typically milder in DM2 than in DM1, but cases of sudden death at relatively early age have been reported, and these severe cardiac manifestations seem to be present in selected families (Schoser et al., 2004b; Udd et al., 2003). Brain functions are usually normal, although an avoidant personality change has been reported on testing scales and mild white matter changes can occur on brain MRI (Franc et al., 2012; Meola et al., 2003; Minnerop et al., 2011).

2.3.2.3 Diagnostics

Clinical examination of a patient is the first step towards the diagnosis of DM1 or DM2 disease. Special attention in suspecting DM2 is drawn to proximal muscle weakness, large calves and muscle pain or stiffness, together with symptoms and signs in the family history. Clinicians also have to keep in mind the variability of the DM2 phenotype and the possibility of diagnosis even in absence of any myotonia (Udd et al., 2011). Serum creatine kinase (CK) levels, a marker for abnormal muscle fibers, may be normal or slightly elevated in DM2 (Udd et al., 2011). For DM1 the clinical diagnostics is usually straightforward, in part due to the fact that patients come very late in the course of the disease for neurological examinations, although patients with late-onset mild oligosymptomatic phenotype might easily remain unnoticed. Studies on muscle biopsy are usually very helpful especially in DM2 in guiding the diagnosis in the right direction. Specific histopathological changes in DM2 muscle biopsy sample include nuclear clump fibers without changes of

chronic neurogenic abnormality, increased number of internal nuclei, a subpopulation of highly atrophic type 2A fibers including nuclear clump fibers (Ashizawa and Sarkar, 2011; Udd and Krahe, 2012). A common difficulty with assessment of these highly atrophic type 2A fibers is that they cannot be identified on conventional fiber type staining such as ATPase histochemistry, but need immunohistochemical staining for fiber-type specific myosin heavy chains (Raheem et al., 2010a). Histopathological changes in DM2 are presented in Figure 5. Histopathological changes in DM1 include increased number of internal nuclei, variation in fiber size and atrophic type 1 fibers, together with sarcoplasmic masses and ring fibers, but muscle biopsy is hardly ever needed for DM1 diagnostics anymore (Ashizawa and Sarkar, 2011).

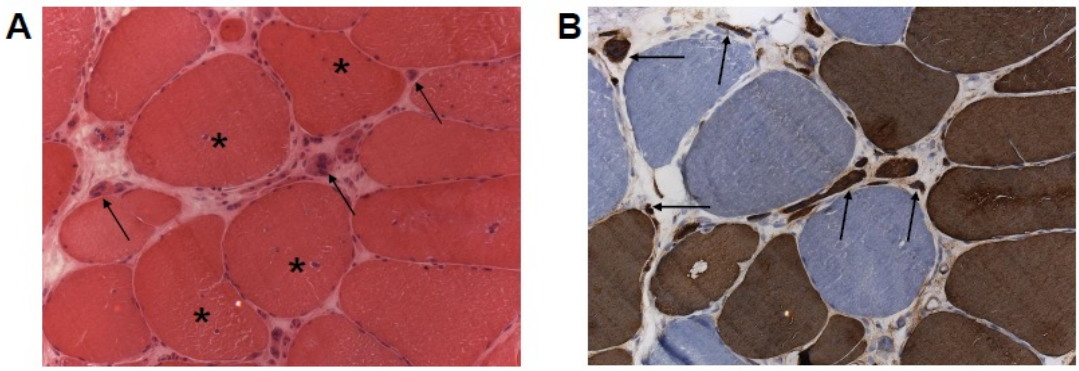

Figure 5. Typical histopathological changes of DM2 disease. A) Increased number of internal nuclei (asterisks) and nuclear clump fibers (arrows) are visible in hematoxylin and eosin (HE) staining. B) Highly atrophic type 2A fibers stain positive (brown) in fast MyHC (arrows).

Because of the variable and heterogeneous symptoms of DM2, the diagnosis cannot be established or excluded on the basis of clinical findings. Therefore the detection of the mutation itself is necessary for accurate and reliable diagnosis of DM2, and also of DM1. Several techniques have been developed to identify the repeat expansions, although the nature of the mutation makes it challenging. These techniques include the detection of the mutation *in situ* by hybridization on the muscle biopsy sections or elongated DNA strands (CISH, FISH and fiber-FISH). More convenient DNA-based methods could also be used: either digested genomic

DNA and probes to detect the mutation (Southern blot) or amplifying the repeat region with PCR-based methods (RP-PCR and TP-PCR).

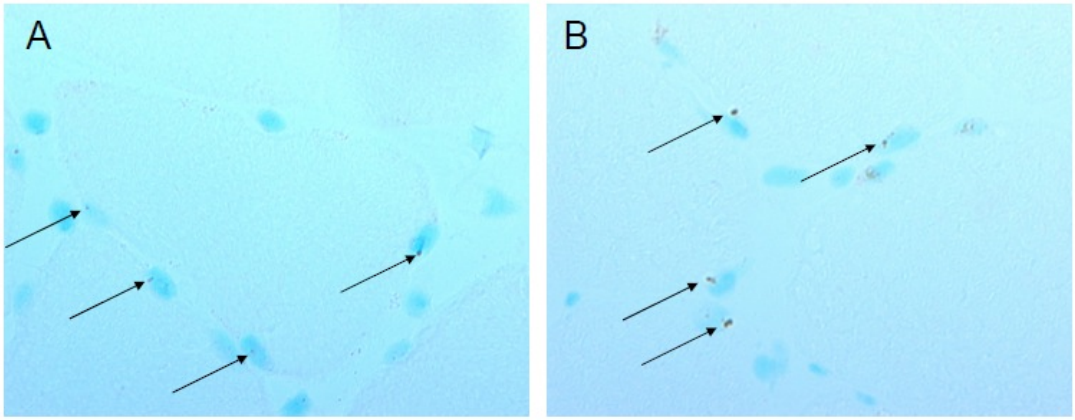

Figure 6. Detection of DM2 mutation by chromogenic *in situ* hybridization (CISH). A) With sense probe a single signal in each nucleus representing the CCTG repeat expansion on genomic DNA is detectable. B) Using anti-sense probe detecting RNA aggregates of transcribed CCTG repeat, stronger signals are obtained. (The figure was kindly provided by Olayinka Raheem.)

***In situ* hybridization techniques.** *In situ* hybridization techniques have been developed for detecting both DM1 and DM2 mutation (Bonifazi et al., 2006; Sallinen et al., 2004). By *in situ* hybridization, the repeat expansion mutation can be seen on muscle biopsy samples using labeled sense and antisense probes to detect the mutation on DNA and the mutant transcript on RNA level, respectively. Because mutant RNA molecules form aggregates and thus outnumber mutant DNA molecules, signals obtained with the antisense probe tend to be stronger, detecting transcribed $(CUG/CCUG)_n$ nuclear foci in the muscle, whereas the sense probe provides only one single signal per nucleus (Sallinen et al., 2004). The visualization of the repeat expansion mutation is obtained either by chromogenic detection of digoxigenin-labeled probes or by using fluorescently labeled probes and requires fluorescent microscopy for visualization. For chromogenic detection the method is called chromogenic *in situ* hybridization, CISH, and the sections are viewed under a bright-field microscope. An example of the detection of DM2 mutation by CISH is shown in Figure 6. The technique utilizing fluorescent detection is called fluorescent

in situ hybridization, FISH. A modification of FISH using stretched DNA molecules for better detection and estimation of the repeat size, fiber-FISH, has also been developed for DM2 (Sallinen et al., 2004). Although these hybridization techniques are quite sensitive, shorter repeats not forming ribonuclear foci are often not detected (Bonifazi et al., 2006). Besides, these techniques are quite laborious and require a muscle sample.

Southern blot analysis. Detection of the DM1 and DM2 mutations on DNA level was first performed by Southern blot analysis (Brook et al., 1992; Fu et al., 1992; Liquori et al., 2001). This method uses enzymatically digested genomic DNA from which the expansion mutation is detected by labeled probes. The advantage is that the size of the expansion can be estimated and the somatic variation of the repeat size is observable. The amount of DNA needed is very high, although by using locked nucleic acid probes the sensitivity can be increased (Nakamori et al., 2009). The most notable disadvantage of the Southern blot analysis is the poor detection of both shorter and large highly heterogeneous repeat expansions from alleles with a high degree of somatic mosaicism (Radvansky and Kadasi, 2010). For detecting DM2 repeat expansion, Southern blot analysis has been reported to fail in detection of the mutation in as many as 20 % of known mutation-positive patients (Day et al., 2003).

PCR-based mutation detection. For reliable detection of DM1 and DM2 mutations from DNA, several assays amplifying the repeat region have been developed. Repeat assay was originally developed to detect the repeat expansion in Huntington's disease and DM1 (Warner et al., 1996), but has been modified to be applicable also for DM2 (Bachinski et al., 2003). The advantage of these PCR-based methods is the low amount of DNA needed and high sensitivity also for short repeat expansions. As a drawback, the size of the repeat expansions cannot be defined. Repeat assays are called either repeat-primed PCRs (RP-PCR) or triplet- or tetraplet-primed PCRs (TP-PCR), but the principles of both assays are the same (Bachinski et al., 2003; Catalli et al., 2010; Day et al., 2003; Falk et al., 2006; Warner et al., 1996). The amplification of the repeat region is designed to utilize three primers in contrast to two flanking primers in a conventional PCR-amplification. The first primer flanks the region on either side of the repeat, the second is designed to attach to the repeat region itself and the third primer is complementary to the universal sequence tail in primer two. One of the primers has

to be labeled for the detection of the results. Usually the label is a fluorescent dye and the detection is performed with automated capillary electrophoresis and appropriate analysis software. A typical pattern of DM2 repeat expansion in RP-PCR is shown in Figure 7. The repeat assay can be designed to amplify the region in either direction. In DM2 the direction may be relevant since the repeat region in *CNBP* is complex with additional repeating elements of polymorphic $(TG)_n(TCTG)_n$ tracts upstream of the expanding $(CCTG)_n$ repeat. The TP-PCR developed by Catalli et al. amplifies the repeat region from the opposite direction with forward primer downstream of the $(CCTG)_n$ repeat (Catalli et al., 2010). It has been suggested that using the repeat-primed PCR methods in a direction that does not include polymorphic $(TG)_n(TCTG)_n$ tracts will lead to more informative results compared to assays amplifying across the $(TG)_n(TCTG)_n$ tract (Radvansky et al., 2011).

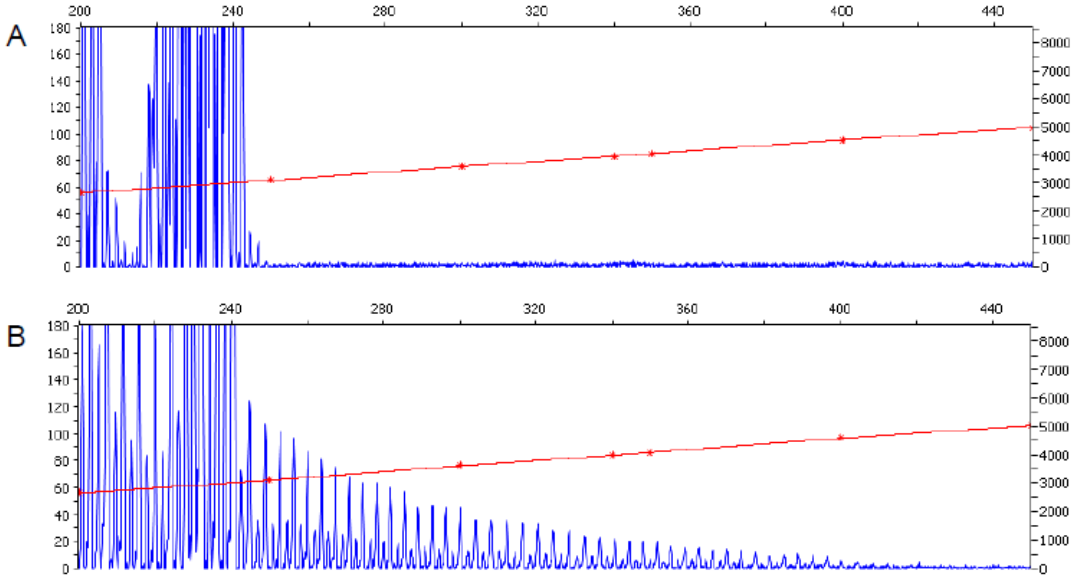

Figure 7. Typical pattern of RP-PCR analysis. A) Negative sample with no DM2 mutation. B) DM2 positive sample in which the pattern of peaks gradually tapers off in intensity.

In diagnostics of DM1 and DM2, DNA-based mutation detection is usually preceded by PCR amplification across the repeat region (Radvansky and Kadasi, 2010). Although it does not detect a mutated allele unless it is very short i.e. <100 repeats, PCR amplification provides a useful method for excluding individuals with

two normal alleles from having the disease, and is thus used as a first step in genetic testing. From this across PCR the number of CTG repeats in DM1 can be determined, but not for DM2, because the repeat region in *CNBP* contains also other polymorphic repeat elements than the CCTG repeat tract. The repeat regions in both *DMPK* and *CNBP* are very variable in size and the heterozygosity in European population is around 80 % and 90 %, respectively (Radvansky and Kadasi, 2010). The detection of two normal-sized alleles excludes the possibility of the disease. The advantage is that the across PCR is very fast and more straightforward than the repeat assays, which are then used for discrimination of carriers of two same-sized normal alleles from those with the expansion mutation.

2.3.2.4 Repeat expansion mutations

Mutations causing DM1 and DM2 are both located in untranslated regions of their respective genes, *DMPK* and *CNBP*. DM1 mutation region consists merely of three repeating nucleotides $(CTG)_n$, although some patients with multiple interrupting GGC and CCG repeats at the 3′-end of the repeat possibly affecting the phenotype have been reported (Braida et al., 2010). In DM2 the $(CCTG)_n$ repeat expansion mutation is part of a complex repeat region of $(TG)_n(TCTG)_n(CCTG)_n$ (Liquori et al., 2001) and in most healthy individuals the repeat region is even longer containing additional $(CCTG)_n$ repeats interrupted by one or more (TCTG) or (GCTG) motifs (Bachinski et al., 2009). DM2 mutation contains large stretches of $(CCTG)_n$ without any interrupting motif and is thus referred to as uninterrupted.

The repeat expansions accounting for DM1 and DM2 are unstable. Variations of the repeats are not only intergenerational, changing the repeat length in subsequent generations, but occur also in somatic cells and even in postmitotic tissue (Lavedan et al., 1993; Liquori et al., 2001). The DM2 mutation tends to grow during the lifetime of a patient. If the repeat sizes increase in subsequent generations, they often correlate with the severity of the disease, causing anticipation, i.e. more pronounced symptoms in subsequent generations. This is compatible with DM1 disease, for which the repeat size can increase especially through maternal transmission, causing earlier onset and more severe disease phenotype in children (Koch et al., 1991). In DM2, on the other hand, anticipation is not the rule. In fact,

its occurrence has been reported only in a few studies of German DM2 families, and that by statistical methods (Schneider et al., 2000; Udd et al., 2003). Although DM2 repeat is unstable and the repeat number may vary from generation to generation in affected families, the repeat sizes generally do not associate with the disease onset or severity (Bachinski et al., 2003; Day et al., 2003). Interestingly, rather than growing in subsequent generations, DM2 mutation repeat length is usually reduced in young patients of the subsequent generation, which may be, at least partly, due to correlation of increased repeat size with age (Day et al., 2003).

The repeat expansion of $(CCTG)_n$ in DM2 is the largest known repeat mutation causing a human disease. Up to 11,000 CCTG repeats have been reported in the affected allele of a DM2 patient (Liquori et al., 2001). The difference between the shortest disease-associated repeat expansion and the largest one is remarkable, since the shortest reported repeat expansion contains only 75 CCTG repeats (Liquori et al., 2001). The average number of repeats in DM2 patients is around 5,000 (Liquori et al., 2001) whereas the number of uninterrupted repeats in normal individuals ranges from approximately 10 to 30 repeats (Bachinski et al., 2009; Todd and Paulson, 2010). The minimum number of repeats necessary to cause a DM2 disease phenotype is somewhat equivocal. In an individual with a repeat allele of 75 CCTG repeats also larger alleles were observed in Southern blot analysis (Dalton et al., 1993; Liquori et al., 2001), and therefore the determination of pathogenicity of the 75 CCTG repeat allele cannot be confirmed. Another observation of a short repeat expansion reported a patient with 100 CCTG repeats and similar pathomechanism including ribonuclear foci with MBNL1 aggregation (Lucchiari et al., 2008). The symptoms in this patient were similar to those seen in an average DM2 patient, which is consistent with the proposition that the repeat sizes in DM2 do not correlate with the disease severity. However, how comprehensively larger expansion alleles were excluded in this patient is not known.

The origin of DM1 and DM2 mutations has been studied by linkage analysis of the affected families. Those studies suggest that both DM1 and DM2 mutations are of European origin (Bachinski et al., 2003; Imbert et al., 1993; Liquori et al., 2003; Neville et al., 1994; Yamagata et al., 1996). Apart from European descent, DM1 has been reported from one Nigerian kindred with suggested *de novo* mutation (Krahe et al., 1995). DM2 mutation has also been found in a non-European population with apparently different origin. Saito et al. reported one Japanese DM2 patient with a

haplotype different from that found in Caucasian DM2 patients (Saito et al., 2008). DM2 mutation has also been reported in one family originated from Morocco and one Afghanistan family, but the haplotypes in these families were found to be similar to the common European founder haplotype, indicating a single ancestral origin (Coenen et al., 2011; Liquori et al., 2003). With the exception of the one case in Japan, all DM2 patients have a common European origin of their mutation.

2.3.2.5 Inheritance

Both DM1 and DM2 are inherited in an autosomal dominant manner. Because of the nature of the mutation, there are some extraordinary features involved in the inheritance pattern as well. As discussed earlier, anticipation is common in DM1, but exceptional in DM2. Because of anticipation, prediction of the phenotype in subsequent generations is very difficult. Most often a severe congenital form of DM1 is transmitted through the affected mother, but also a few cases of paternal transmission have been reported (Di Costanzo et al., 2009; Zeesman et al., 2002). The risk of generating a large repeat expansion causing congenital DM1 is increased if the mother has more than 300 CTG repeats (Cobo et al., 1995; Martorell et al., 2007). It has been suggested that the maternal transmission is caused by a mechanism involving DNA ligase I, which could function in mediating CTG expansions and in avoiding maternal CTG contractions (Tome et al., 2011).

For DM2, on the other hand, the variability of the phenotype is very common, and the phenotype of the offspring of an affected parent is very difficult to predict. In published data the penetrance of the DM2 mutation is considered to be 100 % (Udd et al., 2003).

For both DM1 and DM2, patients homozygous for the repeat expansion have been reported (Schoser et al., 2004a; Zuhlke et al., 2007). The phenotype of homozygous DM1 patients ranges from typical adult-onset DM1 to severe congenital form (Zuhlke et al., 2007), whereas the phenotype of homozygous DM2 patients does not differ so much from the phenotype generally observed in heterozygous patients.

2.3.2.6 Pathomechanism

The main mechanism by which the repeat expansion mutations cause DM1 or DM2 is the toxic effect of the transcribed RNAs containing the expanded mutant CUG or CCUG repeats (Mankodi et al., 2000; Seznec et al., 2001; Udd and Krahe, 2012). Although both repeats are located in untranslated regions of their respective genes and thus are not translated to protein, the mutant repeats are still transcribed to RNA. These mutant RNAs accumulate in the nucleus and form large aggregates that affect normal cellular functioning. This toxic RNA gain-of-function mechanism is believed to underlie the similarities of the multisystemic phenotype of both diseases, because the mutated genes bear no resemblance in function. *DMPK* gene codes for DMPK protein, which is a serine/threonine protein kinase necessary for the maintenance of skeletal muscle structure and function (Harmon et al., 2011; Shaw et al., 1993). *CNBP* on the other hand codes for nucleic acid-binding protein CNBP, which functions as a transcription regulator that is especially involved in sterol-mediated regulation (Rajavashisth et al., 1989). The involvement of RNA transcripts in the pathogenesis was first suggested by fluorescent *in situ* hybridization studies showing CUG or CCUG repeats within ribonuclear foci (Liquori et al., 2001; Taneja et al., 1995). Later the accumulated RNA transcripts were found to associate with other proteins to affect the splicing of several genes. The most important proteins involved in the alternative splicing are MBNL1 and CUGBP1. MBNL1 (Muscleblind-like protein 1) is a regulator of alternative splicing expressed highly in cardiac and skeletal muscle. The RNA aggregates in DM1 and DM2 cells sequester MBNL1 protein, causing a reduction of functional MBNL1. The functional importance of MBNL1 in the course of the disease is supported by the studies on knock-out mice with abolished Mbnl1 function. These mice generated symptoms similar to DM1, including myotonia and cataracts, although without muscular dystrophy (Kanadia et al., 2003). In addition to sequestering MBNL1 protein, mutant RNA transcripts also affect another protein important for the pathogenesis, CUGBP1. Levels of CUGBP1 (CUG-binding protein 1 or CELF1, CUGBP Elav-like family member 1), in contrast to MBNL1, are increased in DM1. In DM2 the abnormality of CUGBP1 is more equivocal with different results reported from different groups. CUGBP1 functions in post-transcriptional regulation, including splicing. The alternative splicing pattern produced by CUGBP1 activation is

antagonistic to the splicing pattern produced by MBNL1. It has been suggested that both the reduction of MBNL1 and increase in CUGBP1 levels in the cells is responsible for the abnormal splicing of several genes in both DM1 and DM2. In addition to MBNL1 and CUGBP1, also Staufen1 has been recently reported to function in splicing regulation. Staufen1 protein levels are increased in skeletal muscle of DM1 patients and it is suggested that it interacts with mutant mRNAs containing expanded $(CUG)_n$ and promotes their nuclear export and translation (Ravel-Chapuis et al., 2012).

In DM1 the splicing pattern is reported to be altered for several genes including skeletal muscle chloride channel (*CLCN1*) (Mankodi et al., 2002), insulin receptor (*INSR*) (Savkur et al., 2001), microtubule-associated protein tau (*MAPT*) (Sergeant et al., 2001), cardiac muscle troponin T (*TNNT2*) (Philips et al., 1998), fast skeletal muscle troponin T (*TNNT3*) (Kanadia et al., 2003), LIM domain-binding protein 3 (*LBD3*) (Lin et al., 2006), sarcoplasmic/endoplasmic reticulum calcium ATPases *ATP2A1* and *ATP2A2*, ryanodine receptor (*RYR1*) (Kimura et al., 2005), myotubularin related protein 1 (*MTMR1*) (Buj-Bello et al., 2002), Glutamate [NMDA] receptor subunit zeta-1 (*GRIN1 or NMDAR1*) and amyloid beta precursor protein (*APP*) (Jiang et al., 2004). In addition, alternative splicing of *BIN1* (Fugier et al., 2011) has also been reported very recently. Alternative splicing in DM1 and DM2 changes the expressed protein isoforms so that they will produce more non-functional fetal/developmental isoforms of the proteins. It has been suggested that some alternatively spliced genes have an effect on the specific phenotypic features in DM1 and DM2. The most studied gene is *CLCN1*, the alternative splicing of which has been shown to cause myotonia in patients. Aberrant splicing of insulin receptor gene is associated with insulin resistance and BIN1 possibly with developmental muscle weakness in congenital and childhood onset DM1 (Fugier et al., 2011; Ravel-Chapuis et al., 2012; Savkur et al., 2001; Savkur et al., 2004). Aberrant splicing for most of the genes abnormally spliced in DM1 has also been reported in DM2. Alternatively spliced genes in DM2 include *CLCN1* (Mankodi et al., 2002), *INSR* (Savkur et al., 2004), *MAPT* (Maurage et al., 2005), *TNNT3* (Salvatori et al., 2009), *LDB3* (Lin et al., 2006) and, interestingly, also *CNBP* (Raheem et al., 2010b). The effects of alternative splicing are similar to those in DM1 except for *TNNT3*, *LDB3* and *CNBP* (Raheem et al., 2010b; Vihola et al., 2010). Aberrant splicing of *TNNT3* and *LDB3* is more pronounced in DM2

compared to DM1, which could at least partly explain the differences between DM2 and DM1, especially the specific involvement of type 2 fibers in DM2, since *TNNT3* is specifically expressed in fast type 2 fibers. (Vihola et al., 2010).

Other suggested pathomechanisms include the effects mediated by functions of the genes carrying the mutations, i.e. *DMPK* in DM1 and *CNBP* in DM2. For DM2 the reduction of CNBP protein levels to 15-50 % in addition to abnormal splicing of *CNBP* has been reported (Raheem et al., 2010b), indicating a CNBP-related function possibly affecting the pathogenesis and eventually the phenotype. CNBP function has also been reported to be involved in translation of certain proteins, by binding the translation factors containing terminal oligopyrimidine (TOP) tract (Huichalaf et al., 2009). In DM2 muscle this binding activity of CNBP is reduced and subsequently the rate of protein synthesis for the proteins downstream of specific translation factors is lowered (Huichalaf et al., 2009). Inhibition of translation has also been reported for DM1, but the mechanism involves CUG repeat-induced stress, the suggestion being that it is related to the progressive muscle loss in DM1 (Huichalaf et al., 2010). In DM1 stress has also been reported to occur through dsRNA -dependent protein kinase PKR. The prolonged activation of PKR by mutant repeat transcripts leads to endoplasmic reticulum stress, which through block of translation initiation or apoptosis results in muscle wasting and weakness (Ikezoe et al., 2007; Udd and Krahe, 2012).

DM1 mutation has effects on the *DMPK* gene itself and also on the gene adjacent to *DMPK*, *SIX5*. The abnormal methylation in DM1 patients has been thought to lead to toxic antisense transcription of *DMPK* and to effects on *SIX5* gene (Lopez Castel et al., 2011; Udd and Krahe, 2012). In addition, studies on transgenic mice lacking functional *Dmpk* indicate specific phenotypic effects including cardiac conduction defects, and mice lacking *Six5* function have been reported to develop cataracts and have reduced male fertility and cardiac dysfunction (Berul et al., 2000; Sarkar et al., 2004; Wakimoto et al., 2002).

Muscle-specific miRNAs (microRNAs, very small regulatory RNA molecules) have been identified as either up- or down-regulated in DM1 and DM2 and might contribute to the overall phenotype (Gambardella et al., 2010; Perbellini et al., 2011; Rau et al., 2011). Although the down-regulation of miR-1 in DM1 and DM2 has been associated with heart defects (Rau et al., 2011), other specific implications of miRNAs to DM phenotype are yet to be discovered.

2.3.2.7 Treatment

There is no curative treatment yet available for either DM1 or DM2. However, many of the symptoms can be treated and severe effects of complications prevented by regular monitoring of the patients. Although the course of the disease cannot be changed, the management of the patients is very important for increasing their quality of life. Counseling and support is also important especially for family planning. Many DM2 patients have mild symptoms and may not need any medication besides having their complications monitored, once they have received the correct diagnoses explaining their symptoms. However, for DM1 patients the treatment of the symptoms has a major effect for their well-being. (Udd and Krahe, 2012)

The most worrisome problem among DM1 and DM2 patients are cardiac defects, which untreated can lead to sudden death (Udd et al., 2003). Careful monitoring by electrocardiography and early insertion of a pacemaker is essential for preventing sudden cardiac deaths. Cataracts can be treated by surgical removal and clinical myotonia, if disabling, by medication. Atherosclerosis in DM2 patients is frequent and can be treated with statins. However, in some DM2 patients with myalgia the statins induce higher levels of CK, indicating myopathy (Udd et al., 2011). Statin treatment has to be carefully monitored to avoid this adverse reaction and if elevation of CK levels is noticed, discontinuing the treatment indefinitely is recommended (Udd and Krahe, 2012; Udd et al., 2011). For treating pain in DM2, no efficient medication exists, although patients have experienced some relief for their pain by non-steroid anti-inflammatory drugs (NSAID) (Suokas et al., 2012).

Several therapy trials are ongoing with focus on elimination of toxic repeat transcripts or on targeting the downstream effects of the repeat transcripts (Udd and Krahe, 2012). The use of antisense oligonucleotides (AON) has been studied in DM1 mouse models and in human cells with promising results of attenuating the toxic RNA repeat transcripts and releasing of MBNL1 from its sequestration in the RNA foci (Mulders et al., 2009; Wheeler et al., 2009). Despite these promising results, AONs have not yet been studied in humans. Additional therapy approaches studied in DM1 mouse models targeting the downstream effects of the repeat transcripts include upregulation of MBNL1 activity and downregulation of CELF1 (Gomes-Pereira et al., 2011). Although these strategies may not be sufficient alone,

their combination could reverse the harmful effects of mutant RNA transcripts on the splicing. Also, reversing the spliceopathy by exon-skipping using AONs for specific target genes has been suggested (Wheeler et al., 2007). This approach would require several different AONs to correct the splicing of several affected effector genes to overcome the multiple phenotypic features of DM1 (Gomes-Pereira et al., 2011).

3. AIMS OF THE STUDY

DM2 is a relatively recently discovered disease and full understanding of the clinical spectrum of the disease has not yet been reached. The disease is difficult to diagnose because of its late onset, the variable clinical phenotypes and presentations of the individual features of the multiorgan phenotype. The comparatively mild clinical presentation in many patients is misleading and complicates the clinical diagnosis. Therefore, genetic methods for reliable diagnosis are necessary. Although DM diseases are reported as the most common adult-onset muscular dystrophies, the frequency of the DM1 and DM2 mutations has not previously been studied. Estimates of disease prevalence are based on clinically or genetically ascertained and diagnosed patients within a studied population. The goal of this study was to gain knowledge on the real mutation frequency of DM2 and DM1 mutations in the population and to understand the clinical spectrum of DM2 disease. Better understanding of the disease frequency and the clinical spectrum will aid health professionals to accurately consider and diagnose DM diseases.

The specific aims of the study were:

1. To ascertain the real frequency of the DM2 and DM1 mutations in the population
2. To clarify the role of modifiers such as heterozygous *CLCN1* mutations for the variable phenotype of DM2 disease
3. To understand the clinical spectrum of DM2 by
    a. analyzing fibromyalgia patients for possible DM2 mutation as the cause of their symptoms
    b. characterizing a new disease caused by short DM2 expansion mutation with distinct pathomechanism

4. SUBJECTS AND METHODS

4.1 Patients and controls

Samples included in this study were DNA samples isolated mainly from peripheral blood leukocytes in addition to some DNA samples isolated from saliva or skeletal muscle. Altogether 460 patient samples and approximately 6000 samples as controls from population cohorts in Finland, Germany and Italy were included.

The study was approved by the institutional review board of Tampere University Hospital. The majority of patient samples had been previously obtained for diagnostic purposes and all patients gave their informed consent prior to their inclusion in the study. Control DNA samples were obtained in research collaboration and they were anonymous.

4.1.1 Co-segregating *CLCN1* mutations among DM2 patients (I)

Patients included in study I were both DM2 and DM1 patients from Finland and Germany. From Germany 100 DM2 DNA samples and 100 DM1 DNA samples were used and from Finland 100 DM2 patients were included. Controls were composed of 100 German and 150 Finnish DNA samples.

4.1.2 DM2 patients diagnosed as having fibromyalgia (II)

Ninety Finnish patients with a fibromyalgia diagnosis meeting strict established criteria were invited and 63 of them consented to provide a blood sample for DM2 genetic testing. A disease control cohort of 70 individuals with long QT syndrome caused by one single Finnish founder mutation, G589D in the *KCNQ1* gene, was included (kindly provided by Prof. Kimmo Kontula and Heikki Swan, MD, PhD) in addition to 200 control DNA samples obtained from healthy Finnish individuals (kindly provided by Prof. Anna-Elina Lehesjoki).

4.1.3 DM2 mutation frequency in the Finnish population (III)

A large number (n = 4,532) of anonymous control DNA samples from Finnish blood donors originally collected for the 'Health 2000 Project' was obtained in collaboration with Prof. Leena Peltonen-Palotie. The study included also a control cohort of 1003 Finnish samples consisting of 606 patients with various non-myotonic NMD, 221 tibial muscular dystrophy (TMD) patients and their 176 healthy relatives. Samples with indications of any myotonic disorder in the NMD patients were carefully excluded. A disease cohort of 93 Italian patients with undetermined non-myotonic proximal myopathy or asymptomatic hyperCKemia was also included.

4.1.4 Short repeat expansion in the *CNBP* gene (IV)

This study was conducted on DNA samples isolated from peripheral blood, saliva or skeletal muscle. The samples were collected from altogether 12 individuals from a Finnish family, but only seven were used in subsequent studies because of the presymptomatic age of five individuals. Muscle biopsy from vastus lateralis muscle was obtained from two patients.

4.2 Methods

The methods used in this study are summarized in Table 2. Methods are described in this section only briefly and more detail can be found in publications I-IV.

Table 2. Methods used in this study.

METHOD	STUDY
DNA isolation	I-IV
Allele sizing by PCR	I-IV
Repeat-primed PCR (RP-PCR)	I-IV
TaqMan 5′ nuclease assay	I
Statistical analyses	I, III
Single genome equivalent amplification	III, IV
Sequencing	III, IV
Genotyping of microsatellites and SNP	III, IV
Histopathological analysis	IV
Chromogenic and fluorescent *in situ* hybridization	IV
SDS-PAGE and Western blotting	IV
Long-range PCR repeat expansion assay and Southern blot	IV
Allele specific expression analysis	IV
Splice variant analysis	IV

4.2.1 DNA isolation (I-IV)

DNA was isolated from peripheral blood and muscle samples with standard methods using Puregene DNA Blood Kit (Gentra Systems, Minneapolis, MN, USA) or UltraClean Blood DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). For isolating DNA from saliva samples, Oragene DNA saliva kits (DNA Genotek, Ottawa, Canada) were used according to manufacturer's instructions.

4.2.2 Allele sizing PCR (I-IV)

PCR across the repeat regions in *CNBP* (DM2) and *DMPK* (DM1) genes was performed under conditions in which only normal-sized alleles are amplified (Liquori et al., 2001). Two normal-sized alleles exclude DM disease whereas one normal sized allele corresponds to either two normal alleles of the same size or one normal allele and one expanded mutant allele. Sizes of the amplification products were determined using fluorescently labeled (FAM) primers in capillary

electrophoresis on an ABI 3100 or 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Results were analyzed with Genotyper or GeneMapper software (Applied Biosystems).

4.2.3 Repeat-primed PCR (RP-PCR) (I-IV)

Repeat expansion mutation in *CNBP* or *DMPK* genes can be detected with RP-PCR. The reaction in which the repeat expansion is amplified uses three primers that are designed to flank the repeat region (primer 1), attach to the repeat itself (primer 2), and attach to the universal tail of primer 2 (primer 3) and further amplify the fragments generated with primers 1 and 2 (Bachinski et al., 2003). Results were obtained by capillary electrophoresis on an ABI 3100 or 3130 Genetic Analyzer (Applied Biosystems) using fluorescently labeled primer 3 and analyzed with Genotyper or GeneMapper software (Applied Biosystems).

4.2.4 TaqMan 5′ nuclease assay (I)

Mutation detection for three selected mutations in the *CLCN1* gene, R894X, F413C and A531V, was performed using TaqMan 5′ nuclease assay (Sequence Detection System). Primers were designed to flank the mutation, and probes to attach to both the mutant allele and normal allele. The probes were labeled with different fluorescent dyes, and only the correct hybridization allowed the release of the corresponding dye and the separation of the mutant and normal allele. Primers and probes were designed using Primer Express Oligo Design software version 1.5 (Applied Biosystems, Foster City, CA, USA) and the results were obtained and analyzed with ABI Prism 7000 analyzer (Applied Biosystems).

4.2.5 Statistical analyses (I, III)

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) statistical software package (Study I) or R version 2.11.1 (GNU Operating System, Free Software Foundation Inc.) statistical computing environment (Study III). One-sided Fisher's Exact Test

was performed to calculate statistical significance in study I. For study III the statistical significance, P-value, was calculated in addition to computing both an equal-tailed (ET) 95% interval estimate (excluding the bottom and top 2.5% of all values) and a maximum likelihood (ML) 95% interval estimate.

4.2.6 Single Genome Equivalent Amplification (III, IV)

Single genome equivalent amplification (small-pool PCR, SP-PCR) was performed as previously described (Bachinski et al., 2009; Coolbaugh-Murphy et al., 2005) to determine the stability of the repeat region. DNA samples were diluted to levels of a single diploid genome (6 pg). Multiple SP-PCRs were performed in a three-primer reaction and labeled amplification products subjected to capillary electrophoresis (ABI 3100 Genetic Analyzer, Applied Biosystems).

4.2.7 Sequencing (III, IV)

The repeat regions in *CNBP* gene were sequenced from DNA isolated from peripheral blood leukocytes to characterize the repeat number and interruptions of the $(CCTG)_n$ repeat. The samples in which the RP-PCR resulted in a pattern consistent with a short repeat allele were sequenced. Alleles were amplified and the products cloned using the StrataClone PCR cloning kit (Agilent Technologies, Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. DNA was prepared from the clones using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA) and plasmid DNA was sequenced directly using ABI BigDye terminator chemistry (Applied Biosystems). Sequences were visualized by capillary electrophoresis on an ABI 3100 Genetic Analyzer (Applied Biosystems) and analyzed using Sequencher software (Gene Codes, Ann Arbor, MI, USA).

4.2.8 Genotyping of microsatellites and SNP (III, IV)

Six microsatellite markers (D3S3606, D3S3607, 571C11_AG1, 221E20-GT1, 814L21-GT1 and 436B3-AG1) around the DM2 mutation locus were genotyped to characterize the haplotype of the short repeat family in comparison to previously

reported Finnish DM2-associated haplotype (Study IV). Markers were genotyped as previously described using PCR and fragment analysis (ABI 3100 or 3130 Genetic Analyzer, Applied Biosystems) (Bachinski et al., 2003; Bachinski et al., 2009). Because the genotyping was performed in two different laboratories (in Tampere and in Houston) the primers used differ slightly between different laboratories. However, the results obtained in one laboratory are comparable.

Single nucleotide polymorphism (SNP), rs1871922, which is in linkage-disequilibrium (LD) with the DM2 repeat expansion mutation, was genotyped for the samples with large, possibly unstable alleles in *CNBP* intron 1 (Study III) and for the samples of short repeat expansion family (Study IV). The SNP locus was amplified by PCR and the products digested with *Hae*III (New England BioLabs, Ipswich, MA, USA). After digestion the samples were electrophoresed through 4% MetaPhor agarose gels (FMC), and visualized by ethidium bromide staining as described previously (Bachinski et al., 2003).

4.2.9 Histopathological analysis (IV)

Muscle biopsy samples were obtained from two family members, the proband and his brother, of the short repeat expansion family. Both samples were from vastus lateralis muscle. Samples were snap frozen and 8-10 μm sections were cut and examined by using standard histochemical stainings. In addition, sections were immunostained according to established protocols for different myogenic antigens including myosin heavy chain isoforms (fetal, neonatal, slow and fast MyHC).

4.2.10 Chromogenic and fluorescent *in situ* hybridization (IV)

Chromogenic *in situ* hybridization (CISH) was performed on frozen muscle sections as previously described (Sallinen et al., 2004). Sense probe $(CCTG)_8$ was designed to attach to the DNA sequence of the *CNBP* repeat expansion and antisense probe $(CAGG)_8$ to attach to the RNA aggregates. After hybridization with both digoxigenin end-labeled sense and antisense DNA oligonucleotide probes, Invitrogen Spot-Light® CISH™ Polymer Detection Kit (Invitrogen Corporation, CA, USA) was used for chromogenic detection. Sections were counterstained with

methyl green zinc chloride (Merck KGaA, Darmstadt, Germany), dehydrated, mounted and viewed under a bright field microscope.

RNA fluorescent *in situ* hybridization (FISH) to detect mutant $(CCUG)_{DM2}$ RNAs in primary patient myoblast cultures was performed with an antisense Cy3-$(CAGG)_{10}$. Briefly, cultures were fixed at 4 % PFA/PBS and washed in PBS, followed by permeabilization in 2 % pre-chilled acetone/PBS. After pre-hybridization the hybridization was performed with the $(CCUG)_{DM2}$ LNA probe (2 ng/µl). Post-hybridization washing was done in 30 % formamide and 2x SSC followed by 1x SSC. Slides were mounted either in SlowFade® Gold antifade reagent with DAPI (Molecular Probes) or used for subsequent IF. Images were acquired using a Nikon 2000U Deconvolution Microscope and processed with AutoQuant's AutoDeBlur software (Silver Spring, MD, USA).

4.2.11 SDS-PAGE and Western blotting (IV)

Muscle biopsies were treated as previously described for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (Haravuori et al., 2001). After SDS-PAGE, proteins were transferred onto a PVDF membrane and immunolabeled with a rabbit polyclonal antibody against CNBP (Chen et al., 2003). Anti-β-actin antibody C4 (Santa Cruz Biotechnology Inc., CA, USA) was used as a loading control. After HRP-conjugated goat anti-rabbit and goat anti-mouse IgG secondary antibodies (Zymed Laboratories Inc., San Francisco, CA, USA) were incubated, the antibody reacting bands were visualized using an enhanced chemiluminescence detection kit (Pierce Biotechnology SuperSignal West femto maximum sensitivity substrate, Rockford, IL, USA).

4.2.12 Long-range PCR repeat expansion assay and Southern blot (IV)

Long-range PCR was performed with primers flanking the DM2 mutation locus. PCR products were separated on 0.7 % agarose gel and transferred to Hybond N+ membranes (Amersham Biosciences, Amersham, UK). Radioactively end-labeled

oligo was used for visualization. The method has been described in detail by Schoser et al. (2004a).

4.2.13 Allele specific expression analysis (IV)

To verify that the short expansion allele of *CNBP* was expressed, reverse-transcriptase (RT) RP-PCR analysis was conducted on the cDNA sample of patient II-3, isolated from skeletal muscle as described previously (Raheem et al., 2010b). If pre-mRNA of mutant *CNBP* is present, a typical pattern in RP-PCR will be detected. To determine whether both *CNBP* alleles were transcribed to pre-mRNA the SNP rs1871922 A>C polymorphism was analyzed by quantitative allele-specific method as described previously (Raheem et al., 2010b).

4.2.14 Splice variant analysis (IV)

Alternative splicing of effector genes is part of DM2 disease pathogenesis. Aberrant splicing was analyzed for *CLCN1*, *INSR*, *ATP2A1*, *RYR1*, *TNNT3* and *TTN* genes from cDNA of patient II-3, isolated from skeletal muscle. Splice variant analysis for all the studied genes was performed using the same protocol as previously described for *TNNT3* (Vihola et al., 2010).

5. RESULTS AND DISCUSSION

5.1 Co-segregating *CLCN1* mutations among DM2 patients (I)

DM2 patients have very variable clinical presentation, as described in the Review of the literature. In addition to the dominant DM2 mutation causing the disease, other genetic factors are possibly contributory to the phenotype. The disease pathomechanism involves alternative splicing of *CLCN1* mRNA towards fetal protein isoform, which accounts for the myotonia symptom in DM2 patients. Mutations in *CLCN1* gene itself cause myotonia congenita, either recessive Becker type or more infrequent dominant Thomsen type. Carriers of recessive mutations usually do not present any symptoms, but findings of subclinical EMG myotonia have been reported (Deymeer et al., 1999). Because of the relatively high frequency of *CLCN1* mutations in the population, this enabled the hypothesis that co-segregating recessive *CLCN1* mutations could act as genetic modifiers in DM2 patients.

5.1.1 Frequency of common *CLCN1* mutations in DM2 patients, controls and DM1 patients

In a cohort of 100 Finnish DM2 patients heterozygous *CLCN1* mutations were found in five samples: three carried the most common R894X mutation and two carried the F413C mutation. In a German cohort of 100 DM2 patients we found heterozygous R894X mutation in four samples in addition to one sample with R894X mutation in a homozygous state. Interestingly, this homozygote patient had not, prior to DM2 diagnosis, been identified as a Becker myotonia patient. Taken together, *CLCN1* mutations were found in 5 % of DM2 patients of either Finnish or German origin. Controls were obtained from Finland, n = 150, and Germany, n = 100, and *CLCN1* mutations were found in three Finnish samples and one German sample. We also studied a cohort of 100 German DM1 patients to determine if the

possible modifier effect of *CLCN1* mutations is specifically related to DM2 or if it is common to both DM diseases. One heterozygote *CLCN1* mutation was observed in the DM1 patient cohort. The results are summarized in Table 3.

Table 3. *CLCN1* mutations in Finnish and German DM2 and DM1 patients and controls.

***CLCN1* mutation**	**DM2 patients (n=200)[a]**	**DM1 patients (n=100)[b]**	**Controls (n=250)[c]**
R894X heterozygotes			
Finnish	3	na	2
German	4	1	1
R894X homozygotes			
Finnish	0	na	0
German	1	0	0
F413C heterozygotes			
Finnish	2	na	1
German	0	na	0
A531V heterozygotes			
Finnish	0	na	0
German	0	na	0
Total	10	1	4
Total (%)	**5**	**1**	**1.6**

[a]Finnish samples n=100, German samples n=100
[b]German samples n=100
[c]Finnish samples n=150, German samples n=100
na not assessed

The mutations in *CLCN1* are relatively frequent. Over 150 different mutations have been reported from all over the gene (Human Gene Mutation Database). The frequency of the most common mutation R894X in the Northern Scandinavian population is reported to be 0.87 % (Sun et al., 2001) and in Finland there are at least 20 different mutations (Neuromuscular Research Unit, Tampere, Finland, unpublished data), thus the frequency of all the *CLCN1* mutations in the Finnish population is much higher. According to our results the population frequency of common *CLCN1* mutations (R894X, F413C and A531V) is 2 % in the Finnish population and 1 % in the German population, albeit none of the German controls had either F413C or A531V mutations.

The *CLCN1* mutation frequency in a cohort of 200 DM2 patients was 5 %, which is three times higher than the mutation frequency of 1.6 % in the combined general

population cohorts. The statistical significance is evident with a p-value of 0.037. Again in a cohort of 100 German DM1 patients, heterozygous *CLCN1* mutation was found in only one patient, which results in a mutation frequency similar to the frequency in normal German population. Because *CLCN1* mutations were found in only 1 % of DM1 patients, the higher frequency of co-segregating *CLCN1* mutations is unique to patients diagnosed with DM2 disease. Because the clinical manifestations in DM1 disease are more obvious, the correct diagnosis is usually achieved and no modifier genes increasing symptoms are needed for reaching the DM1 diagnosis. Similar to the DM1 patients, the frequency of *CLCN1* mutations should also be the same in DM2 patients as in the general population, since the loci of these two genes, *CNBP* and *CLCN1*, are unlinked (*CNBP* maps to chromosome 3q21.3 and *CLCN1* maps to chromosome 7q35). The discrepancy in frequencies among currently diagnosed DM2 patients and controls could be explained by the diagnostics practices of DM2 disease favoring patients with myotonia findings, as is the case with DM2 patients having additional recessive *CLCN1* mutation.

5.1.2 Clinical records of patients with both DM2 and *CLCN1* mutation

To investigate the possible aggravation of DM2 symptoms by co-segregating *CLCN1* mutation, the clinical records of patients with mutation in both genes were retrospectively reviewed. All German patients with both mutations showed very prominent clinical and early-onset myotonia, which is distinct from the average DM2 phenotype. Among DM2 patients in general, the degree of myotonia varies widely from the mild end with some changes only on EMG to clinical myotonia. In addition, myotonia can be absent in a significant proportion of DM2 patients (Udd et al., 2003; Udd et al., 2011). Finnish patients with both DM2 and *CLCN1* mutation did not show as prominent myotonia as the German patients. Even so two of the five Finnish patients showed very prominent clinical myotonia. One of the two had it as the leading symptom at the onset, but the clinical manifestation in the other three did not differ from the variable phenotype of general DM2 disease. However, they all had myotonia either clinically or on EMG, whereas in a notable part of DM2 patients in general myotonia is completely absent. Overall, retrospective clinical analysis of both German and Finnish patients with DM2 and *CLCN1* mutations

indicated a more severe myotonia phenotype compared to general DM2 manifestation. The clinical data is summarized in table 4.

The change in clinical presentation towards the more severe end of the DM2 clinical spectrum explains the difference in frequencies of heterozygous *CLCN1* mutations among DM2 patients compared to controls. DM2 patients with co-segregating and modifying additional gene mutation are more likely to be ascertained and diagnosed with DM2 than patients without other modifying mutation. *CLCN1* was considered a good candidate for a modifying gene because the pathomechanism of DM2 disease involves abnormal splicing and function of ClC-1 protein, which accounts for the myotonia symptom in patients. Variable myotonia in DM2 patients may be due to a variable amount of mis-spliced *CLCN1* mRNA and if the gene in one chromosome carries a mutation, the amount of functional ClC-1 is even less. In conclusion, co-segregating *CLCN1* mutations modify the DM2 phenotype towards more obvious signs of myotonia, and so those patients with both mutations are more likely to have a correct diagnosis than DM2 patients without this modifying effect.

The coexistence of *CLCN1* mutations in a DM2 patient is not a unique observation (Cardani et al., 2012; Lamont et al., 2004; Sun et al., 2011; Udd et al., 2003). The clinical findings with the ten patients characterized with both mutations in the present study are in accord with the results obtained by the other groups.

The results of this study indicate that a large number of DM2 patients without a modifying *CLCN1* mutation may remain undiagnosed, possibly due to less apparent symptoms. Therefore the threshold for clinicians to offer DM2 genetic diagnostics should be lowered.

Table 4. Clinical findings in DM2 and DM1 patients with co-segregating *CLCN1* mutations. Figure adopted from Suominen et al. (2008) with permission of the publisher.

German Patients	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Genotype: DM	DM2	DM2	DM2	DM2	DM2	**DM1**
CLCN1	R894X **/ -**	R894X **/ -**	R894X **/ -**	R894X **/ -**	R894X**/** R894X	R894X **/ -**
Gender	male	female	male	male	male	female
Age at evaluation	26	51	37	45	54	37
Age at onset	12	10	35	14	32	18
Symptom at onset	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia
Years of weakness	-	-	2	3	-	4
Clinical myotonia	+	+	+	++	+++	++
EMG myotonia	++	++	++	++	+++	++
Cataracts	-	-	-	-	na	na
Myalgia	++	++	++	+	++	+
Serum CK value	300-500	300-400	150-221	376-643	390	265-470
Other	proximal muscle hypertrophy	gallstones, myocardial infarction, WML[1] on brain MRI		hypertrophy of calves	muscle hypertrophy	

Finnish Patients	**F1**	**F2**	**F3**	**F4**	**F5**
Genotype: DM	DM2	DM2	DM2	DM2	DM2
CLCN1	R894X **/ -**	F413C **/ -**	R894X **/ -**	R894X **/ -**	F413C **/ -**
Gender	male	female	male	female	male
Age at evaluation	48	58	52	50	65
Age at onset	30	25	51	48	41
Symptom at onset	pains and proximal weakness of upper and lower limbs	proximal weakness and stiffness of lower limbs	radicular pains of upper arm after trauma	stiffness of lower limbs	thoracic pains
Years of weakness	18	33	-	-	-
Grip myotonia	+	+	+	-	-
EMG myotonia	+	++	++	+	+
Cataracts	-	-	na	na	na
Myalgia	++	+	+	+	++
Serum CK value	709-1055	466	710	289	244
Other	hypertrophy of calves	-	-	-	radicular pains and lumbalgia without clear radiculopathy

+: finding/sign present, ++: finding/sign frequent, +++: finding/sign profuse, -: finding/sign not present
For EMG myotonia: +: myotonia present but not in all muscles, ++: present in all muscles tested, +++: profuse in all muscles
[1]WML: white matter lesions, na: not assessed

5.2 DM2 mutations among fibromyalgia patients (II)

Fibromyalgia (FM) is a chronic pain disorder with a relatively high prevalence of 2 % (0.5 % in men and 3.4 % in women) (Wolfe et al., 1995). The core symptoms include chronic widespread pain, generalized stiffness, poor-quality sleep, and fatigue. According to diagnostic criteria elaborated by the American College of Rheumatology in 1990, a fibromyalgia patient by definition has a history of chronic widespread pain defined as pain involving at least three quadrants of the body and the axial skeleton in addition to sensation of pain in at least 11 of 18 tender points (Wolfe et al., 1990). These strict diagnostic criteria were used for selecting the fibromyalgia patients for this study. After this study was published, new FM diagnostic criteria have been suggested that have more emphasis on symptoms other than pain (Wolfe et al., 2010).

5.2.1 DM mutation found in 2 out of 63 fibromyalgia patients

Of the 90 fibromyalgia patients invited 63 participated and were analyzed for DM2 mutation, which was found in two patients (3.2 %). DM2 mutation was also analyzed in 70 patients with long QT syndrome caused by one single Finnish founder mutation, G589D in *KCNQ1* gene (Piippo et al., 2001). No DM2 mutations were found in this disease control cohort or in a control cohort of 200 healthy individuals. The sex of the individuals in neither control cohort is known. Although FM is much more common among women, the sex of the individuals of the control cohorts has no significance since the study concentrates on DM2 disease, which is a genetic disorder not related to sex. The two fibromyalgia patients who tested positive for DM2 mutation were re-examined and their previous records re-evaluated. On re-evaluation they both still met the diagnostic criteria of fibromyalgia and their symptoms were also adequate for the wide spectrum of the DM2 phenotype. The most prominent symptom was muscle pain in addition to muscle stiffness. Age at symptom onset was 38 for patient 1 and 37 for patient 2. Neither of the patients had clinical myotonia, but patient 1 showed myotonia on EMG. Serum CK levels were slightly elevated: 411 IU/L in patient 1, but normal, 60 IU/L, in patient 2. Both patients had a positive family history and in the follow-up studies they were unexpectedly found to be sisters.

5.2.2 Fibromyalgia as a differential diagnostic possibility of DM2

Features of DM2 and fibromyalgia, including myalgic pain and muscle stiffness, are overlapping and indistinguishable. Besides, fatigue and poor-quality sleep have been reported also in DM2 patients (Tieleman et al., 2010). As our findings indicate, DM2 disease appears to be a true candidate in the differential diagnostics of fibromyalgia. A distinguishing symptom to direct the diagnosis towards DM2 would be myotonia, but at least half of the DM2 patients exhibit no clinical myotonia and a considerable proportion lack myotonia even on EMG (Udd et al., 2003; Udd et al., 2011). Proximal muscle weakness of lower limbs as well as the presence of cataracts at a relatively early age in a patient or in his/her family are suggestive of DM2. In addition, elevated CK levels may also guide the diagnosis towards DM2. The most distinct clue for DM2 would be pathologic muscle biopsy findings characteristic of DM2, but muscle biopsies are rarely obtained in fibromyalgia patients.

Fibromyalgia is a frequent condition in the general population in many countries. Therefore it is important to determine whether there are distinct genetic disease entities that go undiagnosed among patients with FM diagnosis. Another aspect of this study is to emphasize the variable phenotype of DM2 disease and the high frequency of DM2 mutation. Prevalence of FM has been estimated at 2 % as studied in a general population of the city of Wichita, KS, USA (Wolfe et al., 1995). According to this estimate there would be some 100,000 FM patients in Finland. The finding of 3.2 % DM2 patients among FM patients would produce a computational number of 3200 DM2 patients in Finland. Compared to a previous prevalence estimate of 1 in 8,000 for both DM1 and DM2 diseases (Harper, 2001), this number of DM2 mutation carriers is much higher and leads to the conclusion that DM2 disease is much more common in the general population and the symptoms may be largely overlooked. In fact, the finding of DM2 mutation among patients diagnosed with fibromyalgia suggests that DM2 disease phenotype can present with symptoms similar to those seen in FM. Clinicians should be aware of this differential diagnostic possibility and consider offering DM2 genetic testing for FM patients, at least for those with EMG myotonia, elevated CK levels or proximal lower limb weakness.

5.3 Frequency of DM2 mutation in the population (III)

Estimation of the prevalence of DM diseases, both DM1 and DM2, dates back to the year 2001 and was based on clinical ascertainment of patients (Harper, 2001). This estimation, 1 in 8,000, is likely based mostly on DM1 patients because of their more defined clinical phenotype compared to DM2. DM1 prevalence has been studied in different populations and the estimates vary a lot from as high as 1 in 475 in a French Canadian population with a founder effect (Bouchard et al., 1989) to a very low prevalence of 0.46 per 100,000 (1 in 217,000) in Taiwan (Hsiao et al., 2003). For DM2 disease the prevalence has not been established because the wide variability of clinical outcome complicates the diagnosis and the number of patients is difficult to assess. To overcome the limitations of clinical diagnostics, we chose the genetic approach to study the frequency of DM2 mutation in the population.

5.3.1 DM mutations in the population cohort

DM2 and DM1 mutations were analyzed by allele sizing and RP-PCR from DNA samples taken from a population cohort of 4,532 healthy blood donors. Results for allele sizes were obtained from 4,508 samples for DM2 and 4,520 samples for DM1. At the *CNBP* locus a single allele was seen in 12.6% (572) of the samples and at the *DMPK* locus in 20.7% (938). RP-PCR from these single allele samples revealed two samples positive for DM2 mutation and also two samples positive for DM1 mutation. Results of all the cohorts studied are presented in Table 5. The number of tri- or tetranucleotide repeats is usually not calculable from RP-PCR, but for shorter repeat expansions at least for DM1 mutation, an approximation can be made from allele size reading. The size of the repeat expansion in one DM1 positive sample was $(CTG)_{80}$, whereas for the other mutation-positive sample the repeat number could not be determined, nor could the repeat sizes of the two DM2 mutations be determined. Both DM2 mutation-positive samples were also analyzed for the SNP rs1871922, and they were both homozygous for the C allele, which is in linkage disequilibrium with the DM2 expansion mutation. The presence of four DM mutations, both DM2 and DM1, in a population cohort of 4,532 samples suggests a significantly higher prevalence (p-value = 0.0027) than the previous estimate of DM disease prevalence of 1 in 8,000.

Table 5. Results of DM mutation analysis for all analyzed cohorts.

		DM2 positive		DM1 positive
Analyzed cohorts	n	n	n	n
General population cohort (FIN)	4,508	2	4,520	2
NMD cohort (FIN)	988	1	991	0
Proximal myopathy or asymptomatic hyperCKemia cohort (I)	93	1	na	na
Total	5,589	4	5,511	2

FIN, Finland; I, Italy. na, not applicable.

In addition to the two DM2 mutation-positive samples, nine samples with an enlarged allele, possibly premutation alleles, were identified in the *CNBP* locus. Five of these showed a pattern consistent with an unstable $(CCTG)_n$ repeat in RP-PCR and all nine were at least heterozygous for the SNP rs1871922 C allele. The material was too scanty for further analysis (e.g. sequencing). The relevance of the premutations in the *CNBP* locus for the carrier is unknown, but it has been suggested that they have evolved from small expansions by unequal crossing-over (Bachinski et al., 2009). As in *CNBP*, large unstable alleles were also found in the *DMPK* locus. One sample with a premutation of approximately $(CTG)_{45}$ and additional 13 samples with alleles ranging from 35 to 40 CTG repeats were identified. The premutations in the *DMPK* locus most likely do not cause any symptoms to the carriers, but may have an impact in the subsequent generations as the premutations are prone to increase in size especially if transmitted by a male carrier (Martorell et al., 2001).

5.3.2 DM mutations in the NMD cohort

Our cohort consisted of 1,003 samples of patients with various neuromuscular diseases, including tibial muscular dystrophy, and healthy relatives of the patients. The cohort was included in the mutation frequency study to investigate the possible modifying effect of DM2 mutation in other neuromuscular diseases. Among 988 samples with available molecular diagnostic DM2 data, one DM2 mutation was found in a patient with a previous genetic diagnosis of TMD. This patient had, in

addition to a weakness of ankle dorsiflexion characteristic of TMD, an unusually marked proximal muscle involvement at the age of 55 years, which suggested a possible influence of the DM2 mutation. The patient had not been studied on EMG, but clinically there was no myotonia. TMD patients presenting with unusual phenotype is not a novel finding although co-segregation with DM2 mutation has not been previously reported. Udd et al. reported in 2005 a cohort of 209 TMD patients of whom 9 % had an unusual phenotype despite an identical founder mutation in *TTN* gene (Udd et al., 2005). The reason for these phenotypic variations remains unknown.

A single allele in the *CNBP* repeat locus was observed in 11 % of the NMD cohort samples and in RP-PCR six large alleles in addition to one mutation-positive case were found. Sequencing of the large allele samples revealed two uninterrupted short repeat expansions of $(CCTG)_{24}$ and $(CCTG)_{25}$, which were also found to be unstable in small-pool PCR. The patient with the $(CCTG)_{24}$ allele had an undetermined asymmetric muscle disorder with pseudohypertrophy of one leg and mild atrophy of the other leg. The patient with the $(CCTG)_{25}$ allele was diagnosed with a mitochondrial DNA 3243A>G mutation causing MELAS syndrome. As these patients did not present any DM2-like phenotype, the short repeat expansions are unlikely to cause any symptoms.

5.3.3 Cohort of proximal myopathy or asymptomatic hyperCKemia

DM2 mutation was analyzed also from the cohort of idiopathic proximal myopathy or asymptomatic hyperCKemia patients consisting of 93 Italian DNA samples. In this cohort one DM2 mutation was identified. This patient showed waddling gait, proximal weakness and Gower's sign at the age of 49 years, whereas CK levels were normal. EMG showed findings consistent with a myopathic pattern without myotonic discharges. As the only sign of DM2 disease in this patient was proximal weakness, it is of interest that this patient proved to have DM2 mutation without other symptoms indicative of DM2. This suggests that DM2 can easily be missed in a neuromuscular diagnostic setting due to incomplete or uncharacteristic phenotypic presentation.

5.3.4 Frequencies of DM2 and DM1 mutations

In the cohorts of 4,510 healthy population samples and 990 NMD patients and their healthy relatives we identified three DM2 mutations and two DM1 mutations. Together these five DM mutations indicate a frequency of 1 DM mutation in 1,100 individuals in the population. Compared to the previous estimate of DM disease prevalence, 1 in 8,000, our finding for DM mutation frequency is significantly higher (p-value = 0.0007). Even when only the DM2 mutation frequency is compared to the previous 1 in 8,000 prevalence estimate, the three mutations in 5,500 samples is still significantly higher (p-value = 0.0326).

The large population cohort consisted of individuals of 18-65 years of age, of whom 45.5 % were male and 54.5 % were female. The sex of the individuals does not have an effect on the results, since the studied mutations (DM1 and DM2) cause a hereditary disease not related to sex. The age is also insignificant for the studied genetic diseases. Whether the carriers of a DM mutation found in the population cohort have or have not the disease symptoms at present is irrelevant, since they will at some point of life develop a disease. The sex and age of individuals in the NMD patient cohort were not analyzed since the frequency of the mutations studied is neither sex nor age dependent.

The finding of three DM2 mutations in a cohort of 5,496 samples, comprising a population cohort of 4,508 samples and neuromuscular disease cohort of 988 samples, suggests a mutation frequency of 1/1,830. This mutation frequency means some 2,700 DM2 mutation carriers in Finland. Because of the single European founder haplotype, the mutation frequency in Finland is expected to largely represent the situation also in other European countries. However, specific historical population bottlenecks and genetic drift may cause somewhat skewed frequencies in different sub-populations, such as the Finnish population.

All mutation carriers, regardless of the expansion size, are expected to develop DM2 symptoms, but because the symptoms begin in adulthood and even as late as after the age of 70, the actual number of DM2 patients is far less than the number of mutation carriers. Nevertheless the mutation frequency of 1/1,830 suggests that the number of DM2 patients is much higher than previously estimated and that many DM2 patients lack a correct diagnosis for their symptoms. Because of the mild phenotype of some DM2 patients, disease-related symptoms may go unnoticed by

the clinician or even by the patient. As a previous study reported, DM2 disease may be easily misdiagnosed as fibromyalgia (Auvinen et al., 2008). The variability of DM2 disease phenotypes is wide and the phenotype may extend beyond the currently known variation. The mutation frequency determined in this study suggests a need for increased consideration of DM2 and molecular diagnostic testing with a rather low phenotypic threshold.

5.4 Proximal myalgic myopathy caused by a short repeat expansion in *CNBP* (IV)

DM2 disease-associated intronic $(CCTG)_n$ repeats are reported to contain from 75 to 11,000 repeats. While some of the shorter repeat alleles may be mosaic for larger repeats, the number of smallest disease-associated repeats remains unknown (Dalton et al., 1993). Premutation alleles are estimated to contain 22-33 uninterrupted repeats (Bachinski 2009) and the upper normal limit of repeats is estimated to be 30 (Todd 2010). In this study we characterized a family with dominant proximal myalgic myopathy using several genetic and protein-based methods.

5.4.1 Disease phenotype and histopathology

The symptoms in the index patient II-1 (in Figure 8) consisted of myalgia, muscle weakness and stiffness, but without clinical or EMG myotonia. When examined at the age of 62 years, no cataracts or other multisystemic features of DM2 disease were observed. Laboratory tests were normal except for slightly elevated serum creatine kinase (CK) levels of 300-400 IU/L that were increased by statins to 700 IU/L (upper normal limit 200 IU/L). Two brothers of the index patient were also symptomatic and clinically their main complaint was myalgic cramps. Patient II-3 was clinically examined while patient II-7 was not available for further studies.

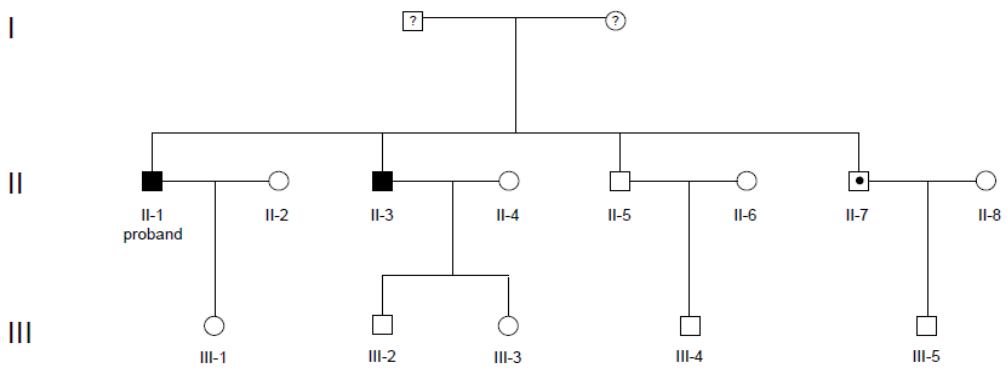

Figure 8. Pedigree of the short $(CCTG)_{DM2}$ expansion family. Affected patients are depicted as black boxes. Patient II-7 is marked with a black dot, because he is a carrier of the mutation, but has not been clinically examined.

Magnetic resonance imaging (MRI) of lower limb muscles of patients II-1 and II-3 showed mild fatty degenerative changes of thigh and calf muscles. Histopathology findings of both patients II-1 and II-3 were evident and characteristic of DM2 with highly atrophic subpopulation of type 2 fibers, nuclear clump fibers and increased number of internal nuclei.

5.4.2 Diagnostics and pathogenesis

The index patient (II-1) was referred to genetic diagnostics of DM2 disease because of the DM2-like features in the muscle biopsy. PCR over the repeat region showed just one normal-sized allele. Mutation analysis with chromogenic *in situ* hybridization (CISH), which is designed to detect the repeat expansions in DNA (sense probe) and accumulated RNA transcripts (antisense probe), was negative. The pattern in RP-PCR, however, was positive but with repeat peaks declining rapidly (Figure 9). This kind of pattern is consistent with a short repeat expansion. The same pattern was observed also in patients II-3 and II-7 and also from the DNA obtained from the index patient's muscle sample. The short repeat result was confirmed by Southern blotting. The exact repeat numbers and the lack of interruptions for the index patient and his brother (II-3) were revealed by sequencing the peripheral blood leukocyte DNA. The structure of the repeat region in the index patient was $(TG)_{19}(TCTG)_7(CCTG)_{55}$ and the affected brother (II-3) had

a similar repeat structure $(TG)_{19}(TCTG)_7(CCTG)_{61}$. The repeats were found to be unstable by SP-PCR. These short repeats resemble the larger expansions causing DM2 as they are also unstable and contain no interruptions.

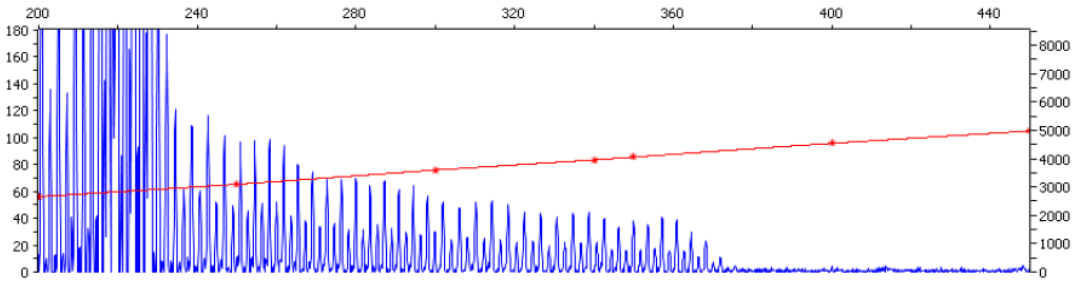

Figure 9. The pattern of a short repeat expansion in RP-PCR. The pattern of peaks starts off similar to a typical DM2 positive pattern (see Figure 7), but the peaks decline abruptly.

In order to study the pathomechanism of this short expansion associated disease, fluorescent *in situ* hybridization (FISH) was performed on the myoblast and fibroblast cells of the index patient. In accordance with the negative CISH result, nuclear RNA foci were absent also in FISH. The repeat seems to be too short to form the aggregates of mutant RNA, which are characteristic of DM2. The shortest reported expansion in a DM2 patient with evidence of nuclear foci and co-localization of MBNL proteins is $(CCTG)_{100}$ (Lucchiari et al., 2008). The clinical phenotype in this patient was within the typical range of DM2, leaving open the question whether the patient also carried larger expansions together with the $(CCTG)_{100}$ allele. Because the ribonuclear inclusions are associated with splicing defects of several genes, their absence would suggest normal splicing and no sequential clinical symptoms. This seems to apply to the short repeat disease. Splicing of several genes misspliced in DM2 (*CLCN1*, *INSR*, *SERCA1*, *RYR1*, *TNNT3* and *TTN*) (Mankodi et al., 2002; Salvatori et al., 2009; Vihola et al., 2010) was normal in patient II-3. Accordingly, none of the affected brothers had myotonia, cataracts, insulin resistance or cardiac defects, suggesting that the molecular pathomechanism is different from the toxic RNA mechanism in DM2.

At the mRNA and protein levels, the expression of *CNBP* has been reported to be significantly reduced in DM2 patients compared to DM1 and normal control individuals (Raheem et al., 2010b). On RP-PCR from cDNA isolated from the muscle sample of patient II-3, a pattern typical of a repeat expansion was observed, suggesting that the mutant allele is transcribed to pre-mRNA as is the case in DM2. However, using the SNP rs1871922 to determine allele-specific pre-mRNA transcript levels, both normal and mutant alleles appeared to be expressed at equal levels, while in DM2 the pre-mRNA from the mutant allele is over-represented. Furthermore, in contrast to DM2, the expression level of CNBP protein in the proband's skeletal muscle tissue was normal. Taken together, the findings of normal CNBP protein levels in muscle and equal representation of both alleles of SNP rs1871922 in pre-mRNA suggest that there is no pre-mRNA processing defect of this short repeat expansion allele. Apparently, the short expansion is not large enough to cause secondary effects on CNBP protein expression by abnormal pre-mRNA processing.

5.4.3 Origin of the short expansion mutation

The flanking haplotype, constructed using six microsatellite markers and one SNP, is completely different from the extended haplotype commonly found in Finnish DM2 patients (Bachinski et al., 2003) (Figure 10). However, the SNP rs1871922 C allele, which is in complete linkage disequilibrium (LD) with the DM2 repeat expansion mutation, was observed to segregate with the short expansion haplotype. These findings either suggest that the mutation derives from an older European haplotype, from which the common extended Finnish DM2 haplotype has been generated, or the C allele as the most frequent allele in the population is a co-incidence and the mutation has originated as an independent event. Studies on DM2 haplotype have suggested that all genetically confirmed DM2 patients of European descent arose from a single ancestral origin (Bachinski et al., 2003; Liquori et al., 2003). In addition, one Japanese DM2 patient with a haplotype different from that found in Caucasian DM2 patients has been reported (Saito et al., 2008). Because most of the microsatellite markers available are relatively far away from the

mutation locus, the prediction of short expansion mutation origin is challenging and definitive conclusions cannot be made based on these results.

Marker	Finnish DM2	Short expansion
D3S3606	170	172
D3S3607	160	164
571C11_AG1	216	221
221E20-GT1	178	184
814L21-GT1	150	148
DM2	exp	short exp
rs1871922	C	C
436B3-AG1	253	249

Figure 10. Haplotypes associated with DM2 disease in Finnish families (yellow) and with the new proximal myalgic myopathy in the studied short (CCTG) repeat expansion family (orange). These allele sizes are obtained in Tampere and show differences compared to allele sizes obtained in Houston.

5.4.4 Name of the new disease: proximal myalgic myopathy (PMM)

We have identified a new disease phenotype, proximal myalgic myopathy (PMM), which is associated with short, unstable $(CCTG)_{55\text{-}61}$ alleles at the DM2 locus. Our finding that expanded $(CCTG)_n$ alleles with as few as 55-61 repeats manifested phenotypically suggests that DM2 alleles in the proto-mutation range may be disease-causing, although the underlying pathomechanism(s) appear to be different compared to typical DM2 disease. Confirmation of this phenotype as a new disease entity requires identification and characterization of additional DM2 proto-mutation carriers, and also warrants clinical re-examination of seemingly unaffected individuals with small expansion alleles, not fulfilling diagnostic criteria for DM2 due to the lack of myotonia and multisystemic symptoms.

5.5 Applicability of the principal methods used

In studies II-IV the RP-PCR was the principal method and was used to analyze the presence of DM2 or DM1 mutation. RP-PCR is a very sensitive method, detecting

almost 100 % of DM2 patients (Tampere Neuromuscular Research Unit, unpublished data) compared to Southern blot analysis, which has been reported to detect only approximately 80 % of DM2 cases (Day et al., 2003). In addition to its sensitivity, the advantages of RP-PCR include the detection of short repeat expansions, and the very low amount of DNA needed for the analysis. As a drawback, the size of the repeat expansion is not definable. For DM2 disease the expansion size has no diagnostic relevance, since the mutation size does not correlate with the disease severity, but in DM1 the expansion size and disease severity, including age of onset, do correlate. In this thesis study the RP-PCR was utilized to detect the mutations for calculating the mutation frequency and not for diagnostic purposes, and thus knowledge of the repeat expansion size is not essential.

For the detection of *CLCN1* mutations in study I a qualitative TaqMan 5′ nuclease assay was selected. This assay is very fast, allowing a large number of samples to be analyzed in the same run, and only a small amount of DNA is needed. The method provides reliable results on detecting specific mutations or polymorphisms from genomic DNA (McGuigan and Ralston, 2002). Compared to detecting the studied mutations by sequencing, TaqMan 5′ nuclease assay is faster but detects only the mutation for which the assay is designed. By sequencing, all the mutations in the amplified region, even those uncharacterized, are found, but the method is quite laborious especially for large numbers of samples. In this study the target was to analyze only the most common known *CLCN1* mutations, and for this purpose the TaqMan 5′ nuclease assay was the most suitable method.

6. CONCLUSIONS AND FUTURE PROSPECTS

DM2 disease is the most common muscular dystrophy in adults. As our studies on the frequency of the disease-causing mutation have indicated, DM2 prevalence may be even higher than that of DM1, which is generally estimated to be the most prevalent muscular dystrophy affecting adults. This new data is important to clinicians for the understanding of the magnitude of the disease prevalence and its significance to the field of neuromuscular diseases. Although DM2 is relatively mild in symptoms and features, the right diagnosis will have substantial meaning for the patients. With a right diagnosis they can start to plan their life with their knowledge of the course of the disease and they will also be eligible to get social benefits. Obtaining the correct diagnosis as early as possible will save the patients from several unnecessary laboratory tests and doctors' appointments that are financed by society.

The very high frequency of the DM2 mutation is the main finding of this thesis. By studying a carefully selected cohort of fibromyalgia patients, 3.2 % of them were found to actually have DM2 disease as the cause of their symptoms. Based on the prevalence estimate of 2 % for fibromyalgia (Wolfe et al., 1995), the observed frequency of 3.2 % for DM2 mutation would correspond to a mutation frequency of 1 in 1,600 for DM2 disease. This is well in accord with the mutation frequency of 1 in 1,830 observed in the large population study with over 5,000 samples. The higher than previously expected frequency of DM2 mutation is also suggested by the study of *CLCN1* mutation carriers among DM2 patients compared to healthy controls and DM1 patients. As the frequency of *CLCN1* mutations in DM2 patients was found to be significantly higher than in controls or DM1 patients, the conclusion of some DM2 patients not getting a correct diagnosis can be drawn. The frequency of mutations in both genes should be independent, and the higher frequency of *CLCN1* mutations among DM2 patients suggests a bias in current clinical understanding of the phenotype of DM2 disease and diagnosis of patients in the severe end of the phenotypic continuum. The delay in receiving the correct diagnosis after onset of

the first symptoms has been reported to be over 14 years for DM2 patients (Udd et al., 2011). Clearly this is too long for the patients to wait. This observation implies that the current understanding of the phenotypic spectrum of DM2 needs to be widened.

Another major finding of this study is the highly variable disease phenotype, which as such is not a new observation, but the widening of the clinical spectrum to the fibromyalgia phenotype is novel data. In addition, the modifier effect of *CLCN1* mutations on the DM2 phenotype is an important observation even if the occurrence of these two gene mutations has also been reported in other studies. Our finding of *CLCN1* mutations aggravating the myotonia symptom in DM2 patients is in accord with other reports (Cardani et al., 2012; Sun et al., 2011).

The variable disease phenotype is further emphasized by the finding of a new disease associated with a short DM2 repeat expansion. The phenotype of this new disease is milder than typical DM2 disease and myotonia is not involved. Also the pathomechanism may be different with no apparent RNA gain-of-function mechanism since no ribonuclear foci were observed. The characterization of this new proximal myalgic myopathy associated with $(CCTG)_{55\text{-}61}$ repeats suggests that even very short repeats can cause a disease phenotype and some correlation between the disease severity and the size of the repeat can be observed. Yet, the mechanism by which this new disease is caused remains to be clarified. In addition, further studies are needed to define the origin of this short expansion mutation, since the genetic markers available at the moment cannot discriminate whether this is a new mutational event or originated from the common European DM2 founder haplotype.

The results obtained in this study are useful for clinicians, geneticists and other researchers studying DM diseases and their pathogenesis and eventually also their possible treatments. Apart from researchers and clinicians, patients will benefit from this study by hopefully receiving the correct diagnosis earlier. New disease phenotypes for DM2 are yet to be found and require careful clinical examination and alertness on the part of the clinicians to recognize possible DM2 mutation carriers.

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High frequency of co-segregating *CLCN1* mutations among myotonic dystrophy type 2 patients from Finland and Germany

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Running title: *CLCN1* mutations in DM2 patients

Abstract

Based on previous reports the frequency of co-segregating recessive chloride channel (*CLCN1*) mutations in families with myotonic dystrophy type 2 (DM2) was suspected to be increased. We have studied the frequency of *CLCN1* mutations in two separate patient and control cohorts from Germany and Finland, and for comparison in a German myotonic dystrophy type 1 (DM1) patient cohort. The frequency of heterozygous recessive chloride channel (*CLCN1*) mutations is disproportionally higher (5%) in currently diagnosed DM2 patients compared to 1.6% in the control population ($p = 0.037$), while the frequency in DM1 patients was the same as in the controls. Because the two genes segregate independently, the prevalence of *CLCN1* mutations in the total DM2 patient population is, by definition, the same as in the control population. Our findings are, however, not based on the total DM2 population but on the currently diagnosed DM2 patients and indicate a selection bias in molecular diagnostic referrals. DM2 patients with co-segregating *CLCN1* mutation have an increased likelihood to be referred for molecular diagnostic testing compared to DM2 patients without co-segregating *CLCN1* mutation.

Keywords: myotonic dystrophy, co-segregation, *CLCN1*, genetic modifier, phenotype variation

Introduction

Myotonic dystrophies are multisystemic neuromuscular disorders. To date, two genetic types have been identified with similar features including autosomal dominant inheritance, muscle weakness, myotonia and multi-organ involvement. Myotonic dystrophy type 1 (DM1, Steinert disease, OMIM #160900) is caused by an unstable (CTG)n repeat expansion in the 3´ untranslated region (UTR) of *dystrophia myotonica-protein kinase* (*DMPK*) on chromosome 19q13.3 [3, 9, 16]. The mutation underlying myotonic dystrophy type 2 (DM2, PROMM, OMIM #602668) is a (CCTG)n tetranucleotide repeat expansion in the first intron of the *zinc finger 9* (*ZNF9*) on chromosome 3q21 [1, 15].

A common molecular pathomechanism based on dominant toxic RNA gain-of-function effects of accumulated mutant pre-mRNA transcripts has been suggested for both DM1 and DM2 [14, 22, 23]. Large amounts of retained (CUG)n/(CUGG)n repeat complexes appear to affect normal cellular functioning by interfering with the proper pre-mRNA splicing of a number of downstream effector genes [11, 17], such as *skeletal muscle chloride channel* (*CLCN1*) [18], *insulin receptor* (*INSR*) [24] and *microtubule-associated protein tau* (*MAPT*) [5, 17, 19, 28]. Clinical features in DM2 are much more variable than in adult onset DM1 [26, 28]. In a single patient any of the core features of the disease, proximal muscle weakness, myotonia and cataracts, may be absent, and signs such as myotonia may even vary over time both when clinically assessed and when recorded by electromyography (EMG) [27]. Molecular modifiers or other mechanisms underlying this variability in phenotype and penetrance of different signs are currently not known. In particular, no correlation of the size of the repeat expansion with the clinical outcome has been shown in DM2 [6].

Several unrelated DM2 families have been reported with heterozygous recessive *CLCN1* mutations [13, 25, 27]. *CLCN1* maps to chromosome 7q35 and when mutated causes myotonia congenita (OMIM #255700 for recessive Becker disease, and #160800 for dominant Thomsen disease). A high prevalence of *CLCN1* mutations in DM2 families evaluated in a myotonia clinic has also been reported [20]. One immediate question from these observations is whether the co-segregation of *CLCN1* mutations with the DM2 mutation modifies the clinical outcome and thus affects the overall ascertainment likelihood of DM2 disease.

In order to study the occurrence of co-segregating recessive *CLCN1* mutations in DM2, we screened 200 mutation-confirmed DM2 patients (100 each from Finland and Germany) for the three most common recessive *CLCN1* mutations in the Finnish population (R894X, F413C and A531V, in the order of frequency). The exact prevalence of these mutations in the population is not well established. A prevalence of 7.3 per 100,000 for clinically determined congenital myotonia (myotonia congenita Becker) patients was reported in northern Finland 1998 [2], which would account for a carrier frequency of recessive *CLCN1* mutations in the population of approximately 2%, when considering the possibility of partially incomplete ascertainment. For a single recessive mutation, R894X, a carrier frequency of 0.87% was estimated in the Northern Scandinavian population [25]. For comparison, we also investigated the frequency of *CLCN1* mutations in 100 DM1 patients from Germany and 250 control samples.

Subjects and methods

The study was approved by the IRB of Tampere University Hospital and all patients gave their informed consent prior to their inclusion in the study. In total, we studied 200 DM2 patients with molecularly confirmed DM2 mutation, of which half were from Finland and half from Germany, for three *CLCN1* mutations (R894X, F413C and A531V). A total of 250 anonymous control samples were also analysed for the same mutations: 150 population control samples were from Finland, and 100 were from Germany. The 100 German patients with DM1 were analysed for *CLCN1* R894X mutation only, because in the German DM2 patients and in the control cohort none of the other *CLCN1* mutations were present. Mutations were analysed using the TaqMan Sequence Detection System (ABI Prism 7000, Applied Biosystems, Foster City, CA, USA) with fluorescent oligonucleotide probes for both mutation and normal sequences. The studied fragments were amplified by PCR using forward and reverse primers (primer and probe sequences are shown in Supplementary Table 1). One-sided Fisher´s Exact Test using the SPSS (version 15.0) statistical software package was performed to calculate statistical significance.

After obtaining the *CLCN1* genotypes for DM2 patients, a retrospective review of reported clinical findings in the DM2 patients with observed co-segregating *CLCN1* mutation was performed.

Results

Genetic analyses

In the cohort of 100 German DM2 patients, four patients were heterozygous carriers and one DM2 patient was homozygous for the *CLCN1* R894X mutation. The homozygous patient, therefore, was affected by two distinct genetic diseases − DM2 and Becker congenital myotonia. These results correspond to a frequency of 5% for DM2 patients showing co-segregation of the *CLCN1* R894X mutation. In the German control population, one R894X heterozygous carrier was identified, which suggests an overall *CLCN1* R894X mutation carrier frequency of 1% in the German population. None of the German DM2 patients or the controls had either the F413C or A531V mutations. In the German DM1 cohort of 100 patients, we found one heterozygous R894X mutation, a carrier frequency of 1% that is identical to that seen in the German control population. Thus, German DM2 patients had a frequency of *CLCN1* co-segregating mutations 5-times higher than both unaffected controls and DM1 patients from the same population. These results are summarized in Table 1a.

In the 100 Finnish DM2 patients, we found five patients segregating a heterozygous *CLCN1* mutation, which corresponds to a co-segregation prevalence of 5% that is equally high as that seen in the German DM2 population. Three patients had the R894X mutation and two had F413C. However, the frequency of recessive *CLCN1* mutation carriers was higher in the Finnish population compared to the German population. In the cohort of 150 Finnish controls, we found two individuals with heterozygous R894X and one individual with heterozygous F413C. For the R894X mutation, these results correspond to similar overall carrier frequency of 1.3% in the Finnish population compared to 1% in the German

population. The F413C mutation was found only in the Finnish population with a carrier frequency of 0.7%. The previously reported A531V mutation in the Finnish population was not observed in any of the combined 250 Finnish DM2 and control individuals analysed. Thus, similar to German DM2 patients, Finnish DM2 patients showed enrichment for co-segregating *CLCN1* mutations -- a 2.5-times higher frequency than unaffected controls from the same population (Table 1b). Table 1c shows the combined data for the two populations studied.

Retrospective review of clinical records

All German DM2 patients with co-segregating *CLCN1* mutation showed very prominent clinical and early-onset myotonia. Myotonia was the leading symptom at an unusually young age for DM2 patients. On EMG, myotonic discharges were constantly obtained in all muscles tested, which is different from average DM2 patients, where EMG myotonia may be highly variable or even absent [27]. The single DM2 patient with the homozygous *CLCN1* mutation had generalized muscle hypertrophy and profuse myotonia both clinically and on EMG, in line with a phenotype of congenital Becker myotonia.

Two of the five Finnish DM2 patients with co-segregating *CLCN1* mutations showed very prominent clinical myotonia, in one as the leading symptom at onset. These patients also had EMG myotonic discharges in all muscles tested. The remaining three patients showed no significant differences compared to the wide variability of clinical phenotypes in older DM2 patients without *CLCN1* mutations. All patients with co-segregating mutations had myotonia either clinically or on EMG, whereas a significant proportion of DM2 patients in general does not show myotonia [27, 28]. The clinical data are summarized in Table 2.

Statistical analysis

The combined German and Finnish DM2 patients (n = 200) and control cohorts (n = 250) provide a calculated statistically significant *p*-value of 0.037 for the disproportion in co-segregating *CLCN1* mutations.

Discussion

Based on previous reports of a few families and a pilot study [13, 20, 25, 27], the frequency of co-segregating recessive *CLCN1* mutations was suspected to be higher in currently diagnosed DM2 patients than in the population at large. Because the loci of these two genes, *ZNF9* and *CLCN1*, are unlinked (*ZNF9* maps to chromosome 3q21.3 and *CLCN1* maps to chromosome 7q35), the frequency of *CLCN1* mutations would be expected to be the same among DM2 patients as in the general population. However, our results in German DM2 patients showed that 5% were carriers of *CLCN1* R894X mutation, whereas the carrier frequency of this mutation in the controls was 1%. Importantly, the R894X mutation was not more frequent in DM1 patients than in controls, suggesting that the severe outcome in adult DM1 is caused by the DM1 mutation alone and that the effects of the co-segregation of *CLCN1* mutations are specific for DM2 patients. Interestingly one DM2 patient had a homozygous R894X mutation and had not, prior to DM2 diagnosis, been identified as a Becker myotonia patient. Considering the large number of different mutations and the frequency of *CLCN1* mutations in the population, screening of the same cohorts with a number of other known mutations would provide more detailed information.

In our Finnish patient cohort, *CLCN1* mutations were also found with the same high frequency of 5% in DM2 patients as in the German DM2 patients. However, the carrier frequency in the Finnish population at large may be somewhat higher than that in the German population. For the R894X mutation we found quite similar frequencies in Finnish and German populations (1.3% and 1%, respectively). Diverging results came for the F413C mutation that occurred in <1% of the Finnish population and not in the German population, suggesting that the total frequency of recessive *CLCN1* mutations may be around 2% in Finland. For the R894X mutation, we found a carrier frequency of 1.3% in Finland, compatible with the previously reported 0.87% in the Northern Scandinavian population [25]. Our findings support previously reported data on higher prevalence of myotonia congenita in Northern Scandinavia [21]. This study did not include rare mutations; therefore, the real frequency of myotonia congenita was likely underestimated. Comparing both Finnish and German DM2 patients to both sets of controls, the disproportion in frequency of recessive *CLCN1* mutations is statistically significant ($p = 0.037$). In the previously reported DM2 families with *CLCN1* mutations, the effect of co-segregation on the clinical outcome was unclear. In this study the number of DM2 patients co-segregating recessive *CLCN1* mutations was not high enough for statistical analysis of the specific clinical findings, but the generalized occurrence of EMG myotonia in every muscle tested in all patients is different when compared to the variability usually encountered in DM2 patients.

Our results are suggestive of an influence of co-segregating *CLCN1* mutations on the DM2 phenotype. Current diagnostic practice favors patients, in whom myotonia is present clinically and/or on EMG, to be referred for DM2 molecular testing. DM2 patients with co-segregating recessive *CLCN1* mutations are thus more likely to be ascertained and diagnosed with DM2 than patients without *CLCN1* mutations. The true frequency of *CLCN1* mutations

in the total DM2 population would be expected to be the same as in the general population. However, the disproportionately higher frequency we have observed among currently diagnosed DM2 patients is indicative of a selection bias in our current diagnostic process (a separate manuscript is in preparation on the clinical phenotype variability among the DM2 patients).

Homozygous and compound heterozygous mutations in the *CLCN1* gene cause autosomal recessive myotonia congenita [12]. Carriers are usually unaffected, but findings of subclinical EMG myotonia have been reported [7]. Mutations causing the autosomal dominant form of myotonia congenita [10] are very rare and were therefore not considered in this study, although the R894X mutation is known to occur, albeit rarely, in families with an apparently dominant inheritance pattern. However, the possibility of a co-segregating DM2 mutation was not evaluated in these families [8]. Both in DM1 and DM2, loss of chloride channel protein has been demonstrated. The underlying major cause is the abnormal splicing of *CLCN1* pre-mRNA, which leads to non-functional protein due to premature termination codons [4, 18].

The proportion of mis-spliced *CLCN1* mRNA in DM2 patients is very high and the amount of CLCN1 protein in the muscle cell membrane is reduced leading to decreased chloride conductance and electrical instability [4, 18]. It is possible that the amounts of mis-spliced CLCN1 channels are highly variable in different DM2 patients, even over longer time periods in the same patient, which may account for the observed variability of myotonic symptoms. In DM2 patients with heterozygote *CLCN1* mutation, abnormal splicing will affect both the normal and the mutant *CLCN1* allele, leading to even more loss of functional channel than in patients with either DM2 or *CLCN1* mutation alone. Whether the 5% DM2-*CLCN1* double

mutants have measurably less chloride channel protein than the 95% DM2 patients without *CLCN1* mutation remains to be investigated. In the single DM2 patient with homozygous *CLCN1* mutation, the loss of channel is determined by the *CLCN1* mutation, and the superimposed DM2 related splicing defect will most likely not decrease the chloride channel further. However, other DM2-related problems such as late proximal muscle weakness and other multi-organ abnormalities will probably change the patient's prognosis in late adulthood. The disproportionately higher frequency of co-segregating *CLCN1* mutations in the currently diagnosed DM2 patients compared to the control population suggests that a large number of DM2 patients without *CLCN1* mutation may remain undiagnosed, possibly due to less apparent symptoms.

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Table 1a. The number of *CLCN1* mutations found in the German cohorts.

CLCN1 mutation	DM2 patients (n = 100)	DM1 patients (n = 100)	Controls (n = 100)
R894X			
heterozygotes	4	1	1
homozygotes	1	0	0
F413C	0	na	0
A531V	0	na	0
Total	5	1	1
Total (%)	**5%**	**1%**	**1%**

na: not assessed

Table 1b. The number of *CLCN1* mutations found in the Finnish cohorts. All the mutations are heterozygous.

CLCN1 mutation	DM2 patients (n = 100)	Controls (n = 150)
R894X	3	2
F413C	2	1
A531V	0	0
Total	5	3
Total (%)	**5%**	**2%**

Table 1c. Occurrence of *CLCN1* mutations in the combined cohorts

CLCN1 mutations	DM2 patients (n = 200)	DM1 patients (n = 100)	Controls (n = 250)
Total (%)	**5%**	**1%**	**1.6%**

Table 2. Clinical findings in DM2 and DM1 patients with co-segregating *CLCN1* mutations.

German Patients	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Genotype:						
DM	DM2	DM2	DM2	DM2	DM2	**DM1**
CLCN1	*R894X/wt*	*R894X/wt*	*R894X/wt*	*R894X/wt*	*R894X/R894X*	*R894X/wt*
Gender	male	female	male	male	male	female
Age at evaluation	26	51	37	45	54	37
Age at onset	12	10	35	14	32	18
Symptom at onset	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia
Years of weakness	-	-	2	3	-	4
Clinical myotonia	+	+	+	++	+++	++
EMG myotonia	++	++	++	++	+++	++
Cataracts	-	-	-	-	na	na
Myalgia	++	++	++	+	++	+
Serum CK value	300-500	300-400	150-221	376-643	390	265-470
Other	proximal muscle hypertrophy	gallstones, myocardial infarction, WML[1] on brain MRI		hypertrophy of calves	muscle hypertrophy	

Finnish Patients	**F1**	**F2**	**F3**	**F4**	**F5**
Genotype:					
DM	DM2	DM2	DM2	DM2	DM2
CLCN1	*R894X/wt*	*F413C/wt*	*R894X/wt*	*R894X/wt*	*F413C/wt*
Gender	male	female	male	female	male
Age at evaluation	48	58	52	50	65
Age at onset	30	25	51	48	41
Symptom at onset	pains and proximal weakness of upper and lower limbs	proximal weakness and stiffness of lower limbs	radicular pains of upper arm after trauma	stiffness of lower limbs	thoracic pains
Years of weakness	18	33	-	-	-
Clinical myotonia	+	+	+	-	-
EMG myotonia	+	++	++	+	+
Cataracts	-	-	na	na	na
Myalgia	++	+	+	+	++
Serum CK value	709-1055	466	710	289	244
Other	hypertrophy of calves	-	-	-	radicular pains and lumbalgia without clear radiculopathy

+: finding/sign present, ++: finding/sign frequent, +++: finding/sign profuse.
For EMG myotonia: +: myotonia present but not in all muscles, ++: present in all muscles tested, +++: profuse in all muscles.
-: finding/sign not present, na: not assessed.
[1]WML: white matter lesions.

Supplementary Table 1. Primers and probes (5´ to 3´) for TaqMan analysis of *CLCN1* mutations.

CLCN1	**R894X**	**F413C**	**A531V**
Forward primer	tcttcagctacagaaggccattg	ttgtcattgcctcattcacctt	ccctaacctacaggcgtattcct
Reverse primer	cctcaggcaggttccagttc	gtgctgtttcctctttaccatgtg	tggagactgtgtgggaaacg
Probe 1	6-FAM-acgacttcaactcgaaa	6-FAM-tgggtcaattcatggc	6-FAM-cagcagcgctgac
Probe 2	VIC-cttcaacttgaaagagtac	VIC-tcaatgcatggctgga	VIC-agcagtgctgactgg

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Myotonic Dystrophy Type 2 Found in Two of Sixty-Three Persons Diagnosed as Having Fibromyalgia

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Because of its high prevalence, fibromyalgia (FM) is a major general health issue. Myotonic dystrophy type 2 (DM2) is a recently described autosomal-dominant multisystem disorder. Besides variable proximal muscle weakness, myotonia, and precocious cataracts, muscle pain and stiffness are prominent presenting features of DM2. After noting that several of our mutation-positive DM2 patients had a previous diagnosis of FM, suggesting that DM2 may be misdiagnosed as FM, we invited 90 randomly selected patients diagnosed as having FM to undergo genetic testing for DM2. Of the 63 patients who agreed to participate, 2 (3.2%) tested positive for the DM2 mutation. Their cases are described herein. DM2 was not found in any of 200 asymptomatic controls. We therefore suggest that the presence of DM2 should be investigated in a large sample of subjects diagnosed as having FM, and clinicians should be aware of overlap in the clinical presentation of these 2 distinct disorders.

Fibromyalgia (FM) is a common chronic pain syndrome characterized by diffuse musculoskeletal aches, stiffness, and exaggerated tenderness. Classification criteria were elaborated by the American College of Rheumatology in 1990 (1). The pathogenesis of FM is incompletely understood. Most investigators favor the view that the major cause of FM is dysfunctional pain processing in the central nervous system (CNS) (2). Muscle histologic data are mixed, but predominantly demonstrate nonspecific changes (3). Core symptoms are chronic widespread pain, generalized stiffness, poor-quality sleep, and fatigue. Chronic widespread pain is defined as pain involving at least 3 quadrants of the body and the axial skeleton, and tenderness of at least 11 of 18 specified tender points (1). Symptoms wax and wane in intensity over days and weeks, and flare with increased exertion, lack of sleep, and exposure to temperature changes and other changes in weather conditions, as well as to psychological stressors. Persistent widespread muscle pain is quite common in the general population, with a prevalence of ~10%, while the prevalence of FM has been estimated to be as high as 2–4% (0.5% in men and 3.4% in women) (4).

The myotonic dystrophies are the most common muscular dystrophies in adults. They are multisystem disorders with autosomal-dominant inheritance, progressive muscle weakness and atrophy, myotonia, precocious cataracts, cardiac conduction abnormalities, endocrinologic and gastrointestinal involvement, and CNS symptoms (5). Myotonic dystrophy type 1 (DM1; Steinert disease) was first described more than 100 years ago (5). In 1992 the genetic cause of DM1 was clarified as an expansion mutation of an unstable trinucleotide (CTG) n repeat in the 3′-untranslated region of *DMPK* (the gene for myotonic dystrophy protein kinase) in chromosome 19q (6). Once genetic testing for DM1 became available, it was found that some patients with similar features did not have the DM1 mutation (7–9). In the vast majority of these DM1 mutation–negative families, the clinical features proved to be linked to

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Table 1. Clinical manifestations of myotonic dystrophy type 2

Core features	
Clinical myotonia	Present in fewer than half
Myotonia on electromyography	Absent or variable in many patients
Muscle weakness	Onset may occur after age 60–70 years
Cataracts	Present in a minority of patients
Localization of muscle weakness	
Facial weakness, jaw muscles	Usually absent
Bulbar weakness, dysphagia	Absent
Respiratory muscles	Exceptional cases
Distal limb muscle weakness	Flexor digitorum profundus on testing, only in some patients
Proximal limb muscle weakness	The main disability in most patients
Sternocleidomastoid weakness	Prominent in few patients
Muscle symptoms	
Myalgic pain	The most disabling symptom in many patients
Muscle strength variations	Can be considerable
Visible muscle atrophy	Usually absent
Calf hypertrophy	Present in half or more
Muscle biopsy findings	
Fiber atrophy	Highly atrophic type 2 fibers
Nuclear clump fibers	Scattered early, before weakness is clinically evident
Cardiac arrhythmias	From absent to severe
Brain	
Tremors	Prominent in many patients
Behavioral change	Not apparent
Hypersomnia	Infrequent
Cognitive decline	Not apparent
Other features	
Anticipation	Exceptional
Childhood-onset central nervous system involvement	Absent
Congenital form	Absent

another locus in chromosome 3q21, which has been designated myotonic dystrophy type 2 (DM2). DM2 is caused by a large (CCTG) n repeat expansion mutation in intron 1 of the zinc-finger protein 9 gene (*ZNF9*) (10).

The clinical picture of DM2 is generally milder and more variable than that of adult-onset DM1 (Table 1). Prominent symptoms in DM2 are pain and muscle stiffness (11–13). Muscle pain in DM2 can be very disabling, especially in individuals who perform physically demanding work.

We noted that a considerable number of our patients in the Jyväskylä Central Hospital region who had DM2 as documented by genetic testing had a previous diagnosis of FM (5 of 36 [14%]) (Auvinen S: unpublished observations). Therefore, we undertook a study of the occurrence of the DM2 mutation in patients with previously diagnosed FM.

METHODS AND RESULTS

Patients. The study was approved by the Institutional Review Board of Jyväskylä Central Hospital and carried out according to the Helsinki Declaration. The Rheumatology Center in the Jyväskylä Central Hospital region has long been involved in FM research and has a database of patients with a standardized FM diagnosis (14). Ninety patients were randomly selected from the database to participate in the study. Of the 90 patients invited, 63 consented to provide blood samples for genetic testing for the DM2 mutation. Six of the 90 patients who were invited were men (6.7%) and 84 were women (93.3%), and the mean age was 45.7 years. Proportions were similar among the group of 63 patients who were studied (4 men [6.3%], 59 women [93.7%]; mean age 49.3 years).

As a disease control cohort, 70 patients with long QT syndrome caused by one single Finnish founder mutation, G589D in the *KCNQ1*gene (15), were tested for the DM2 mutation as a possible modifier gene because of the large variation of the phenotype despite identical channel mutation. A control population of 200 healthy individuals (400 chromosomes) was also analyzed for the DM2 mutation.

Genetic analyses. DNA extracted from peripheral blood leukocytes was used for diagnostic polymerase chain reaction (PCR) (16,17). First, PCR across the DM2 (CCTG) n repeat in the first intron of *ZNF9* was used to identify individuals with 2 heterozygous normal alleles. Samples with only 1 allele were further analyzed by repeat-primed PCR specific for the DM2 mutation, to distinguish mutated expansion alleles from normal homozygous alleles (17). The results from both screening and repeat-primed PCR were obtained using fluorescent fragment analysis with capillary electrophoresis (ABI 3130) and analyzed with GeneMapper software (Applied Biosystems, Foster City, CA).

Results. In the group of 63 patients diagnosed as having FM, we identified 2 patients (3.2%) with the DM2 mutation (see below). No DM2 mutations were identified in the cohort of 70 patients with long QT syndrome or in the 200 healthy controls.

After the DM2 genetic diagnosis was obtained, patients with negative findings were informed about the test result. The 2 patients who tested positive for the DM2 mutation were clinically reexamined, and their previous records were reevaluated. Both patients were also referred for genetic counseling.

CASE REPORTS

Patient 1. Patient 1, a 48-year-old woman, had been diagnosed as having FM at the age of 45 years. She reported having experienced some muscle stiffness since her teens and had difficulty with physical exercise, but there was no proven relationship of these symptoms to the current illness. Since the age of 38, she had had muscle pain, provoked by exercise. Besides myalgia and stiffness, she had difficulty bending her knees, climbing stairs, and working with her arms elevated. Even daily housekeeping activities provoked widespread muscle

Table 2. Clinical findings in the 2 fibromyalgia-diagnosed patients who were found to have myotonic dystrophy type 2

	Patient 1	Patient 2
Age at fibromyalgia diagnosis, years	45	39
Age at evaluation, years	48	45
Age at symptom onset, years	38	37
Symptoms at onset	Muscle pain and muscle stiffness	Muscle pain and muscle stiffness
Years of subjective weakness	5	7
Clinical myotonia	Absent	Absent
Myotonia on electromyography	Present and widespread	Absent
Cataracts	Absent	Absent
Myalgia	Present and widespread	Present and widespread
Serum creatine kinase, units/liter (normal 35–210)	411	60
Serum gamma glutamyl transferase, units/liter (normal 10–75)	76	Not tested
Other	Cholecystectomy at age 39 years	Sensorineural hearing loss, excessive sweating, tremor of hands

pain and prevented gainful employment. On reexamination, after the positive DM2 result, very mild ptosis and slightly slow opening of the fist without any activation or percussion myotonia were observed. Her walking was stiff, but no increased muscle tone or weakness was detected upon testing of individual muscles. There was neither proximal muscle atrophy nor calf hypertrophy. Without knowledge of her positive genotype for the DM2 mutation, her clinical signs would easily have been overlooked. Her mother had developed muscle stiffness and walking difficulties at a later age, and many of her 8 siblings were reported to have similar muscle symptoms.

Patient 2. Patient 2, a 45-year-old woman, had been diagnosed as having FM at the age of 39 years, and sensorineural hearing loss was observed 2 years later. She reported having had muscle stiffness and difficulty with physical exercise since she was a teenager, but these early symptoms cannot be proven to be related to the current illness. Chronic lumbago had started at the age of 20 years and worsened during her pregnancies. Since the age of 37, she had had diffuse muscle pain and could not work with her arms elevated. Climbing stairs was difficult, and she reported having hand tremors and excessive sweating. She had falls, pains in her limbs, fatigue, and palpitations. Her working capacity had been extensively evaluated 1 year prior to this study; this included a full neurologic examination, without abnormal findings recorded. On reexamination after the positive DM2 result, her walking was found to be stiff, but results of manual strength testing of individual muscles were normal. Her calf muscles were large, and she had no clinical myotonia. When she was asked about her family, it was discovered, surprisingly, that she was the sister of patient 1.

The clinical characteristics of the 2 patients with DM2 are summarized in Table 2.

DISCUSSION

In accordance with our initial hypothesis, we identified patients with DM2 from among a cohort of patients in whom FM had been diagnosed at a specialized tertiary referral center. Indeed, the features of myalgia and muscle stiffness in DM2 as described in current diagnostic criteria (13) are in many respects indistinguishable from those recorded in FM. FM is frequent in the general population, with the prevalence of diagnosed cases as high as 1/25–1/50 in many countries (4). Thus, it is of obvious interest to determine whether there are specific, genetically determined disease entities that go undiagnosed among patients who have been diagnosed as having FM. This is of particular importance with regard to progressive and genetic disorders such as DM2, in which the prognosis, and clinical and social consequences for patients and their families, differ from those associated with FM.

In this pilot study, we found that 2 of 63 patients diagnosed as having FM (3.2%) had DM2. Even if the frequency of undiagnosed DM2 among patients with FM were 10-fold lower than that recorded in the present analysis, the total number of known cases of DM2 would be increased by tens of thousands, which would prompt a profound revision of the current estimates of DM2 prevalence (11,12). There are no exact data available on the prevalence of the DM2 mutation in Finland or elsewhere, but even a 10-fold higher regional prevalence than the current estimate that the frequency is similar to

that of DM1 (12/100,000) would not explain this finding of a 3.2% frequency of DM2 among patients diagnosed as having FM.

Are there clinical clues to identify DM2 among patients who have been diagnosed as having FM? Widely fluctuating muscle pain and stiffness, which may be provoked by exercise and other stressors, occur in both disorders. Clinical findings may be minor in both disorders, e.g., muscle tissue changes are not seen in FM, and muscle atrophy is not found in DM2. However, both of our patients who were found to have DM2 presented with several extramuscular symptoms, including fatigue, irritable bowel, and numbness, that are typical in FM, and both clearly fulfilled classification criteria for FM even at reevaluation after DM2 had been diagnosed.

One symptom suggestive of DM2, if present, is myotonia. However, half or more of DM2 patients exhibit no clinical myotonia, and a considerable proportion lack myotonia even on electromyography (EMG) (13). Proximal weakness of the lower limbs is another clue pointing to DM2. Furthermore, if present at a relatively early age, cataracts in the subject or family members, as well as elevated serum creatine kinase (CK) and/or gamma glutamyl transferase levels, are certainly suggestive of DM2. Since not all patients with DM2 exhibit myotonia on EMG, EMG is not a perfect screening method. Even when myotonia is present, multiple muscle insertions may be needed in order for it to become apparent on EMG, which is a fairly invasive and unpleasant procedure. The early onset of muscle stiffness reported by both patients with DM2 described herein is unusual. Onset of muscle stiffness in adulthood is more common, and in women, exacerbation during pregnancy is frequently reported.

Taken together, our findings indicate that DM2 appears to be a true candidate in the differential diagnostic of FM. Molecular genetic DNA testing is the gold standard to distinguish DM2 from FM. Serum CK elevation is nonspecific and is sensitive to even moderate physical exercise or minor muscle trauma. Moreover, in many patients with DM2, serum CK levels remain within the normal reference range. Nevertheless, in adult patients with myalgia and elevated CK levels, referral for genetic testing for DM2 should be considered. We suggest that the frequency of DM2 should be investigated in a larger sample of subjects who have been diagnosed as having FM, and clinicians should be aware of overlap in the clinical presentation of these 2 distinct disorders.

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AUTHOR CONTRIBUTIONS

Dr. Auvinen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Auvinen, Hannonen, Udd.

Acquisition of data. Auvinen, Suominen, Hannonen, Bachinski.

Analysis and interpretation of data. Auvinen, Suominen, Hannonen, Bachinski, Krahe, Udd.

Manuscript preparation. Auvinen, Suominen, Hannonen, Krahe, Udd.

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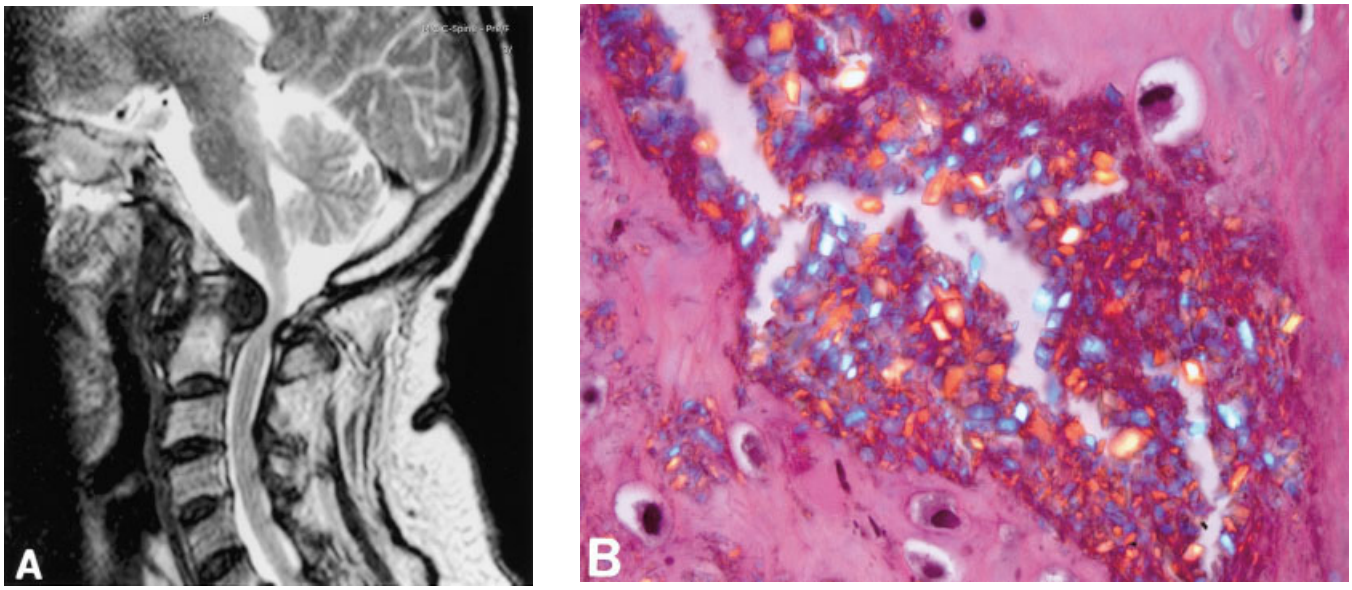
Clinical Images: Compressive cervical myelopathy due to calcium pyrophosphate



The patient, a 64-year-old man, presented with a 4-month history of neck pain, with weakness and paresthesia of both legs and the left arm. Hoffmann's sign was present on the left. He reported having no prior arthritis or joint pain. Radiography of the spine showed calcinosis surrounding the odontoid process. Magnetic resonance imaging revealed a 15-mm hypointense soft tissue mass above the odontoid, causing foramen magnum stenosis and cord compression at the cervico–medullary junction (**A**). An occipital-to-C2 fusion was performed, followed by stereotactic, transoral decompression of the spinal cord using an operating microscope. A soft tissue mass suggestive of pannus was removed, together with the odontoid process. On polarized light microscopy, the mass was found to contain multinodular crystal deposits embedded in fibrocartilaginous tissue, with little inflammation. The crystals were predominantly rhomboid, with positive birefringence, consistent with calcium pyrophosphate (**B**). The patient recovered uneventfully and was discharged home.

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Population frequency of myotonic dystrophy: Higher than expected frequency of myotonic dystrophy type 2 (DM2) mutation in Finland

Running title: Population frequency of myotonic dystrophy

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SUMMARY

Myotonic dystrophy is the most common adult-onset muscular dystrophy with an estimated prevalence of 1/8,000. There are two genetically distinct types, DM1 and DM2. DM2 is generally milder with more phenotypic variability than classic DM1. Our previous data on co-segregation of heterozygous recessive *CLCN1* mutations in DM2 patients indicated a higher than expected DM2 prevalence. The aim of this study was to determine the DM2 and DM1 frequency in the general population, and to explore if the DM2 mutation functions as a modifier in other neuromuscular diseases, to account for unexplained phenotypic variability. We genotyped 5,535 Finnish individuals: 4,532 normal blood donors, 606 patients with various non-myotonic NMD, 221 tibial muscular dystrophy patients and their 176 healthy relatives for the DM2 and DM1 mutations. We also genotyped an Italian idiopathic non-myotonic proximal myopathy cohort (n=93) for the DM2 mutation. In 5,496 samples analyzed for DM2, we found three DM2 mutations and two premutations. In 5,511 samples analyzed for DM1, we found two DM1 mutations and two premutations. In the Italian cohort we identified one patient with a DM2 mutation. We conclude that the DM2 mutation frequency is significantly higher in the general population (1/1,830; *p*-value=0.0326) than previously estimated. The identification of DM2 mutations in NMD patients with clinical phenotypes not previously associated with DM2 is of particular interest and in accord with the high overall prevalence. Based on our results, DM2 appears more frequent than DM1, with most DM2 patients currently undiagnosed with symptoms frequently occurring in the elderly population.

Keywords: myotonic dystrophy, mutation frequency, prevalence, population

INTRODUCTION

Myotonic dystrophy is an autosomal dominant multi-systemic neuromuscular disorder. Two genetically distinct diseases with clinical similarities but also distinct differences have been identified. Myotonic dystrophy type 1 (DM1, Steinert´s disease, MIM #160900) is caused by a $(CTG)_n$ expansion mutation in the 3´ UTR of *DMPK* (*dystrophia myotonica protein kinase*) in chromosome 19q13.3 [1-3]; myotonic dystrophy type 2 (DM2, MIM #602668) is caused by a $(CCTG)_n$ expansion mutation in the first intron of *ZNF9* (*zinc finger protein 9*) in chromosome 3q21 [4, 5]. Similar clinical features of DM1 and DM2 include myotonia and limb muscle weakness, pronounced distal in DM1 and proximal in DM2, and multi-organ involvement including cataracts, insulin resistance, elevated liver enzymes, male hypogonadism and cardiac conduction defects. Muscle atrophy, facial weakness, ptosis and frontal baldness are very prevalent in DM1, while muscle pain and hypertrophy of calf muscles are more characteristic for DM2. Serum CK levels are usually elevated. Overall, clinical manifestations in DM2 appear to be more variable and generally milder than those in classic adult-onset DM1 (for review see references [6] and [7]).

In DM1 but not in DM2, the length of the expansion mutation generally correlates with the severity of the disease. Normal individuals have 5-37 $(CTG)_n$ repeats at the *DMPK* locus and the number of repeats in DM1 patients varies from mild late-onset cases with ≥50 repeats to the most severe congenital form of DM1 containing up to 4,000 $(CTG)_n$ repeats [1]. The four main categories of DM1 based on clinical outcome are (1) congenital, (2) childhood-onset, (3) classic adult-onset, and (4) late-onset/oligo-symptomatic. The distinction is not absolute but rather a continuum generally correlating with increasing length of the $(CTG)_n$ expansion, which tends to increase through successive generations causing genetic anticipation.

The DM2 expansion mutation in intron 1 of *ZNF9* consists of several normally polymorphic elements first described as $(TG)_n(TCTG)_n(CCTG)_n$, but variations with short intervening cryptic repeats (TCTG and/or GCTG) disrupting the $(CCTG)_n$ tract have also been reported [4, 5, 8, 9]. It has been proposed that only uninterrupted $(CCTG)_n$ repeats are associated with the DM2 phenotype [5, 8, 9]. The range of $(CCTG)_n$ repeats in patients varies between 55 and 11,000, but, unlike DM1, the threshold defining the difference between normal and disease-causing repeats has not yet been firmly established [5, 9]. For DM2 correlation between repeat length and disease severity or age-of-onset has not been demonstrated. Phenotypic variability and the large size of most mutant repeats make this determination more complicated.

Before the identification of their respective genetic mutations, the combined prevalence of DM1 and DM2 has generally been estimated at 1 in 8,000 (12.5 per 100,000) based on clinical ascertainment of patients. Since this estimate likely based mostly on more severely affected DM1 patients, DM1 is considered to be the most frequent muscular dystrophy in adults [10]. However, in different populations prevalence estimates vary widely: In an Italian population from the regions around Padova and North-West Tuscany a prevalence of 9.31 per 100,000 inhabitants was reported [11], while a slightly higher prevalence (11.95 per 100,000) was reported in Northern-Ireland [12], and lower in Belgrade (Serbia) 5.3 per 100,000 [13]. In Jewish communities an average prevalence of 15.7 per 100,000 was reported in a study including 416 DM1 patients of which 307 were diagnosed genetically and 109 clinically [14]. The same study reported even higher prevalences in two Jewish subgroups: 20.0 per 100,000 in Sephardim/Oriental Jews and 47.3 per 100,000 in Yemenite Jews. Among Jews, Ashkenazi had the lowest prevalence with 5.7 per 100,000. A relatively high prevalence of DM1 in the smaller Istria region in Croatia has been reported (18.1 per 100,000) based on clinical

ascertainment and genetic confirmation [15]. The highest known prevalence has been reported in the French Canadian population (1 in 475) due to a founder effect [16]. In non-European populations much lower prevalence rates have been observed, for example a prevalence of 0.46 per 100,000 in Taiwan has been estimated based on DM1 patients and families identified by genetic analysis during 1990-2001 (96 subjects belonging to 26 families) [17, 18].

For DM2 there are currently no established prevalence estimates. DM2 is generally thought to be rarer than DM1, but large scale population studies to confirm this have not been carried out. In Finland 56 new DM2 patients were genetically diagnosed in 2004-2005, and a prevalence estimate of 1/10,000 based on molecular genetic testing has been reported for the Central Finland hospital district [19]. However, new clinical ascertainment data suggest at least a two-fold higher prevalence in Central Finland (unpublished data). In Germany 267 mutation-verified DM2 molecular diagnoses were made between 2003 and 2005 compared to 277 DM1 diagnoses within the same period [19]. These data support the notion that DM2 is more frequent than previously thought, possibly even as frequent as DM1. Because of a shared ancestral founder haplotype common to all Caucasian DM2 patients [4], there is no *a priori* reason to expect the frequency of DM2 mutation to be highly variable in different European populations.

Because of the wide spectrum of milder phenotypic presentations in DM2, the prevalence of the disease is not easy to estimate clinically. Tissue-related differences in repeat instability, modifier genes, or other mechanisms may influence the length of $(CCTG)_n$ repeats needed to elicit symptoms in a given patient and within different organs. In DM2 there is no known correlation between repeat size and disease severity. Our experience clearly indicates a higher prevalence of very late onset and mild manifestations of DM2 than previously reported [20].

Because the first descriptions of the clinical phenotype were based on families collected for linkage studies, there may have been an intrinsic ascertainment bias for more severe disease manifestations, resulting in a lack of very mild cases in the phenotypic spectrum. Our objective in this study was to determine the overall frequency of the DM2 and DM1 mutations in the general population.

METHODS

Subjects

To study the frequency of DM2 and DM1 expansion mutations, we analyzed 4,532 Finnish population control DNA samples obtained from anonymous blood donors. The Finnish population is a historically isolated population with very specific features regarding accumulation of certain recessive disorders based on limited numbers of founder populations and bottlenecks. For dominant disorders the outcome is somewhat different and the Finnish population is expected to be a relatively representative Caucasian population in regard to the DM2 mutation, because of the ancestral founder haplotype existing in all Europe. The material was selected because of the availability of suitable and high-quality samples. In order to study the frequency of the DM mutations and to explore whether the presence of the DM2 (or DM1) mutation might function as a modifier in other neuromuscular diseases (NMD), we also studied an additional 1,003 Finnish samples (862 independent chromosomes) consisting of 606 patients with various non-myotonic NMD, 221 tibial muscular dystrophy (TMD) patients, and 176 healthy relatives of the TMD patients from previous linkage studies. We also studied a cohort of 93 Italian patients with undetermined non-myotonic proximal myopathy or asymptomatic hyperCKemia, which were genotyped for

the DM2 mutation only because of their proximal muscle phenotype. The study was approved by the IRB of Tampere University Hospital.

Methods

DNA isolated from peripheral blood leukocytes was genotyped for both the DM2 and DM1 mutations as previously described [4, 21-23]. Briefly, first DM2 and DM1 repeats were amplified by PCR across the repeat under conditions where only normal-sized alleles are amplified. Samples with a single allele (either two normal alleles of identical size, or only one normal allele due to resistance of expanded mutant alleles to PCR) were further studied using repeat-primed PCR (RP-PCR). The results for both steps were obtained using fluorescent fragment analysis by capillary electrophoresis (ABI 3100 or ABI 3130 Genetic Analyzer) and analyzed using Genotyper or GeneMapper software (Applied Biosystems, Foster City, CA). For the two DM2 premutation alleles from the NMD cohort, single genome equivalent amplification (small-pool PCR) was performed as previously described to determine the stability of the repeat region [9, 24], and sequencing of the repeat region to characterize the repeat number and interruptions of the $(CCTG)_n$ repeat in *ZNF9*, as previously described [9]. A single nucleotide polymorphism (SNP, rs1871922) in linkage-disequilibrium (LD) with the DM2 repeat expansion mutation, was also genotyped as previously described [4, 9] for the samples with large, possibly unstable DM2 alleles in *ZNF9*.

Statistical analyses

To determine statistical significance in the general population cohort, we modeled the presence of a DM mutation (either DM1 or DM2) as a binomial random variable, where in 4,510 tries we observed 4 mutation-positive cases. Using a likelihood function for the detection rate, p, we computed both an equal-tailed 95% interval estimate (excluding the

bottom and top 2.5% of all values) and a maximum likelihood 95% interval estimate (all values in the interval have a likelihood above a threshold value). Computation of these estimates used the fact that the likelihood function is proportional to a beta(4+1,4506+1) distribution. We then determined whether 1/8,000 falls in either interval, and computed the chance that ≥4 mutations would be seen in 4,510 trials if this were in fact the true mutation rate.

Statistical significance in the combined general population and NMD cohorts was modeled similarly using a likelihood function that was proportional to a beta(5+1,5495+1) distribution, where in 5,500 tries we observed 5 mutation-positive cases. We again determined whether 1/8,000 falls in either interval, and computed the chance that ≥5 mutations would be seen in 5,500 trials if this were in fact the true mutation rate.

Similarly, to determine statistical significance for the DM2 mutation alone in the general population cohort, we modeled the mutation frequency using a likelihood function that was proportional to a beta(3+1,5497+1) distribution, where in 5,500 trials we observed 3 mutation-positive cases. We then determined whether 1/8,000 falls in either interval, and computed the chance that ≥3 mutations would be seen in 5,500 trials if this were in fact the true mutation rate.

RESULTS

General population cohort

The population cohort consisted of 4,532 anonymous blood donor DNA samples. Results for allele sizes were obtained from 4,508 samples for DM2 and 4,520 for DM1 giving very low

failure rates (0.53% for DM2 and 0.26% for DM1). A single allele was seen in 12.6% (572) of samples at the *ZNF9* locus and in 20.7% (938) of samples at the *DMPK* locus. Using RP-PCR on these single-allele samples, two DM2 and two DM1 mutation-positive samples were identified (Table 1). One of the DM1 mutations had a $(CTG)_{80}$ repeat allele, while for the other the exact repeat number of the expanded allele could not be determined. For the two DM2 mutations the exact $(CCTG)_n$ repeat number could not be determined with the methods used. Both DM2 mutation-positive samples showed homozygosity for the C allele of SNP rs1871922 in LD with the DM2 expansion mutation.

In addition to the normal and mutant alleles, a small number of enlarged alleles that fall in the range between normal and mutant were identified. Such enlarged alleles were found in *ZNF9* in nine samples, of which five showed a pattern consistent with an unstable $(CCTG)_n$ repeat on RP-PCR. These nine samples were genotyped for SNP rs1871922 and the majority (n = 6) were homozygous (C/C) and three samples were heterozygous (A/C).

In *DMPK* one premutation allele of approximately $(CTG)_{45}$ repeats was identified and in 13 other samples alleles in the range of 35-40 repeats were identified by PCR across the repeat region. In seven additional samples RP-PCR suggested an enlarged allele of unknown repeat size.

NMD patient cohort

Among 988 samples with available molecular diagnostic DM2 data, one DM2 mutation was found (Table 1; Figure 1). Eleven percent (11%) of the individuals studied showed one single allele for the repeat tract in intron 1 of *ZNF9*. Possible large alleles were detected in six individuals by RP-PCR. Sequencing of these DM2 repeat alleles revealed two samples with

uninterrupted short repeat expansions of $(CCTG)_{24}$ and $(CCTG)_{25}$ repeats. These expanded alleles were also found to be unstable in small-pool PCR, and the samples were heterozygous for SNP rs1871922 (A/C) [9]. The patient with a DM2 mutation was previously diagnosed with genetically verified tibial muscular dystrophy, but with unusually marked proximal muscle involvement. Clinically the patient had no myotonia. EMG studies were not performed, and the patient is no longer available for follow-up studies. Of the two patients with uninterrupted unstable $(CCTG)_{24-25}$ tracts, one was diagnosed with a mitochondrial DNA 3243A>G mutation causing MELAS (Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes) syndrome [25, 26], the other patient had an undetermined asymmetric muscle disorder with pseudohypertrophy of one leg and mild atrophy of the other leg. In light of the lack of any DM-like phenotype, the co-segregating uninterrupted unstable $(CCTG)_{24-25}$ tract did not appear to have any discernable effect on the phenotype in these two patients.

Among 991 samples with available molecular diagnostic DM1 data, there were no DM1 mutations (Table 1). One borderline pre-mutation allele of 37 repeats was found in one individual. Nineteen percent (19%) of samples were homozygous for the DM1 repeat in *DMPK* showing one allele size in the locus.

In conclusion, combining our general population cohort and the NMD cohort we found three DM2 mutations in 5,496 individuals and two DM1 mutations in 5,511 individuals. Based on these results the frequency of DM mutations is approximately 1 in 1,830 for DM2 and 1 in 2,760 for DM1. Because all patients with myotonia were excluded from the NMD cohort and DM mutations segregate independently from the underlying cause of disease in these patients, this cohort can be considered an unselected population with respect to both DM mutations.

Cohort of proximal myopathy or asymptomatic hyperCKemia

A cohort consisting of 93 Italian patients with idiopathic proximal myopathy or asymptomatic hyperCKemia was analyzed for the DM2 mutation by PCR across the DM2 repeat and RP-PCR. In this cohort, one DM2 mutation-positive patient was found (Table 1). This patient showed waddling gait, proximal weakness and Gower's sign at age 49, while CK levels were normal. EMG showed findings consistent with a myopathic pattern without myotonic discharges.

Statistical analyses

The previously reported prevalence for DM is 1 in 8,000 (0.000125 = 1.25E-4) [10]. Using a likelihood function to model the presence of a DM mutation (either DM1 or DM2) as a binomial random variable in 4,510 anonymous blood donor samples with available genotypes (general population cohort), the observed frequency of four mutation-positive carriers was significantly higher than expected by chance (point estimate (PE): 8.9E-4, equal-tailed 95% interval (ET) [3.6E-4, 2.27E-3], maximum likelihood 95% interval (ML) [2.7E-4, 2.09E-3], p-value = 0.0027). Similarly, the observed frequency of five mutation-positive carriers in the combined general population and NMD cohorts (n = 5,500) was significantly higher than expected by chance (PE: 9.1E-4, ET: [4.0E-4, 2.12E-3], ML: [3.2E-4, 1.98E-3], p-value = 0.0007). The frequency of the DM2 mutation by itself was also significantly more frequent (PE: 5.45E-4, ET: [1.98E-4, 1.59E-3], ML: [1.30E-4, 1.44E-3], p = 0.0326 for the general population and NMD cohorts combined). These results are presented in Table 2. Starting with the null hypothesis that the true rate of the DM prevalence is 1/8,000 and testing this by measuring the status of 5,500 individuals with a required a type I error $\alpha \leq 0.05$, observing ≥ 3 mutation-positive DM cases causes us to reject the null hypothesis. If the true rate is 5/5,500

(or 1/1,100), we have 87.5% power to correctly identify a difference. Thus, our data clearly indicate that DM (DM1 and DM2 combined) and DM2 alone are significantly more frequent than could be expected for the population based on previous estimates.

DISCUSSION

Early estimates of disease frequency for DM relied on clinical ascertainment of patients and have resulted in a calculated overall prevalence of 1/8,000 [10]. Since the advent of molecular genetic testing for DM1, several prevalence studies have been reported [11-15, 17], but so far no large-scale population studies have been performed. Most of the reported studies were based on the number of diagnosed patients related to the corresponding total population in a certain region. In regions with good diagnostic services, this type of ascertainment may be close to 100% for the subcategories congenital and adult-onset DM1. However, the actual DM1 mutation frequency may be higher due to the known existence of a large group of clinically undiagnosed oligosymptomatic mild forms and some undiagnosed childhood-onset forms. For DM2, clinically based ascertainment of patients is even more difficult because of the large phenotypic variability and a large number of individuals with milder symptoms who remain undiagnosed. Since the availability of DM2 molecular diagnostics, our experience indicated that DM2 is far more common than previously estimated [27, 28]. Milder phenotypes with prominent myalgia may easily be misdiagnosed as fibromyalgia [27], and patients with onset of slowly progressive proximal muscle weakness after age 70 may not be referred for neuromuscular investigations. Further evidence for a large proportion of undiagnosed DM2 patients came from our study on diagnosed DM2 patients showing a disproportionally high number of co-segregating heterozygous recessive *CLCN1* mutations [28]. This study directly suggested that DM2 patients with co-segregating *CLCN1* could be more easily identified and

diagnosed than DM2 patients without the modifier allele, and consequently that the majority of DM2 patients remains undiagnosed even in clinical centers with considerable experience with DM2.

Our present study investigated the prevalence of the DM2 and DM1 mutations in a cohort of 5,535 individuals consisting of anonymous blood donors and patients with various determined and undetermined neuromuscular disorders with exclusion of any myotonic symptoms. The finding of three DM2 mutations in this cohort suggests a mutation frequency of 1 in 1,830, which is >4-fold higher than any previous estimates. In the same cohort we identified two DM1 mutations suggesting a mutation frequency of 1 in 2,760 in the general population. Compared to previous prevalence estimates for DM, this is almost 3-fold higher. These results are significantly different to the previous DM prevalence estimate of 1/8,000 (4/4510, *p*-value = 0.0027 for the general population cohort, and 5/5500, *p*-value = 0.0007 when general and NMD cohorts are combined).

The higher than expected DM1 mutation frequency is probably due to a large number of smaller expansion alleles which are known to be asymptomatic or cause very mild symptoms that can be confused with normal aging in elderly individuals [29]. Clinically this may indicate that a number of elderly patients with cataracts or ptosis may, in fact, have DM1 as the underlying cause. However, if these patients do not develop other symptoms indicative of DM1 during their lifetime there is no need to change clinical practice.

With DM2 the situation is different, however. Because all DM2 mutation carriers are expected to develop disease symptoms, and patients currently remain undiagnosed, their symptoms are currently incompletely understood. This may cause significant differences in

DM2 ascertainment in different populations, because the prevailing practice for directing a patient for genetic testing of DM2 mutation may vary. In the most typical families selected for linkage studies, the DM2 mutation was 100% penetrant. However, it is not settled if this is true in all situations or if the phenotype can extend beyond the currently known variation. Since the DM2 mutation may also cause cardiac conduction defects and sudden cardiac death as early as middle age [30], the timely identification of DM2 mutations in carriers is of major clinical importance. Mutation carriers may present to a wide range of clinical specialities: neurology, cardiology, internal medicine, ophthalmology, rheumatology, endocrinology, etc. The mutation frequency determined in this study suggests a need for increased consideration of DM2 as a possible cause of symptoms. At least those patients undergoing neurological evaluation should be assessed by molecular diagnostic testing with a rather low phenotypic threshold [8].

Because the DM2 mutation has a single European founder haplotype [4], the frequency of DM2 in the Finnish population is suggestive for a high mutation frequency also in other Caucasian populations. However, specific historical population bottlenecks and genetic drift may cause somewhat skewed frequencies in different sub-populations. Samples from healthy blood donors are expected to be relatively representative of the population at-large from which they are drawn.

Uninterrupted unstable DM2 repeat tracts of $(CCTG)_{24}$ and $(CCTG)_{25}$ repeats were found in two individuals from the NMD cohort. One of these patients had a diagnosis of molecularly verified MELAS syndrome, while the other had an undetermined asymmetric muscle hypertrophy in one leg and atrophy in the other. However, deleterious consequences of these small expansion alleles on the phenotype in these individuals are unlikely. In the general

population cohort we found nine samples with possibly unstable enlarged alleles, suggesting a DM2 premutation. These nine samples in addition to the two small expansion samples found in the NMD cohort were all either heterozygous or homozygous for the C allele at SNP rs1871922, which is in LD with the DM2 haplotype [4]. The occurrence of such uninterrupted unstable repeat tracts is compatible with the recent identification of DM2 premutation alleles [9].

The single DM2 mutation in the NMD cohort was found in a patient with a genetically confirmed diagnosis of tibial muscular dystrophy. Besides the conventional weakness of ankle dorsiflexion this patient had an unusually marked proximal muscle involvement at the age of 55 years. The DM2 mutation mainly affects proximal muscles, suggesting that the marked proximal muscle involvement in this particular DM2-positive TMD patient could be associated with the co-segregating DM2 mutation, although its impact cannot be definitively determined. Most TMD patients have a highly selective involvement of the anterior compartment muscles of the lower legs and marked proximal lower limb weakness occurring only after age 70-75. In a study of 209 TMD patients, 9% had very unusual phenotypes, including onset in proximal muscles, despite an identical *TTN* founder mutation in all patients [31]. The reason for these phenotypic variations remains unknown and was not associated with a co-segregating DM2 mutation. In the Italian cohort with 93 patients of undetermined non-myotonic proximal myopathy or asymptomatic hyperCKemia, one patient proved to have a molecular diagnosis of DM2. The fact that myotonia is frequently absent in DM2 patients, even on EMG examination, is not a new observation [7, 19]. However, the identification of DM2 in a patient without otherwise suggestive features of the disease is of interest and suggests that the DM2 diagnosis may easily be missed in a neuromuscular diagnostic setting because of incomplete or uncharacteristic phenotypic expression.

Two DM1 mutations were identified in the general population cohort. A $(CTG)_{80}$ allele was identified for one individual, while for the other size could not be determined. Two premutation alleles were found in both the population cohort [$(CTG)_{45}$] and the NMD cohort [$(CTG)_{37}$].

Taken together our data indicate that mutations for DM (DM1 and DM2) are much more prevalent than previously estimated, and that DM2 may even be the most common inherited muscle disease in European populations. The results also indicate that the vast majority of DM2 patients currently remain undiagnosed. At least two conclusions can be drawn: first, DM2 patients with symptoms for myalgia, muscle weakness in advanced age, insulin resistance or cardiac conduction abnormalities are currently not correctly identified, and secondly, a more comprehensive study of the natural history to characterize the entire spectrum of disease presentations is urgently needed for DM2.

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Conflict of interest statement:

We declare that we have no conflicts of interest.

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Figure Legend

Figure 1. Representative results for DM2 mutation genotyping by PCR and RP-PCR. PCR across the $(CCTG)_{DM2}$ in intron 1 of *ZNF9* showed either a single amplifiable allele (A) or two amplifiable alleles (B), which excludes DM2 mutation. A patient with DM2 mutation from the population cohort showed a peak pattern in *ZNF9* RP-PCR characteristic for the DM2 $(CCTG)_n$ repeat expansion mutation (C and D). In the zoomed-in figure (D) adjacent stutter peaks can be seen with base pair difference of 4 bp. Enlarged unstable (but not actually expanded) DM2 alleles have a distinct pattern in RP-PCR (E and F). In the zoomed-in figure (F), the stutter peaks can be seen, but the pattern ends rapidly. Figures (G) and (H) show a typical example of a DM2 negative sample in RP-PCR. Stutter peaks characteristic to DM2 mutation are not seen in the zoomed-in figure (H).

Table 1. Summary of samples analyzed for DM2 and DM1 mutation. General population and NMD cohorts were analyzed for both DM2 and DM1 mutations; the proximal myopathy or asymptomatic hyperCKemia cohort was analyzed for the DM2 mutation only.

	DM2 $(CCTG)_n$		DM1 $(CTG)_n$	
Samples analyzed*	**n**	**DM2 positive n**	**n**	**DM1 positive n**
General population cohort (FIN)	4,508	2	4,520	2
NMD cohort (FIN)	988	1	991	0
Proximal myopathy or asymptomatic hyperCKemia cohort (I)	93	1	na	na
Total	5,589	4	5,511	2

*FIN, Finland; I, Italy. na, not applicable.

Table 2. Results of the statistical analysis. The presence of DM1 and DM2 mutations together or DM2 mutation alone is modeled as a binomial random variable to determine statistical significance compared to previously reported prevalence for DM, which is 1 in 8,000 (0.000125 = 1.25E-4).

Statistical analysis	**PE**	**ET**	**ML**	**p-value**
Presence of DM mutations in general population cohort (4 in 4,510)	8.9E-4	[3.6E-4, 2.27E-3]	[2.7E-4, 2.09E-3]	0.0027
Presence of DM mutations in combined general population and NMD cohorts (5 in 5,500)	9.1E-4	[4.0E-4, 2.12E-3]	[3.2E-4, 1.98E-3]	0.0007
Presence of DM2 mutation in combined general population and NMD cohorts (3 in 5,500)	5.45E-4	[1.98E-4, 1.59E-3]	[1.30E-4, 1.44E-3]	0.0326

DM, DM1 and DM2 combined; PE, point estimate; ET, equal-tailed 95% interval; ML, maximum likelihood 95% interval

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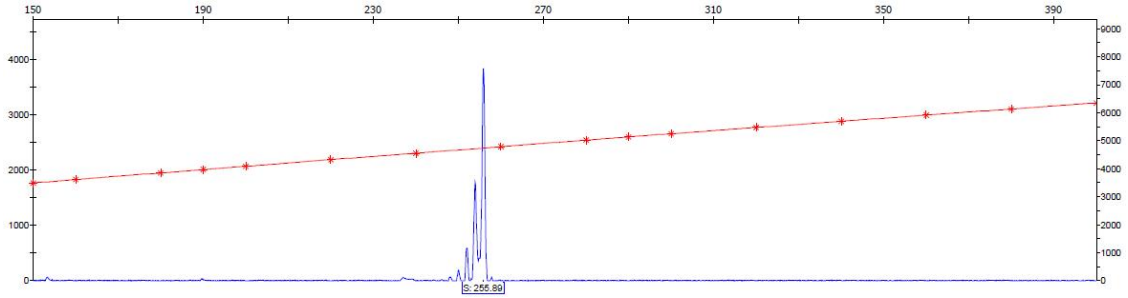
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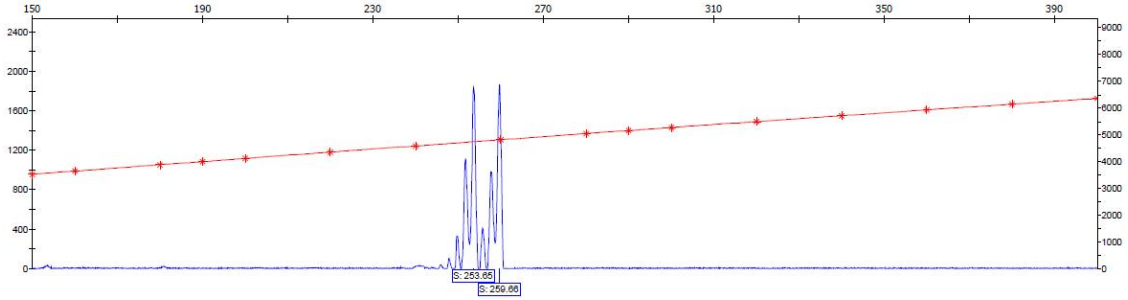
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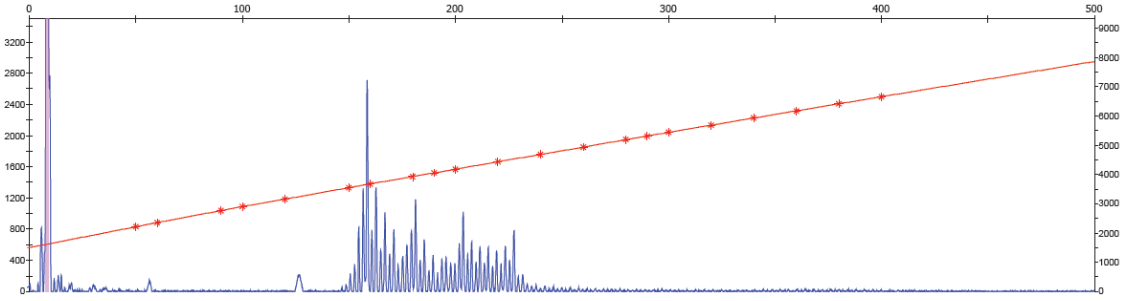
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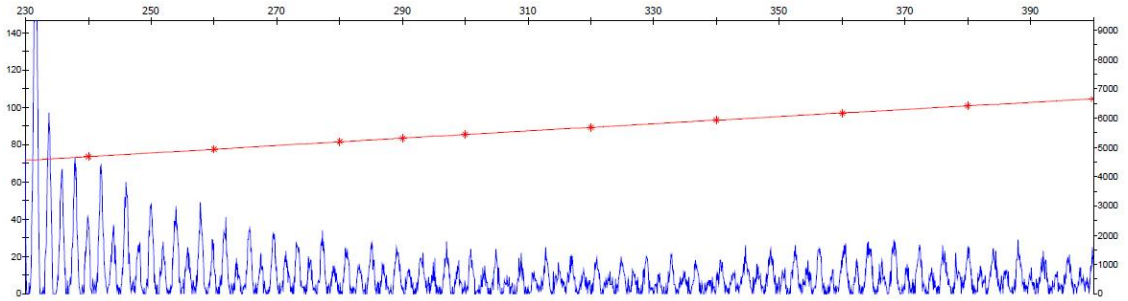
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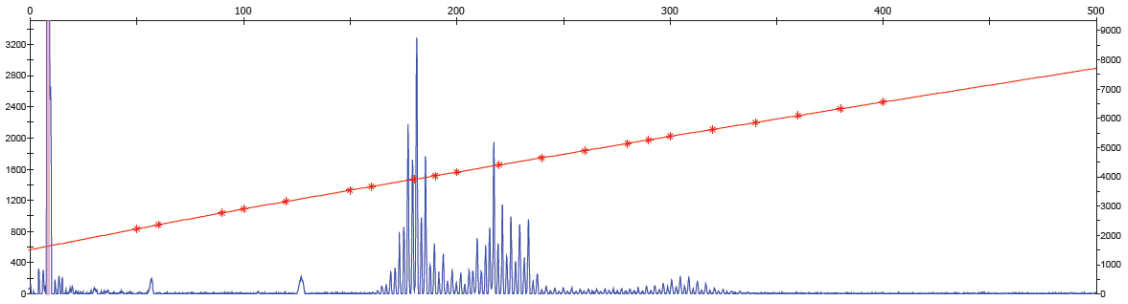
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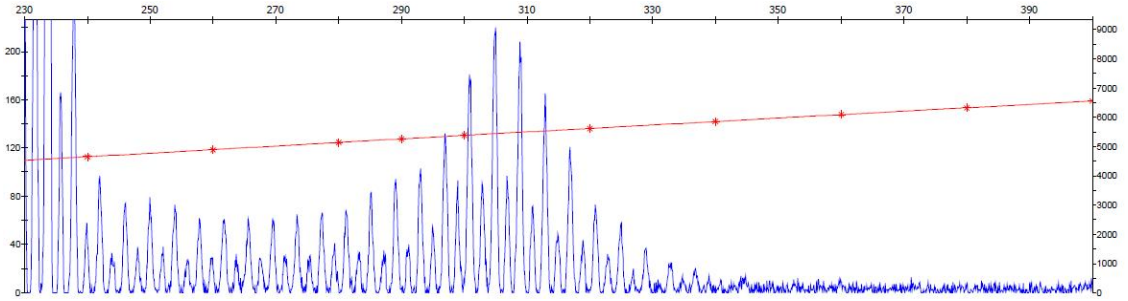
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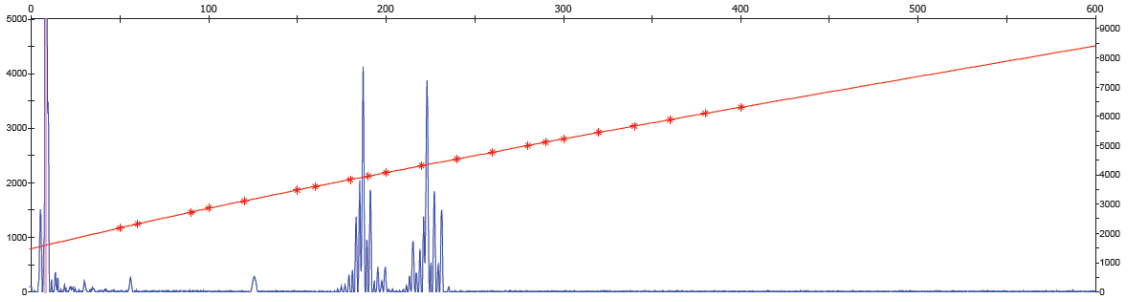
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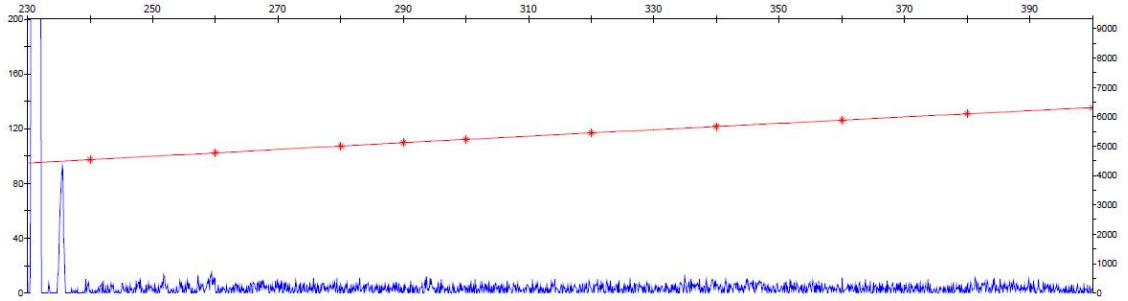
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Proximal myalgic myopathy associated with short mutant $(CCTG)_{DM2}$ alleles

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ABSTRACT

Mutant expansions of a $(CCTG)_n$ tetranucleotide repeat in the first intron of *CNBP* (*ZNF9*) cause myotonic dystrophy type 2 (DM2). The boundaries of normal, pre-mutation, and pathogenic repeat size are not precisely defined. Expansions as large as 11,000 repeats have been reported, while the smallest disease-associated alleles range from 75 repeats, some of which may be mosaic for larger repeats. Expansions as short as $(CCTG)_{100}$ form diagnostic ribonuclear foci, but uncertainties remain regarding the phenotypic outcome of shorter mutant expansions. We describe a kindred segregating mutant alleles of 55 to 61 uninterrupted $(CCTG)_n$ associated with a new type of disease resembling DM2, characterized by lack of the basic fundamental findings of DM2: EMG-myotonia, abnormal splicing of the chloride channel, and ribonuclear foci. Clinical manifestations include late-onset myalgic pains, proximal muscle weakness, muscle stiffness, and mild calf hypertrophy. Histopathology showed some features characteristic of DM2 with highly atrophic type 2 fibers, nuclear clump fibers and increased numbers of internal nuclei. Mutant $(CCTG)_{55-61}$ alleles were unstable as in typical DM2 patients with larger expansions. Based on the lack of both ribonuclear foci and aberrant splicing of genes usually misspliced in DM (including *CLCN1*, *INSR* and *TNNT3*), the underlying pathomechanisms appear to be distinct from DM2 disease. We have identified a new disease phenotype distinct from myotonic dystrophy: a proximal myalgic myopathy, associated with short, unstable $(CCTG)_{55-61}$ alleles at the DM2 locus.

INTRODUCTION

Myotonic dystrophy type 2 (DM2, MIM ID #602668) is one of the most common adult-onset muscle diseases in many European populations.[1] It is caused by a $(CCTG)_n$ tetranucleotide repeat expansion mutation in intron 1 of *CNBP* (cellular nucleic acid-binding protein or *ZNF9*, zinc finger protein 9).[2,3] Inheritance of this multisystemic disorder is autosomal dominant, and DM2 shares some clinical and molecular similarities with myotonic dystrophy type 1 (DM1, MIM ID #160900), which is caused by a $(CTG)_n$ trinucleotide repeat expansion mutation in *DMPK*.[4-6] However, there are significant differences between DM1 and DM2. In DM1, disease severity correlates with expansion size, whereas no genotype/phenotype correlation has been observed in DM2. Clinical presentation in DM2 is generally milder and more variable compared to adult onset DM1. Anticipation, which is common in DM1, is not a regular feature of DM2.[7] The $(CCTG)_{DM2}$ expansion is somatically unstable and tends to increase with age rather than between generations.[2] Usually the repeat length is reduced when transmitted to subsequent generations.[8]

Clinical findings in DM2 include adult or late-onset proximal muscle weakness, myalgic pains, muscle stiffness, cataracts, myotonia, tremor, cardiac conduction problems, and endocrinological abnormalities.[9] While many of these symptoms are also found among DM1 patients, there are important contrasting findings. Muscle weakness in DM1 is more distal than proximal. In DM2 histopathology shows atrophic and nuclear clump type 2

fibers, whereas type 1 fibers are more hypotrophic in DM1.[10] Myalgic muscle pain is often the main clinical problem for DM2 patients.[11]

In both DM1 and DM2, the expansion mutations are located in the untranslated regions of their resident genes.[2,4-6] It is commonly believed that the similarities in clinical features result from similar underlying mutations and molecular pathomechanisms. Both mutant repeats are transcribed into $(CUG)_{DM1}$/$(CCUG)_{DM2}$ RNAs that aggregate in ribonuclear inclusions and interfere with the splicing of down-stream effector genes by dysregulating the functions of RNA-binding proteins, including members of the muscleblind family (MBNL) and CELF1.[12-14] However, recent reports suggest that additional molecular pathomechanisms are involved, which affect translational regulation and global protein turnover.[15,16] Moreover, in DM2 CNBP (ZNF9) mRNA and protein levels are reduced, and changes in the protein expression of the resident gene may partially explain the differences between DM1 and DM2.[17]

Disease-associated $(CCTG)_{DM2}$ expansions are uninterrupted and unstable.[2,3] For DM2 patients of European descent a single ancient founding mutation has been suggested based on microsatellite and SNP analysis.[3] The upper normal number of repeats in the population is estimated to be 30[18,19] and normal $(CCTG)_n$ repeats always include at least one interruption. Premutation alleles are suggested to consist of $(CCTG)_{22-33}$ repeats with no interruptions.[18] Here, we describe a kindred segregating unstable, uninterrupted $(CCTG)_{55-61}$ alleles associated with a new form of late-onset myalgic myopathy disease linked to the DM2 locus.

METHODS

Subjects

This study included individuals from a Finnish family in which the index patient presented with adult-onset myopathy. The pedigree of the family is shown in Figure 1. Blood or saliva samples were collected from 12 family members after obtaining informed consent, and DNA was isolated according to standard procedures. Muscle biopsy, electromyography (EMG) and lower limb muscle magnetic resonance imaging (MRI) were obtained from the index patient (II-1) and his symptomatic brother (II-3); both also underwent extensive clinical, neurological, and ophthalmological examinations including measurement of creatine kinase activity and electrocardiograms.

Muscle biopsy methods

Histopathology. The muscle biopsy samples were obtained from vastus lateralis of the proband (II-1) and his brother (II-3). Samples were snap-frozen and 8-10 μm sections were cut and examined using standard histochemical methods, including hematoxylin & eosin (H&E), Gomori trichrome, reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) and combined succinate dehydrogenase-cytochrome oxidase (SDH-COX). In addition, 6 μm sections were immunostained for different myogenic antigens including myosin heavy chain isoforms (fetal, neonatal, slow and fast MyHC MHC class I and II). Immunohistochemical stainings were performed on the BenchMark (Roche Tissue Diagnostics/Ventana Medical Systems Inc.) immuno-stainer using its official protocols with incubation of primary antibodies for 30 minutes in 37˚C and

visualized with a peroxidase based detection kit (UltraView Universal DAB detection kit, Roche Tissue Diagnostics/Ventana Medical Systems Inc.).

Chromogenic and fluorescent *in situ* hybridization, and immunofluorescence. Chromogenic *in situ* hybridization was performed on frozen muscle sections as previously described.[20] After hybridization with both digoxigenin end-labeled sense $(CCTG)_8$ and antisense $(CAGG)_8$ DNA oligonucleotide probes, Invitrogen Spot-Light® CISH™ Polymer Detection Kit (Invitrogen Corporation, CA 93012, USA) was used for chromogenic detection of the bound probe according to the manufacturer's instructions. Sections were counterstained with methyl green zinc chloride (Merck KGaA, Darmstadt, Germany), dehydrated and mounted. The sections were viewed under a bright field microscope.

RNA fluorescent *in situ* hybridization (FISH) to detect mutant $(CCUG)_{DM2}$ RNAs in primary patient myoblast cultures was performed with an antisense Cy3-$(CAGG)_{10}$ or a 5'-TYE563-$(CAGG)_5$-3' locked nucleic acid (LNA) probe (Exiqon, Woburn, MA). Briefly, cultures were fixed for 30 min at 4 % PFA/PBS at 4°C and washed three times in PBS, followed by permeabilization in 2 % pre-chilled acetone/PBS for 5 min at room-temperature and washed once with PBS. Pre-hybridization was performed in 40 % formamide in 2x SSC for 10 min, followed by hybridization with the $(CCUG)_{DM2}$ LNA probe (25 ng/µl) in buffer (40 % formamide, 2x SSC, 0.02 % BSA, 66 ng/µl yeast tRNA, 10 % dextran sulfate, 2 mM vanadyl ribonucleoside complex), overnight at 45°C. Post-hybridization washing was performed twice with 2x SSC for 5 min at room temperature,

then with 40 % formamide in 2x SSC at 60°C for 30 min, followed by two final washes in 1x SSC at 37°C for 15 min each. Slides were washed with PBS and then mounted either in Prolong® Gold antifade reagent with DAPI (Invitrogen). Images were acquired using a Nikon 80i Deconvolution Microscope and processed with AutoQuant's AutoDeBlur software (Silver Spring, MD, USA).

For combined RNA-FISH/ immunofluorescence (IF), cells grown on coverslips first underwent RNA-FISH as described above and were then permeabilized again with 0.5 % Triton X-100 in PBS at 4°C for 5 min and blocked with 1 % BSA/PBS for 1 hr at room temperature. Cells were incubated with anti-MBNL1 antibody (A2764, 1:5,000) overnight at 4°C, then washed with 1x PBS for 5 min three-times. Alexa Fluor® 647 Donkey Anti-Rabbit IgG (1:1,000) was applied to the cells as secondary for 1 hr at room temperature. Cells were washed with 1x PBS for 5 min three-times and mounted in Prolong® Gold antifade reagent with DAPI (Invitrogen). Imaging was as described for RNA-FISH alone.

SDS-PAGE and Western blotting. Muscle biopsies were treated as previously described for SDS-PAGE and Western blotting.[21] After SDS-PAGE, proteins were transferred onto PVDF membrane and immunolabeled with a rabbit polyclonal antibody against CNBP.[22] Anti-alpha tubulin antibody (Abcam, Cambridge, UK) was used as a loading control of samples on the same blot. After HRP-conjugated goat anti-rabbit and goat anti-mouse IgG secondary antibodies (Zymed Laboratories Inc., San Francisco, CA) were incubated, the antibody reacting bands were visualized using an enhanced

chemiluminescence detection kit (Pierce Biotechnology SuperSignal West femto maximum sensitivity substrate, Rockford, IL, USA).

Molecular genetic methods

Genotyping of DM2 mutation. DNA isolated from peripheral blood leukocytes (PBL) was genotyped for the DM2 mutation as previously described.[3] The DM2 repeat expansion was also analyzed using recently reported new tetraplet-primed PCR (TP-PCR) method comparable to RP-PCR analysis.[23]

Long-range PCR repeat expansion assay (LR-RA) and Southern blot. Long-range PCR across the DM2 mutation locus and visualization using a radioactively end-labeled oligo was performed as previously described.[24]

Sequencing. To characterize the repeat number and interruptions of the $(CCTG)_n$ repeat in *CNBP* the repeat regions of the proband II-1 and his brother II-3 were sequenced from DNA isolated from PBL as previously described.[18] Briefly, alleles were amplified and the products cloned using the StrataClone PCR cloning kit according to manufacturer's instructions. DNA was prepared from the clones using the QIAprep Spin Miniprep kit and plasmid DNA was sequenced directly using ABI BigDye terminator chemistry. Sequences were visualized by capillary electrophoresis on an ABI 3100 Genetic Analyzer and analyzed using Sequencher software.

Single genome equivalent amplification. For the proband and his brother II-3 a single genome equivalent amplification (small-pool PCR, SP-PCR) was performed and described previously to determine the stability of the repeat region.[18,25]

Genotyping of microsatellites and a SNP. Six microsatellite markers (D3S3606, D3S3607, 571C11_AG1, 221E20-GT1, 814L21-GT1 and 436B3-AG1) near the DM2 mutation locus and one single nucleotide polymorphism (SNP, rs1871922) in linkage-disequilibrium (LD) with the DM2 repeat expansion mutation, were genotyped to characterize the haplotype of the short expansion family in comparison to a previously reported Finnish DM2 associated haplotype as previously described.[3,18]

Allele-specific CNBP expression analysis. To verify that the short expansion allele of *CNBP* was expressed, reverse-transcriptase (RT) RP-PCR analysis was conducted on patient II-3. The cDNA sample was prepared from RNA isolated from skeletal muscle as described previously.[17] If pre-mRNA of mutant *CNBP* is present, a typical pattern in RP-PCR is detected. To determine whether both *CNBP* alleles were transcribed to pre-mRNA, the SNP rs1871922 A>C polymorphism was analyzed by a quantitative allele-specific method as described previously.[17]

Splice variant analysis. Alternative splicing of effector genes is characteristic of DM2 and has been reported for several genes, including *CLCN1*[26] *INSR*,[27] *LDB3*,[28] *MAPT*,[29] and *TNNT3*.[30] We analyzed aberrant splicing of *CLCN1*, *INSR*, *SERCA*, *RYR1*, *TNNT3*

and *TTN* genes from patient II-3 cDNA isolated from skeletal muscle as previously described.[31]

RESULTS

Clinical investigations and histological findings

The most prominent symptom of the proband (II-1) was myalgic pains with onset at 38-yrs of age. Proximal lower limb muscle weakness occurred after age 50-yrs, and he also complained of muscle stiffness. On clinical examination there was mild calf hypertrophy but no evident muscle atrophy at the age of 62-yrs. Cardiac arrhythmia, brain involvement, insulin resistance or frontal balding were not observed. Ophthalmological examination revealed some minor posterior lens opacities that were not defined as cataracts. Serum creatine kinase (CK) levels were slightly elevated, 300-400 IU/L, and increased by statins to 700 IU/L (upper normal limit 200 IU/L). Other laboratory tests (hemoglobin A1c, thyroid stimulating hormone, glutamyltransferase, urate and atrial natriuretic peptide) were normal. The patient had no clinical myotonia. EMG was abnormal showing clearly increased insertional activity, repetitive discharges and a few fibrillations, small motor unit potentials, but no electrical myotonia. Magnetic resonance imaging (MRI) axial sections of lower limb muscles showed mild fatty degenerative changes of thigh and calf muscles (Figure 2A). Muscle biopsy findings were characteristic for DM2 with highly atrophic subpopulation of type 2 fibers, nuclear clump fibers and increased number of internal nuclei. The highly atrophic and nuclear clump type 2 fibers were also reactive for neonatal myosin heavy chain (MyHC) as with DM2 (Figure 2B). These muscle biopsy findings prompted the genetic analysis for DM2.

Two brothers of the proband also carried mutant alleles with slightly different repeat lengths. One of them (II-3) was clinically evaluated and had myalgic cramps and muscle stiffness since the age of 40-yrs. His CK levels were normal and liver enzyme levels were just above the upper normal limit. He had no calf hypertrophy. Muscle biopsy showed the same abnormalities as for the proband: increase of internal nuclei with some highly atrophic and nuclear clump type 2 fibers that were reactive for neonatal myosin. On muscle MRI minor early fatty degenerative changes were seen in the soleus muscles. The second brother (II-7) reportedly suffers from myalgic cramps, but he was not available for clinical examinations. Both parents died early, around their 60s, of coronary heart disease without further data on neuromuscular symptoms, although the father had muscle pains in his shoulders interpreted as rheumatic.

In situ hybridization studies

Chromogenic *in situ* hybridization (CISH) with sense and antisense probes was performed on the proband's muscle sample.[20] No mutant $(CCTG)_n$/$(CCUG)_n$ were detectable with either probe (data not shown). In order to increase sensitivity and specificity, we performed RNA-FISH alone using antisense Cy3-$(CAGG)_{10}$ and TYE563-$(CAGG)_5$ LNA probes on myoblast and fibroblast cell cultures of the proband (data not shown). We also performed combined RNA-FISH/MBNL1 IF using the LNA probe (Figure 3A). No ribonuclear inclusions of co-localizing $(CCUG)_n$ RNA and MBNL1 protein were detectable by either method in the short expansion patient.

Molecular genetic characterization of the short $(CCTG)_n$ repeat expansion

Molecular genetic diagnostics of the proband's PBL DNA for the $(CCTG)_{DM2}$ mutation revealed only one amplifiable allele. On RP-PCR, a pattern consistent with short $(CCTG)_{63\text{-}66}$ expansion was seen (Figure 3B). The identical short expansion pattern of RP-PCR was also observed in the DNA sample isolated from proband's skeletal muscle tissue (Figure 3B). In a typical DM2 full expansion the pattern in RP-PCR consists of extended repeat peaks at 4-bp intervals, which gradually taper off in intensity in contrast to a short expansion pattern in which the pattern of peaks end abruptly (Supplementary Figure 1).[1] Mutation diagnostics was performed on samples of individuals II-1, II-2, II-3, II-5, II-6, II-7 and II-8. In addition to the proband (II-1), a short expansion was observed in patients II-3 and II-7. Samples II-2, II-5, II-6 and II-8 were negative for the mutation. To verify the short repeat result in RP-PCR, we analyzed the proband's DNA sample by LR-RA and Southern blot. Again, the pattern by Southern blot was compatible with a short $(CCTG)_{60\text{-}70}$ repeat expansion without any signs of large DM2 repeats present (Figure 3C). In order to define the exact number of CCTG repeats and the structure of the repeat, we sequenced the mutant alleles of both the proband (II-1) and his brother (II-3). Sequence analysis of multiple clones of the mutant allele in the proband's PBL DNA revealed a short repeat expansion of $(TG)_{19}(TCTG)_7(CCTG)_{55}$ as the predominant mutant allele. The affected brother (II-3) had a similar repeat structure $(TG)_{19}(TCTG)_7(CCTG)_{61}$ in his PBL DNA. These uninterrupted short expansion alleles were found to be unstable with repeats from 55 to 66 in single genome equivalent amplification (SP-PCR) analysis.[18] In addition to the previously performed RP-PCR mutation analysis,[18] we also analyzed the mutation using the recently developed tetraplet-primed PCR (TP-PCR[23]). The pattern obtained with TP-PCR in the proband was similar to the typical DM2-

positive pattern obtained by TP-PCR. The peaks did not extend as far as with a typical DM2-positive sample, but otherwise the pattern was not distinguishable from the typical positive pattern (data not shown).

Genotyping of microsatellites and a SNP rs1871922

To determine the haplotype of the chromosome segregating the short expansion alleles, we genotyped six microsatellite markers flanking the $(CCTG)_{DM2}$ repeat mutation. The region comprises approximately 2 Mb, and the distances from the DM2 mutation have previously been described.[3] The haplotype associated with the short $(CCTG)_{55\text{-}61}$ expansion was completely different from the previously observed extended microsatellite haplotype in Finnish DM2 families (Figure 4). However, the C-allele of the rs1871922 SNP, previously shown to be in LD with the DM2 repeat expansion mutation,[3] segregated with the short expansion mutation in our family.

Western blot studies

In DM2 disease CNBP protein levels in skeletal muscle are reduced approximately 15 % to 50 %.[17] In contrast, the expression level of CNBP protein in the proband II-1 and patient II-3 skeletal muscle tissue was normal (Figure 5A).

Allele specific expression analysis

On RP-PCR from cDNA isolated from patient's II-3 muscle sample, a pattern of stutter peaks typical for a repeat expansion was observed (Figure 5B). This indicated that the mutant allele is transcribed to pre-mRNA as is the case in DM2. Using the SNP

rs1871922 to determine quantitative allele-specific pre-mRNA transcript levels, both normal and mutant alleles appeared to be expressed at equal levels.

Splicing of down-stream effector genes

Alternative splicing was assessed for *CLCN1*, *INSR*, *SERCA1*, *RYR1*, *TNNT3* and *TTN*, which have been identified as misspliced in DM2.[26,30,31] We analyzed cDNA isolated from skeletal muscle sample of patient II-3, and only normal transcripts were observed for all of the studied genes. Figure 6 shows results for *TNNT3*, *ATP2A1* and *CLCN1*.

DISCUSSION

We describe a disease that is distinct from myotonic dystrophy type 2 (DM2) because several molecular findings characteristic of DM2, including $(CCUG)_{DM2}$ ribonuclear foci and toxic RNA-induced missplicing are absent. The disease segregates with short $(CCTG)_{55\text{-}61}$ expansion alleles at the DM2 locus, which (presumably) are not large enough to cause aggregations of mutant transcripts (foci) and thus no sequestration of MBLN1 and no splicing defects. The phenotype in family members carrying the mutation could be examined in two brothers, II-1 and II-3. The proband had somewhat more pronounced symptoms with myalgia, muscle weakness and stiffness, when compared to his brother whose main complaint was myalgic cramps. Overall, the clinical presentation was relatively mild, at least during the first six decades of life.

Compared to DM2 disease, the underlying disease mechanism(s) appears to be distinct. Although histopathology findings similar to DM2 are present in the muscle biopsy, ribonuclear inclusions containing mutant transcripts and sequestered MBLN1 characteristic of DM2 and DM1 are not. In fact, the muscle biopsy findings were the only reason for genetic DM2 testing. The shortest reported expansion in a patient with evidence of nuclear foci and co-localization of MBNL proteins is $(CCTG)_{100}$ and even in that case the possibility of undetected longer repeats cannot be fully excluded.[32] The clinical phenotype in that patient was within the typical range of DM2. The presence of ribonuclear inclusions in DM2 disease is associated with splicing defects of several down-stream effector genes.[17,26,27,29-31,33] Ribonuclear inclusions were not detected in

muscle cell cultures of the $(CCTG)_{55}$ proband, and no missplicing of DM2-associated effector genes (*CLCN1*, *INSR*, *SERCA1*, *RYR1*, *TNNT3* and *TTN*) was observed. The absence of both ribonuclear inclusions and missplicing is consistent with the lack of clinical or electrical myotonia, cardiac involvement, and insulin resistance, suggesting that the molecular pathomechanism is different from the toxic RNA mechanism in DM2. The most likely explanation for lack of missplicing is that RNA transcripts of short $(CCTG)_{55\text{-}61}$ expansion alleles do not aggregate as foci and do not sequester MBNL protein. Formation of ribonuclear foci detectable by *in situ* hybridization may be expansion size-dependent, and $(CCTG)_{55\text{-}61}$ alleles may be below the threshold.

At the mRNA and protein levels, *CNBP* expression has been reported to be significantly reduced in DM2 patients compared to DM1 and normal control individuals.[17] However, CNBP protein levels in the skeletal muscle of the proband II-1 were within normal range. Taken together, the findings of normal CNBP protein levels in muscle and equal representation of both alleles of rs1871922 in pre-mRNA suggest that there is no pre-mRNA processing defect as in DM2 disease, due to the short mutant repeat allele. Because in DM2 patients with full expansion mutation the processing of CNBP pre-mRNA is abnormal, the observed equal representation of both pre-mRNA alleles in the proband II-1 provides further evidence that no larger CCTG expansions are present that would have been missed by the other assays. This short expansion is small enough to allow normal CNBP protein expression by bnormal pre-mRNA processing.

The pattern of the short expansion in RP-PCR is clearly different compared to the pattern observed in DM2 patients. As shown in Supplementary Figure 1 the short expansion pattern shows an abrupt decline of peaks as opposed to the DM2-positive pattern of continuous peaks, indicating the absence of any larger alleles in the samples of patients carrying a short expansion. In addition, all different sized fragments in Southern blot analysis are clearly much shorter than those in the DM2 patient also confirming the absence of any larger alleles in the short expansion patient. Interestingly, the flanking microsatellite marker haplotype is completely different from the extended haplotype previously identified in Finnish DM2 patients.[3] However, the C allele of SNP rs1871922, which is in complete LD with the DM2 repeat expansion mutation, was also observed to segregate with the short expansion haplotype. These findings suggest that either the short mutation derives from an older European haplotype, from which the common extended Finnish DM2 haplotype derived, or the association with the C allele at SNP rs1871922 is coincidental, because the C allele is the most common allele in the population, which would indicate this is a *de novo* mutation. Studies on DM2 haplotype have suggested that all genetically confirmed DM2 patients of European descent arose from a single ancestral origin.[3,34] To date, only one single Japanese DM2 patient with a haplotype completely different from that found in Caucasian DM2 patients has been reported.[35]

Using a repeat expansion sizing scheme similar to that adopted for DM1,[36,37] short $(CCTG)_{55\text{-}61}$ expansion alleles fall between the pre- and full mutation range and may constitute protomutations. Our findings at the DM2 locus are reminiscent of the situation at the *FMR1* locus, where premutation alleles of $(CGG)_{50\text{-}200}$ repeats cause allelic, yet

distinct diseases, fragile X tremor/ataxia syndrome (FXTAS) and premature ovarian failure (POF1), compared to full mutations with >$(CGG)_{200}$ that cause fragile X-syndrome.[38,39]

In conclusion, we have identified a distinct disease, proximal myalgic myopathy (PMM) caused by short, unstable $(CCTG)_{55-61}$ alleles at the DM2 locus. Our finding that expanded $(CCTG)_n$ alleles with as few as 55-61 repeats manifested phenotypically suggests that DM2 alleles in the proto-mutation range are disease-causing, although with different yet unknown pathomechanism(s) compared to DM2 disease. Confirmation of this disease as a new entity requires identification and characterization of additional $(CCTG)_n$ proto-mutation carriers, and also warrants re-examination of individuals with small expansion alleles as well as careful molecular characterization of myalgic patients with mild CK elevation and DM2-like pathology.

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Conflict of interest statement:

We declare that we have no conflicts of interest.

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Figure legends

Figure 1. Clinical pedigree of the short $(CCTG)_{DM2}$ expansion kindred. Proband is marked with an arrow.

Figure 2. (A) Muscle MRI of lower limbs showing diffuse fatty degenerative changes in semimembranosus (a) and vastus lateralis muscles (b) on the thigh level, and more replacement in right medial gastrocnemius and soleus muscles in the calf (c) of proband II-1. Arrows indicate the affected muscles.
(B) Histological changes in the proband's vastus lateralis muscle. Nuclear clump fibers (arrows) and some internal nuclei (asterisk) are seen in HE staining (a). Highly atrophic type 2 muscle fibers (b) express neonatal MyHC (brown fibers). These fibers were also detected in MyHC double staining (red color, arrows) in (c) and (d).

Figure 3. (A) Combined $(CCUG)_n$ RNA-FISH and MBNL1 IF. Fluorescent *in situ* hybridization (FISH) was performed with an antisense $(CAGG)_5$ LNA probe on a primary myoblast cell culture of the proband. Co-localization of $(CCUG)_n$ RNA (red foci in FISH) and MBNL1 protein (green foci in IF) in ribonuclear inclusions (yellow foci in Merge) are only detectable for the typical DM2 patient, but not for the short expansion patient (PMM-II-1) or the normal control.
(B) Repeat-primed PCR (RP-PCR) results of the proband's DNA samples isolated from either PBL or vastus lateralis muscle. The pattern with a secondary peak reflecting the

mutant allele combined with abrupt decline of the fragment peaks around the size of 390 bp is characteristic of short repeat expansions (see also Supplementary Figure 1).
(C) Southern blot analysis of proband. The pattern is compatible with a short $(CCTG)_{60\text{-}70}$ repeat expansion. Arrows indicate the smears, which present different fragment sizes. The fragments are longer in the typical DM2 patient than in the patient II-1. N, normal sample without DM2 mutation; DM2, DM2-positive control; II-1, patient II-1 carrying the short expansion $(CCTG)_{55}$; λ-marker, DNA size standard.

Figure 4. Pedigree and flanking haplotype of family segregating a short unstable $(CCUG)_{DM2}$ expansion mutation. Inferred genotypes are in parentheses. "?" indicates unknown genotype; "!!" indicates Mendelian inconsistency. The mutation is segregating on the black haplotype. For some individuals, the predominant expansion size in PBL could be determined by PCR. Where the expansion was too large to size by PCR, it is indicated by a "?". Non-Mendelian segregation was observed in individuals II-1 and II-3 for the expansion allele. For haplotype analysis six microsatellite markers and one SNP in a region comprising 2 Mb and flanking the $(CCTG)_{DM2}$ in intron 1 of *CNBP* were genotyped. Distances (Mb) of microsatellite markers from the $(CCTG)_{DM2}$ expansion: D3S3606 -1.750, D3S3607 -1.652, 571C11_AG1 -0.978, 221E20-GT1 -0.362, 814L21-GT1 -0.124 and 436B3-AG1 +0.216.

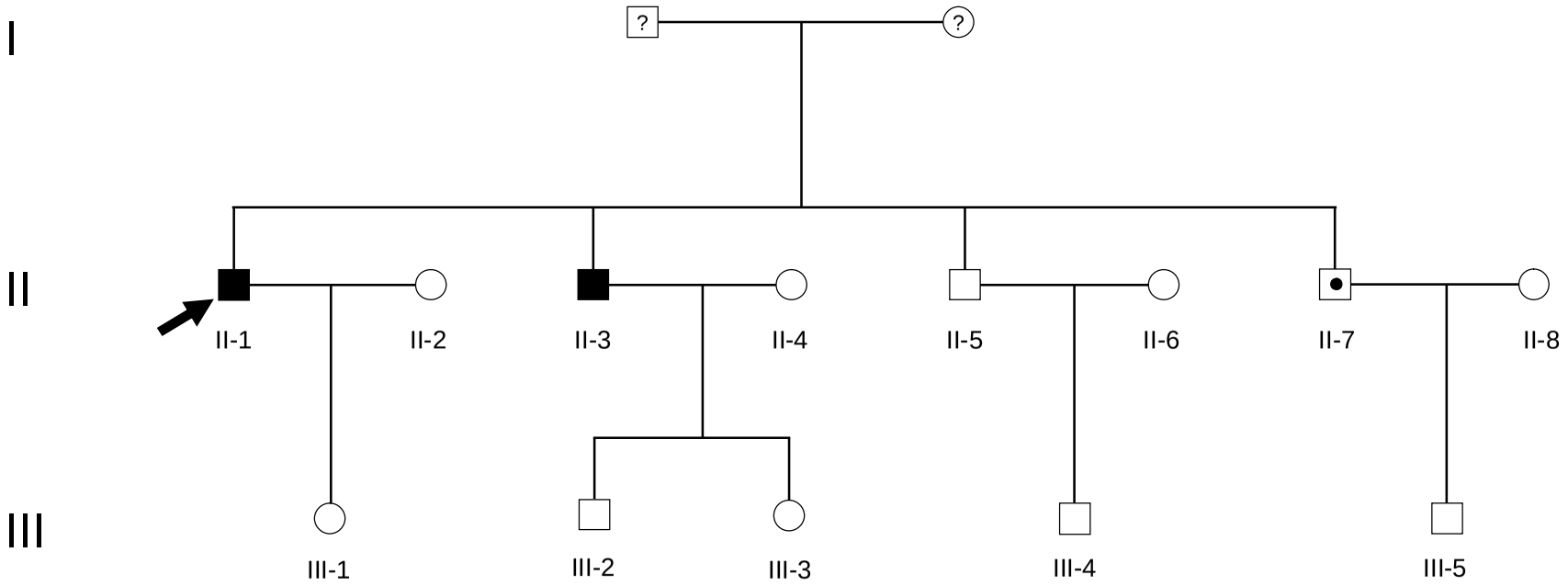
Figure 5. (A) Western blot analysis of CNBP protein levels. Expression levels of CNBP protein in the skeletal muscle of patients II-1 and II-3 were similar to the normal muscle.

In both DM2 patients, a clear reduction compared to normal muscle and patients II-1 and II-3 was seen. Alpha-tubulin was used as a loading control.
(B) RT-RP PCR of DM2 repeat showing expression of the mutant allele. Panels show electropherograms of genomic DNA (gDNA) and myoblast cDNA from PMM-II-3 amplified with a repeat-anchored reverse primer $(CAGG)_5$ with a 5' FAM label. Intensities are on the y-axis and mobility units on the x-axis. (a) genomic DNA from patient PMM_II-3. Red arrow indicates modal size of the small DM2 expansion allele, which is followed by stutter peaks at 4-bp intervals. (b) cDNA from PMM_II-3 showing amplification of pre-mRNA with a pattern identical to genomic DNA, including the expansion allele and stutter peaks. (c) cDNA from a typical large expansion DM2 allele showing stutter peaks. (d) cDNA from a normal individual: the signal ends without the typical stutter pattern.

Figure 6. Analysis of fetal splice isoforms in patient with small expansion. Panels A-C show percent inclusion of alternative exon(s) (y-axes) of *CLCN1* (A), *TNNT3* (B) and *ATP2A1* (C) in skeletal muscle from DM2 (n=5 or 6), PMM Patient II-3 (in duplicate), normal adult (n=2) and fetal (n=1) as determined by peak height in fluorescent quantitative RT-PCR. Panel (D) shows representative electropherograms for *ATP2A1* with size in base pairs (x-axis) and intensity units (y-axis). Red peaks are 400 HD internal size standard. The arrow points to the fetal isoform, 234 bp, consisting of exons 21 and 23. The adult isoform, 276 bp, contains exons 21, 22 and 23.

Supplementary Figure 1. Representative results of DM2 RP-PCR analysis. (A) Normal sample with no DM2 mutation. (B) Typical DM2 patient. Pattern of peaks gradually taper off in intensity. (C) Short repeat expansion patient. The peak pattern shows an abrupt decline as opposed to the typical DM2-positive pattern of continuous peaks.

1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II
III
?
?
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III-1
III-2
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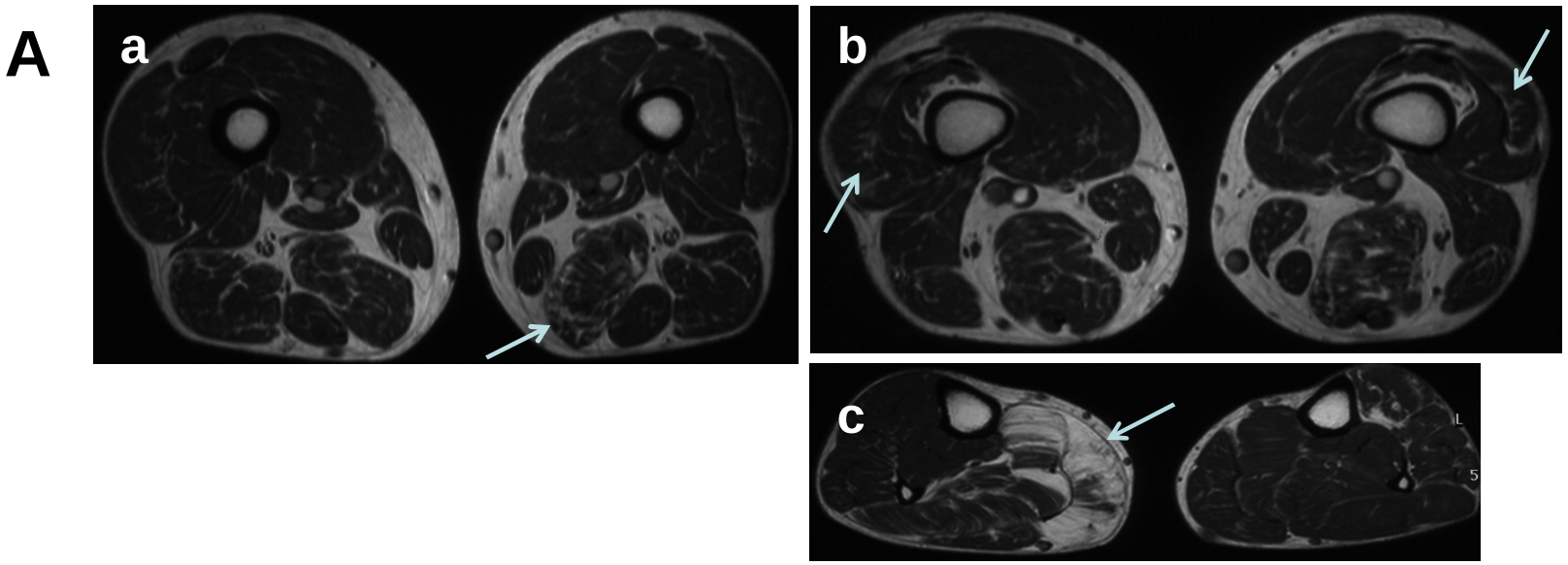
III-4
III-5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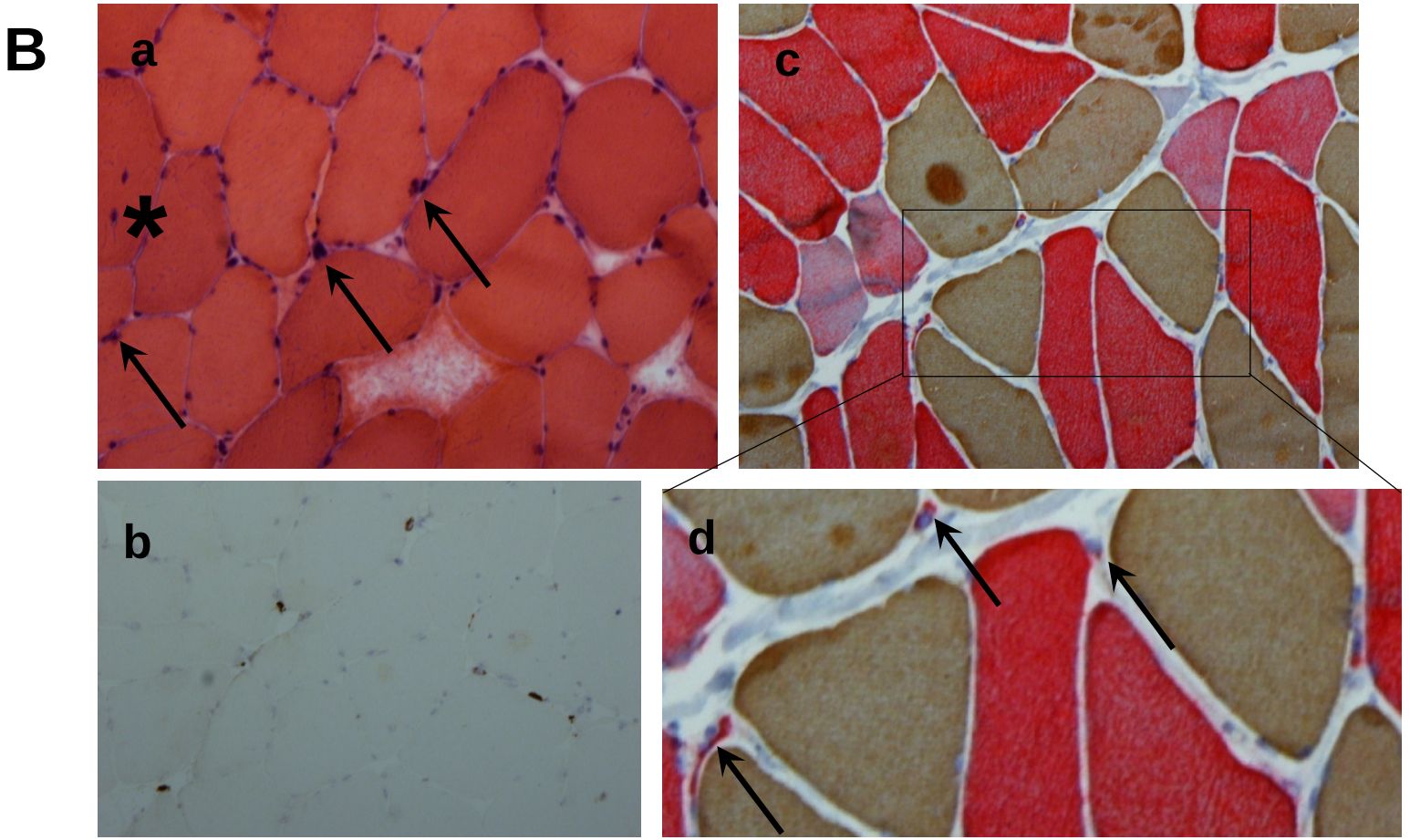
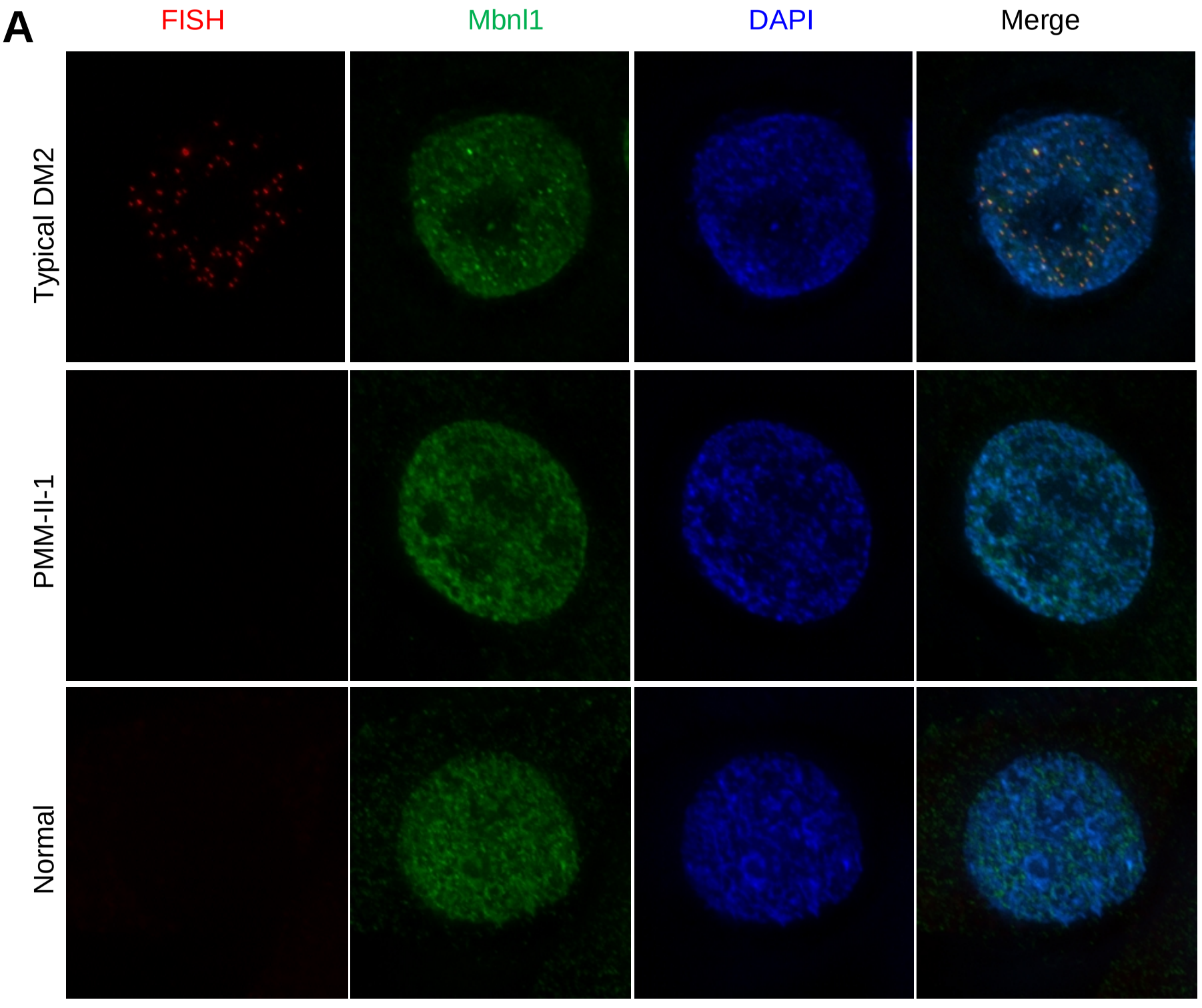
b
c


B
a
c
b
d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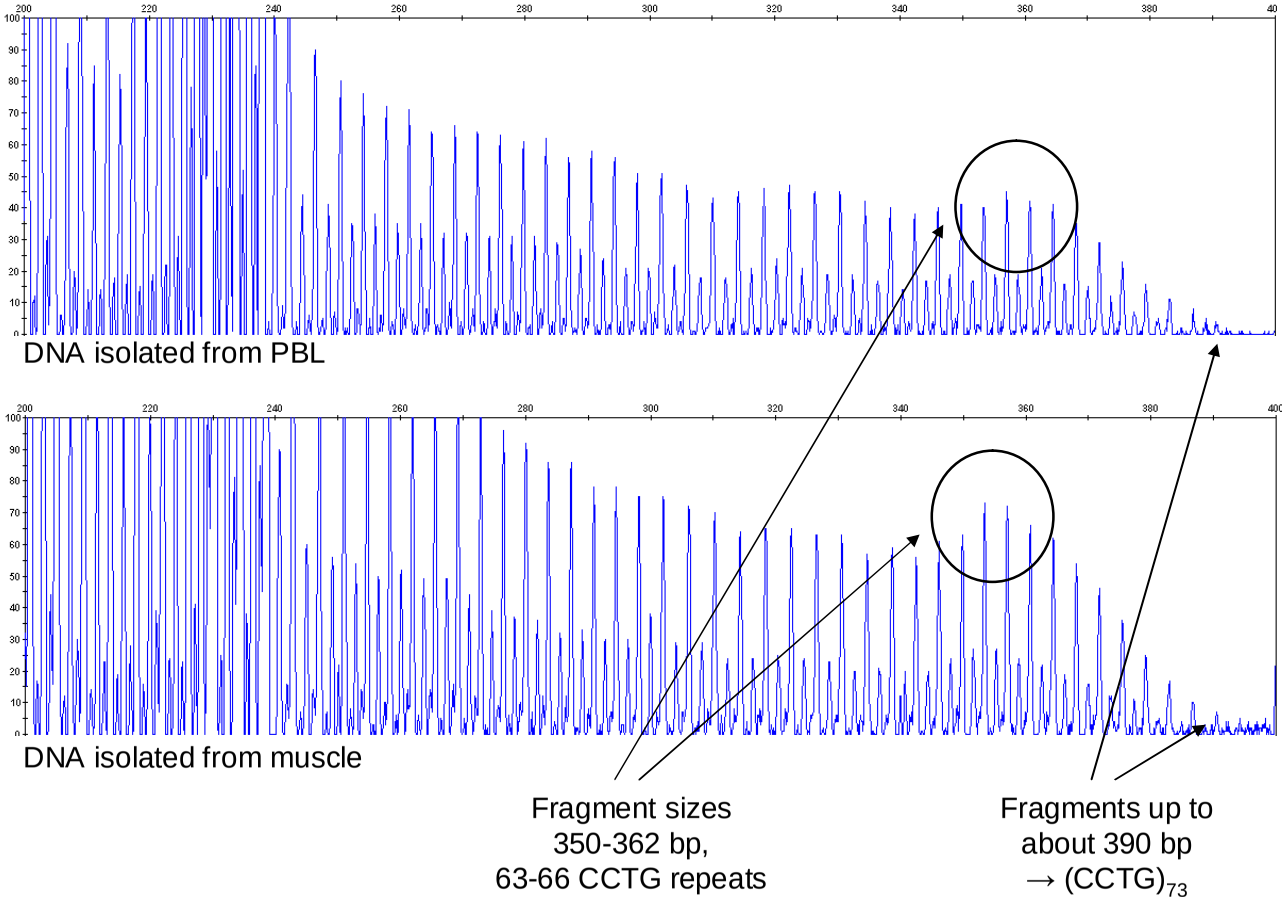
Mbnl1
DAPI
Merge
Typical DM2
PMM-II-1
Normal

B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DNA isolated from muscle
Fragment sizes
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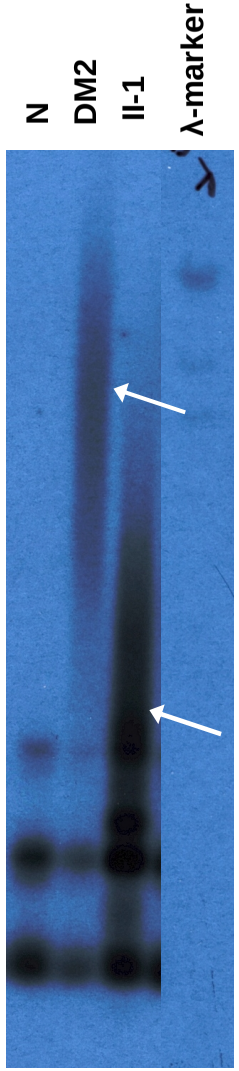
63-66 CCTG repeats
Fragments up to
about 390 bp
→ (CCTG)73

C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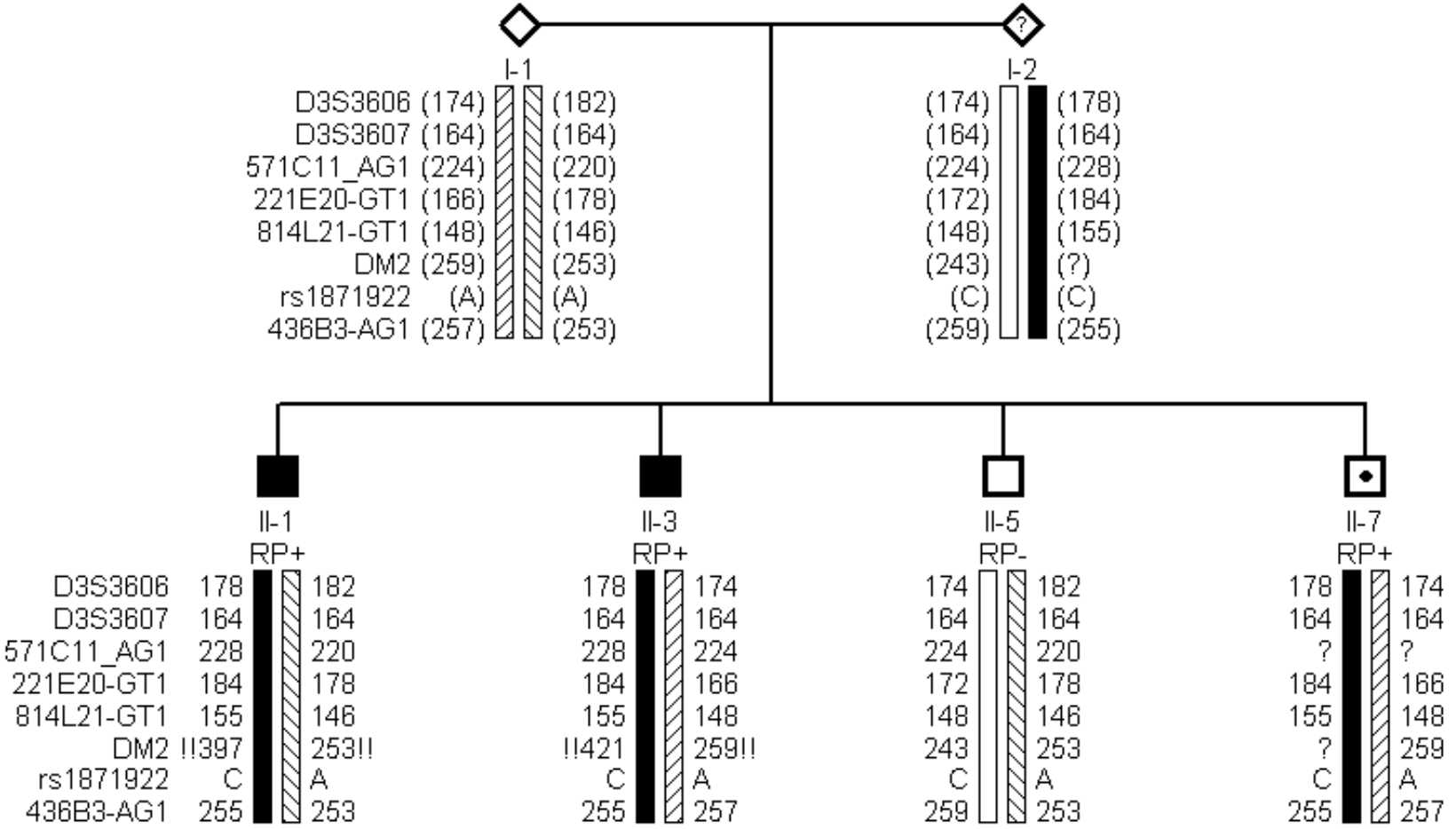
DM2
II-1
λ-marker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D3S3607 (164) (164)
571C11_AG1 (224) (220)
221E20-GT1 (166) (178)
814L21-GT1 (148) (146)
DM2 (259) (253)
rs1871922 (A) (A)
436B3-AG1 (257) (253)
I-2
(174) (178)
(164) (164)
(224) (228)
(172) (184)
(148) (155)
(243) (?)
(C) (C)
(259) (255)
II-1
RP+
D3S3606 178 182
D3S3607 164 164
571C11_AG1 228 220
221E20-GT1 184 178
814L21-GT1 155 146
DM2 !!397 253!!
rs1871922 C A
436B3-AG1 255 253
II-3
RP+
178 174
164 164
228 224
184 166
155 148
!!421 259!!
C A
255 257
II-5
RP-
174 182
164 164
224 220
172 178
148 146
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C A
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II-7
RP+
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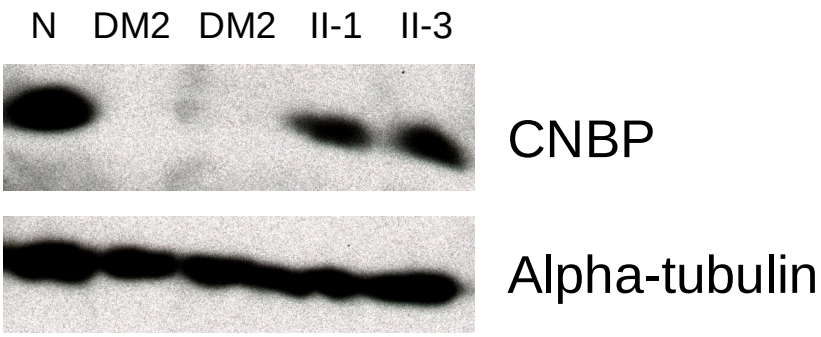
164 164
? ?
184 166
155 148
? 259
C A
255 257

5

A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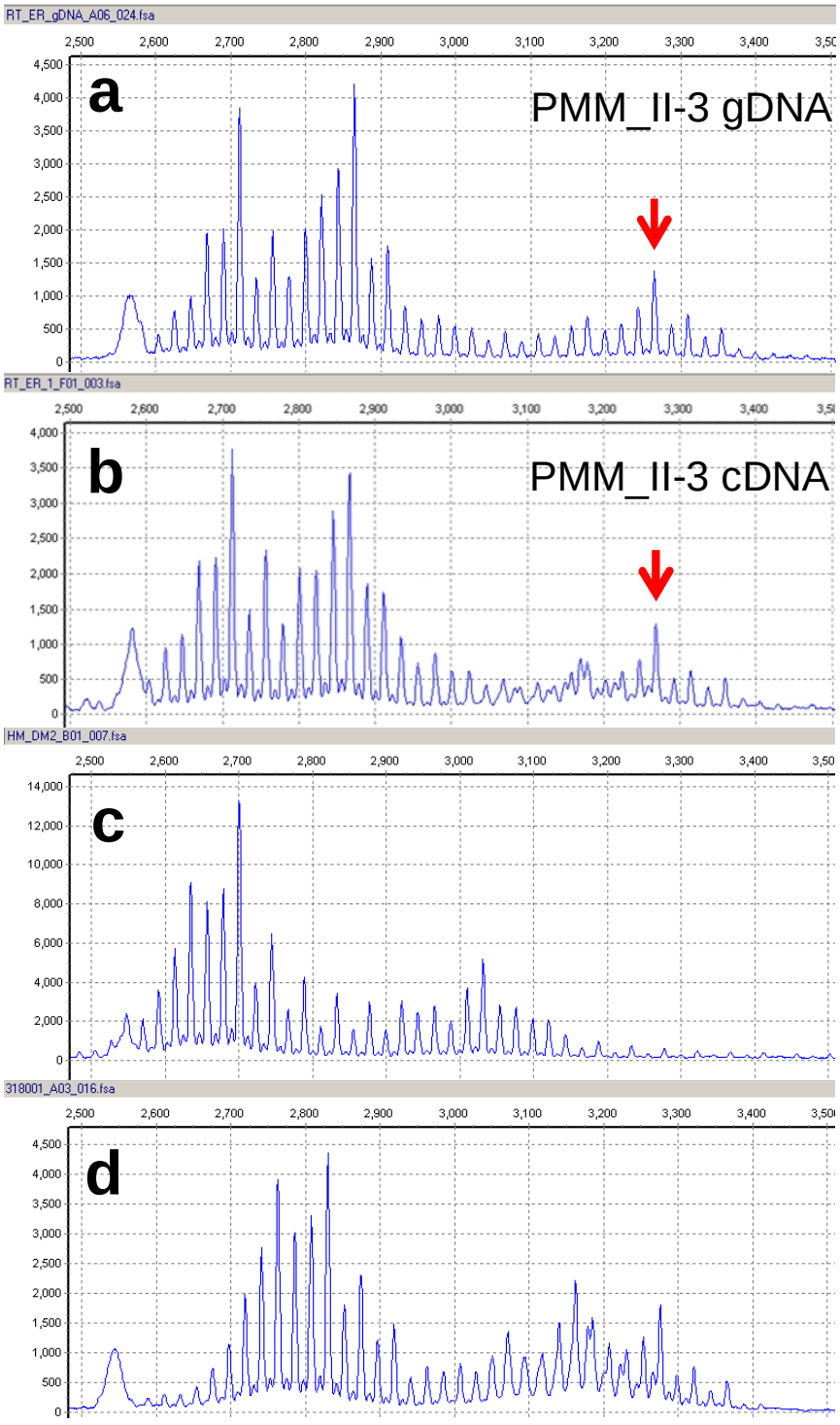
DM2
DM2
II-1
II-3
CNBP
Alpha-tubulin

B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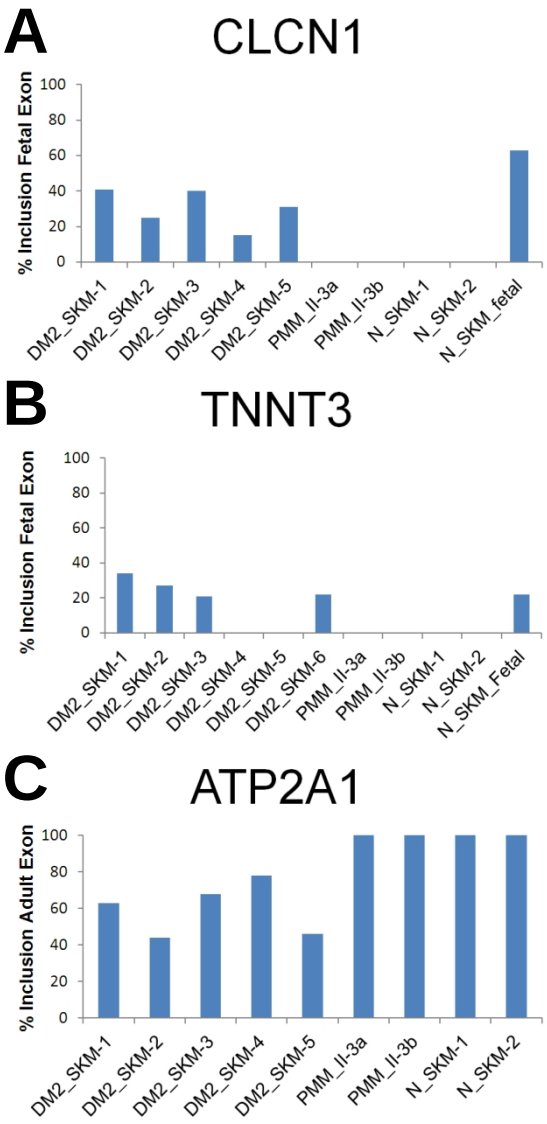
d

6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CLCN1
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80
60
40
20
0
DM2_SKM-1
DM2_SKM-2
DM2_SKM-3
DM2_SKM-4
DM2_SKM-5
PMM_II-3a
PMM_II-3b
N_SKM-1
N_SKM-2
N_SKM_fetal
B
TNNT3
% Inclusion Fetal Exon
100
80
60
40
20
0
DM2_SKM-1
DM2_SKM-2
DM2_SKM-3
DM2_SKM-4
DM2_SKM-5
DM2_SKM-6
PMM_II-3a
PMM_II-3b
N_SKM-1
N_SKM-2
N_SKM_Fetal
C
ATP2A1
% Inclusion Adult Exon
100
80
60
40
20
0
DM2_SKM-1
DM2_SKM-2
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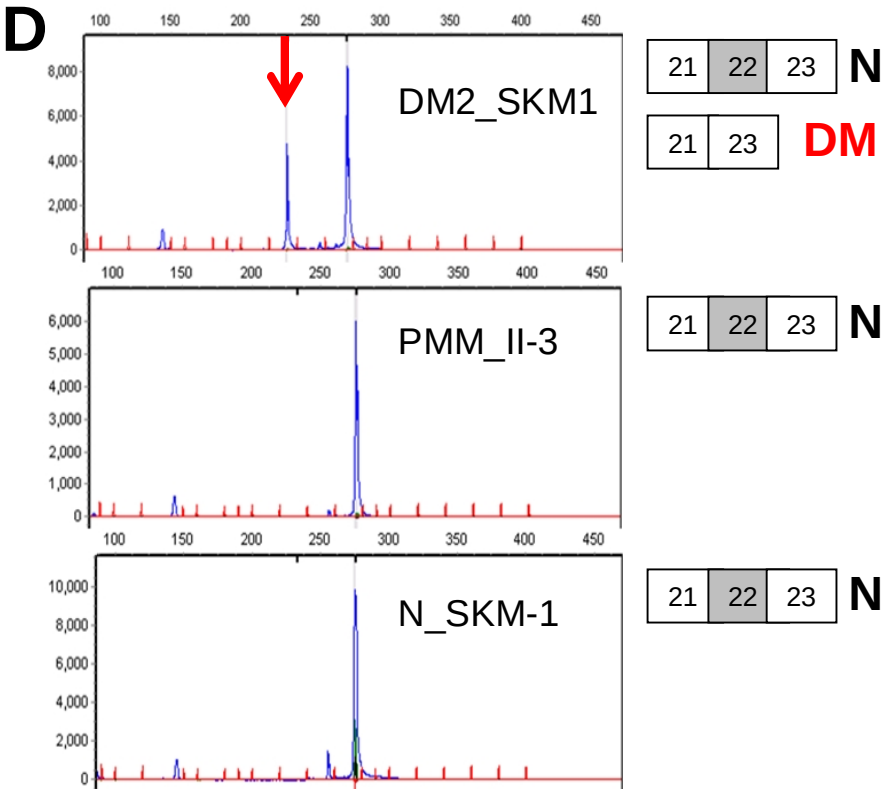
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23
N
21
23
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N

Supplementary figure 1