Myotonic Dystrophy

Disease Mechanism, Current Management and Therapeutic Development

Masanori P. Takahashi
Tsuyoshi Matsumura
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Editors
Masanori P. Takahashi
Department of Functional Diagnostic Science
Osaka University Graduate School of Medicine
Osaka
Japan

Tsuyoshi Matsumura
Department of Neurology
National Hospital Organization Toneyama National Hospital
Osaka
Japan

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Preface

Myotonic dystrophy (DM) is an uncharacteristic form of muscular dystrophy. Despite being a type of muscular dystrophy, the distribution of the affected muscles in DM greatly differs from that of general muscle disorders, which usually affect proximal muscles. The myotonic phenomenon, from which the disease's name is derived, is another cardinal feature. In addition, this condition not only involves the skeletal muscles, but also affects various organs throughout the body. This makes the overall clinical features of individual patients difficult to detect even for neuromuscular specialists. However, the multi-organ nature of the condition means that clinicians from various medical specialties may encounter it. Furthermore, patients are sometimes unaware of their symptoms and problems, which may prevent them from receiving appropriate care and lead to unexpected complications.

Along with other forms of muscular dystrophy, the understanding of DM pathology has greatly advanced since the discovery of its responsible genes. In 1992, the first genetic cause was identified as an abnormal expansion of CTG repeats located at the 3′-untranslated region of the *DMPK* gene. It was subsequently revealed that the second type of DM, DM2, is caused by the similar abnormal expansion of CCTG repeats located in intron 1 of the *CNBP* gene. The finding that similar clinical feature was caused by abnormal repeats in the untranslated region of two different genes, and also observed in experimental animals that expressed expanded repeats, has led to the notion that DM is caused by abnormal RNA. It was further shown that disruption of normal RNA regulation (splicing) in various genes occurs in DM, leading to the failure of multiple organs, even though the disease is caused by only one gene. Such deeper understanding of the underlying disease mechanisms prompted the therapeutic development and clinical trials that are currently being planned and implemented.

In the era of translational medicine, awareness of the importance of understanding DM and establishing a standardized approach to medical care has increased. It is essential to understand the burden and natural history of the disease and to accurately evaluate the effects of intervention. Furthermore, a comprehensive evaluation of therapeutic efficacy and adverse effects would become considerably difficult if the complications are not accurately recognized and addressed during routine

clinical practice. An accurate understanding of the overall picture of DM is required for both determining what is the optimal current strategy and how we can expand the therapeutic armamentarium in the future.

In Japan, a multidisciplinary approach to muscular dystrophy management has been in place for more than half a century. Clinical research has been extensively carried out at a specialized ward established at the National Hospitals with central involvement of the research consortium supported by the Ministry of Health, Labour and Welfare. As a result of this multidisciplinary approach, which includes respiratory and myocardial protection therapy, patients with Duchenne muscular dystrophy have experienced a marked improvement in their survival prognoses and ability to carry out their daily activities. While extensive clinical research on DM has also been conducted, awareness of its results by overseas audiences is low since most of them are reported in Japanese.

We believe that it is timely that a book by 13 Japanese experts that covers the results of clinical research on DM in Japan and the latest insights regarding disease mechanism, functional impairment, and disorders of various organs be published. We would like to express our deep appreciation of the authors as well as other colleagues and collaborators who contributed to the publishing of this book and hope that it will help clinicians and researchers to acquire a more comprehensive understanding of DM.

Finally, we would like to mention that many of the authors of this book have been mentored by the late Dr. Mitsuru Kawai, the former director of NHO Higashisaitama National Hospital, via the aforementioned research groups and similar academic activities. He was a great clinician and also an excellent mentor of ours. He translated *Myotonic Dystrophy* by Professor Peter Harper for Japanese patients and their families. He always taught us to keep exploring what we can do for patients as clinicians. Dr. Kawai's passing in 2016 was a huge loss to the Japanese muscular dystrophy healthcare community. It is our mission to continue his work through our consistent efforts, and it is in Dr. Kawai's memory that we would like to offer this book with our thoughts and gratitude.

Osaka, Japan — Tsuyoshi Matsumura
Osaka, Japan — Masanori P. Takahashi
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Chapter 1
Genetics of Myotonic Dystrophy

Tohru Matsuura

Abstract Myotonic dystrophy (dystrophia myotonica, DM) is the commonest form of muscular dystrophy affecting adults. This multisystem disorder typically affects the skeletal muscle and is characterized by weakness, wasting, and myotonia; other systemic involvement includes ocular, cardiac, endocrine, and central nervous system dysfunction. DM is classified into two main subtypes: type 1 (DM1) and type 2 (DM2) based on mutations in the dystrophia myotonica protein kinase (*DMPK)* gene and CCHC-type zinc-finger cellular nucleic acid-binding protein (*CNBP*) formerly known as the zinc finger 9 (*ZNF9)* gene, respectively. The multisystem phenotype of DM1 and DM2 is due to the presence of expanded repeats and the attendant effects. DM1 occurs due to the persistence of harmful effects of untranslated RNA transcripts of CTG trinucleotide repeat, which are located in the 3′-untranslated region of the *DMPK* gene on 19q13. DM2 results from the toxic effects of the untranslated RNA transcripts of CCTG tetranucleotide repeat, which are located in the primary intron of the *CNBP* gene, on chromosome 3q 21.3. A diagnosis of myotonic dystrophy can be made clinically based on presentation with characteristic features and a positive family history. However, molecular genetic testing for an expanded CTG repeat in the *DMPK* gene is the gold standard for definitive diagnosis of DM1. If DM1 testing is negative, testing for the CCTG repeat in the CNBP gene is then considered appropriate to establish a diagnosis of DM2.

Keywords Myotonic dystrophy · DM1 · DM2 · *DMPK* · *ZNF9* (*CNBP*) · Expanded repeats · Molecular genetic testing

T. Matsuura
Division of Neurology, Department of Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan
e-mail: tohrum@jichi.ac.jp

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Abbreviations

CNBP	Cellular nucleic acid-binding protein
DM	Myotonic dystrophy
DMPK	Dystrophia myotonica protein kinase
MBNL	Muscle blind-like
PROMM	Proximal myotonic myopathy
RNA	Ribonucleic acid
ZNF9	Zinc-finger nuclease 9

1.1 Introduction

Myotonic dystrophy (dystrophia myotonica, DM) is an autosomal dominant multisystem disorder and is the commonest form of muscular dystrophy in adults. The condition is clinically and genetically heterogeneous, typically affecting the skeletal muscle with characteristic paradoxical weakness, wasting, and myotonia [1]. Features of multisystem involvement include ocular, cardiac, endocrine, and central nervous system manifestations. And thus, affected individuals may present with early cataract, cardiac conduction abnormalities, insulin resistance, infertility, sleep disorders, and cognitive dysfunction.

Also, severe developmental disability has been reported in a severe congenital form of DM1 [2]. These multiorgan clinical features account for the initial presentation of such individuals to various medical subspecialties including internal medicine, cardiology, ophthalmology, endocrinology, and even pediatrics, before eventually undergoing specialist neurology review. DM is classified into two main forms, myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2), based on the genetic alteration and clinical features. Showing specifically wide variability in terms of severity and age of onset, the disease can also broadly be divided into congenital and adult forms.

The genetic defect in DM1 is a result of mutation in the dystrophia myotonica protein kinase (*DMPK*) gene with amplified trinucleotide CTG repeats in the 3' untranslated region. Disease severity varies with the number of repeats. In DM2 the defect is in the CCHC-type zinc-finger cellular nucleic acid-binding protein (*CNBP*) formerly known as the zinc-finger 9 (*ZNF9*) gene, with tetranucleotide CCTG repeats. Both of them are caused by microsatellite repeat expansions in noncoding regions of the genome giving rise to RNAs having a toxic gain of function among microsatellite expansion diseases encompassing more than 20 neurological disorders, including Huntington's disease and various spinocerebellar ataxias (Fig. 1.1). The multiorgan phenotype of DM1 and DM2 results from the presence of these expanded repeats and the attendant effects. DM1 occurs due to the persistence of harmful effects of untranslated RNA transcripts of CTG-trinucleotide repeats, which are located in the 3′-untranslated region of the DMPK gene on 19q13. DM2 results from the toxic effects of the untranslated RNA transcripts of CCTG-tetranucleotide repeats, which are located in the primary intron of the CNBP gene, on chromosome 3q 21.3.

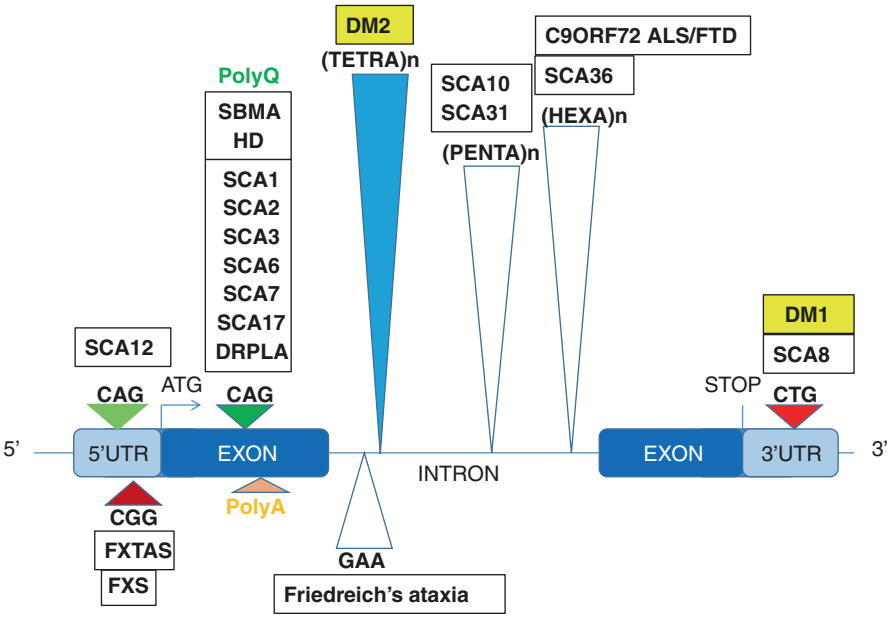

Fig. 1.1 Diseases caused by expanded microsatellite repeats. Abbreviations: *SCA* spinocerebellar ataxia, *FXS* fragile X syndrome, *FXTAS* fragile X-associated tremor/ataxia syndrome, *SBMA* spinal and bulbar muscular atrophy, *HD* Huntington's disease, *DRPLA* dentatorubral-pallidoluysian atrophy, *DM* myotonic dystrophy, *ALS/FTD* amyotrophic lateral sclerosis and frontotemporal dementia

The gene mapping, molecular genetics, genotype-phenotype correlation, and population genetics/epidemiology of DM are further expanded below, with discussions on the various phenotypes of DM1 and DM2 and the genetic basis for investigation and diagnosis.

1.2 Genetics

DM1 and DM2 are genetically distinct and are both inherited in an autosomal dominant manner and present with somewhat overlapping phenotypic abnormalities. The diseases may show variable penetrance, with characteristic increasing severity of phenotype over subsequent generations.

The underlying mechanism of the pathogenesis of DM is still unclear. However, studies show a role for the proliferation of transcription products in mutant forms of defective genes. The two genetically distinct subtypes, DM1 and DM2, are caused by similar noncoding repeat expansions in different genes.

In DM1, expanded repeats occur in CTG in the 3′-UTR of *DMPK* gene. In DM2, expanded repeats occur in the CCTG region in the primary intron of the *CNBP* gene (formerly known as zinc-finger protein 9; *ZNF9*) [3–5]. The DM1 and DM2 phenotypes result from abnormalities in the transcribed RNA equivalents, CTG-to-CUG for DM1 and CCTG-to-CCUG for DM2.

In DM1 and DM2, the abnormal RNA transcripts remain untranslated. They exert a harmful effect on other genes not unique to the DM1 or DM2 locus and aggregate other proteins involved in alternative RNA splicing. These proteins are known as muscle blind-like (MBNL) proteins and CUG-BP and ETR-3-like factors (CELF) [4, 6–8]. MBNL proteins are highly conserved across species including man.

The *MBNL* gene encodes MBNL proteins, responsible for muscle development and photoreceptor neuron differentiation in the eye. Thus, absence of the MBNL gene results in blindness and muscle defects. In patients with DM1 and DM2, MBNL proteins accumulate in the RNA foci due to the activity of the mutated RNA transcripts of CUG or CCUG repeats. Because of this sequestration, the amount of MBNL proteins available for proper function is reduced. A fetal pattern of target transcripts results due to the mutant RNA-induced shift in splicing from normal to abnormal. These mutant RNA transcripts further exert an adverse effect on RNA-binding protein activity, leading to errors in transcript splicing and defects in the function of several genes such as the bridging integrator 1 gene (BIN1) [9], cardiac troponin T [10], insulin receptor [11], and the skeletal muscle chloride channel [12]. These could result in the typical features of abnormalities in cardiac conduction, myotonia, and insulin resistance seen in individuals with DM [4, 6, 7].

1.3 DM1

The genetic defect in DM 1 results from a heterozygous trinucleotide repeat expansion (CTG)n in the 3′ UTR of the DMPK gene on chromosome 19q13. Repeat lengths in excess of 50 CTG repeats are considered pathogenic [13].

DM1 phenotypes can be classified as congenital, childhood-onset, adult-onset "classic DM1," late-onset/asymptomatic, and premutation DM. Table 1.1 presents an overview of these phenotypes, clinical findings, and CTG length [14].

Clinical features are slowly progressive, and clinical severity varies broadly ranging from asymptomatic to severe. Symptoms usually appear in the second and third decades of life for the most common classic form of DM. Molecular pathways are still unclear.

1.3.1 Mapping

The myotonic dystrophy locus was among the earliest human disease loci to be assigned a chromosome by linkage analysis. Linkage was first suspected between the Lutheran blood group (Lu) and the secretor (Se) loci by Mohr [15]. It was later discovered that complement component 3 (C3) was linked to Lu-Se-DM [16, 17]. C3 had earlier been assigned to chromosome 19 by somatic hybrid studies, and thus this linkage indicated that DM is also on chromosome 19. Subsequently, positive LOD scores were discovered for serum C3 and another chromosome 19 locus, peptidase D

Table 1.1 Summary of myotonic dystrophy type 1 phenotypes, clinical findings, and CTG length

Phenotypes	Clinical findings	CTG length	Age of onset
Congenital	Infantile hypotonia	>1000	Birth
	Respiratory failure		
	Learning disability		
	Cardiorespiratory complications		
Childhood onset	Facial weakness	50–1000	1–10 years
	Myotonia		
	Low IQ		
	Conduction defects		
Adult-onset "classic DM1"	Weakness	50–1000	10–30 years
	Myotonia		
	Cataracts		
	Conduction defects		
	Insulin resistance		
	Respiratory failure		
Late onset/asymptomatic	Mild myotonia	50–100	20–70 years
	Cataracts		
Pre-mutation	None	38(35)–49	N/A

(PEPD). PEPD was also assigned to chromosome 19 by somatic cell hybrid studies, and a close linkage was demonstrated between PEPD and DM locus with a LOD score of 3.51 at a recombination fraction of 0.00 [16]. This provided regional assignment and further confirmed the Lu-Se-DM assignment to chromosome 19. Restriction fragment length polymorphism analysis with an associated C3 probe showed evidence of linkage with DM with a LOD score of +3.36 at a recombination fraction of 0.05 in males [17]. Further gene mapping of chromosome 19 with regard to DM revealed suppression of recombination near the centromere, and linkage studies assigned DM a location in the centromere region of chromosome 19 [18].

RFLPs were designated to the D19S19 locus that is linked to DM (maximum LOD score of 11.04 at a recombination fraction of 0.0). The genomic clone LDR152 (D19S19) was reported to be tightly linked to DM with a maximum LOD score of 15.4 at a recombination fraction of 0.0 (95% confidence limits 0.0–0.03) [19, 20].

Friedrich et al. conducted linkage studies in three large kindreds, using RFLPs related to *C3* and the chromosome 19 centromeric heteromorphism as genetic markers, and excluded DM from the 19cen-C3 segment by three-point linkage analysis, thus strongly reinforcing the assignment to the proximal long arm of chromosome 19 [21].

It was conclusively established that the DM gene lies in region 19q13.2–q13.3. The APOC2 and CKM were identified as the closest proximal markers located approximately 3 cM and 2 cM from DM, respectively, in the order cen–APOC2–CKMM–DM. Furthermore, among 12 polymorphic markers on 19q, 10 were revealed to be proximal to the DM gene, and 2 were distal to DM, PRKCG (176980), and D19S22 (located at a distance of approximately 25 cM and 15 cM, respectively, from DM) [22].

1.3.2 Molecular Genetics of DM1

Previously known as Steinert disease, DM1 is inherited in the non-Mendelian autosomal dominant pattern, with variable penetrance and transmission between mother and child. A low frequency of about 5–34 copies of the trinucleotide repeats is typical in the general population. In patients with DM1, a greater number of repeats, ranging from upward of 50 copies, are reported [6, 23]. This may explain the parallel seen between the varying severity of DM with the age of onset and the number of repeats. These expanded repeats are unstable and exhibit intergenerational expansion; further expansion may occur during meiosis with an increase in the repeat size during parent-to-child transmission within successive generations [24]. Thus, at-risk offspring may inherit sizably longer repeat lengths than those present in the transmitting parent. Based on these, DM1 is described as showing a characteristic anticipation and exhibiting parental gender effect/maternal bias.

Anticipation is a phenomenon where the increase in repeat size during parent-to-child transmission within successive generations results in increasing disease severity and decreasing age of onset in successive generations. This anticipation results because DMPK alleles of over 34 CTG repeats in length are unstable. Most often a child with early-onset, severe DM1 (i.e., congenital DM1) has inherited the expanded DMPK allele from the mother [25, 26, 27]. Although anticipation typically occurs in maternal transmission of the disease, anticipation with paternal transmission is also possible [25, 28]. Disease severity is directly proportional to the number of repeats: normal individuals have 5–34 repeats, individuals with mild disease have 50–150 repeats, patients with classic DM have 100–1000 repeats, and patients with congenital DM exhibiting over 2000 repeats. The size of the CTG repeat appears to increase over time in the same individual. Somatic instability of the repeat is also recognized as intra-tissue, inter-tissue, and cell-type variability over a patient's life time [29, 30].

Congenital DM is seen solely when the affected parent is the mother. This maternal bias occurs because expansion of alleles with 40–80 repeats typically occurs in paternal transmissions; however, expansion is seen only in alleles longer than 80 repeats when transmission is maternal. The frequency of repeat contractions is approximately 4.2–6.4% [13].

In congenital DM cases, the CTG-repeat lengths are unusually high, at >1000, and as high as 4000 expanded repeats can occur [6]. Fewer repeat lengths (730–1000) have been reported, but the affected infants may present with infantile hypotonia, mental retardation, and respiratory dysfunction. A decrease in the CTG-repeat size (intergenerational contraction) has been also reported in about 6.4% of transmissions [31]. A paternal factor may contribute to the dynamics of intergenerational contraction, observed in the expanded CTG repeats in DM1. Among the French-Canadian DM1 population, about 7.4% display intergenerational contractions, during transmission. All the cases in this cohort were transmitted from father to child [31]. The intergenerational contraction seen in DM1 could occur within some [32] or all [31] related siblings in a family.

Historically, the isolation of a human genomic clone that detected novel restriction fragments specific to persons with myotonic dystrophy was reported in 1992 [33]. An *Eco*RI polymorphism with two alleles was seen in normal persons, but in most affected individuals, one of the normal alleles was replaced by a larger unstable fragment. Fragment length showed significant variation within families as well as between unrelated affected individuals. The unstable nature of this region was thought to explain the characteristic variation in severity and age at onset of DM.

Subsequently, a DNA fragment was detected that was larger-sized in DM1 affected persons compared with normal sibs or controls. The sequence containing this fragment was located in chromosome 19 and was flanked by two tightly linked markers, ERCC1 (126380) proximally and D19S51 distally [34]. The essential region between the above-mentioned markers was cloned in a 700-kb contig comprising overlapping cosmids and yeast artificial chromosomes. The central part of the contig bridged an area of about 350 kb between two flanking crossover borders. This segment, which presumably contained the DM gene, has been extensively characterized. Two genomic probes and two homologous cDNA probes were situated within approximately 10 kb of genomic DNA and detected an unstable genomic segment in myotonic dystrophy patients. The length variation in this segment showed similarities to the instability seen in the fragile X locus (300624). The discovery of these changes in repeat expansion in families with fragile X syndrome strongly suggested the possibility that a similar mutation with unstable microsatellite expansion might be involved in the pathogenesis of DM. Subsequently, the CTG expansion was identified [5]. The authors proposed that the length variation was consistent with a direct role in the pathogenesis of DM.

Typically, the size of the pathognomonic CTG triplet repeat is larger in DM patients than in unaffected individuals [22]. The sequence shows high variability among normal populations. Unaffected individuals carry 5–27 copies. Patients with mild disease have 50 or more repeats, and patients with the phenotypically more severe classic DM typically carry repeat expansions of up to several kilobase pairs.

The length of the CTG repeat correlates with the incidence of severe congenital DM. Furthermore, mothers of individuals with congenital DM were found to have higher than average CTG repeat lengths [35].

Recently, using triplet-primed PCR (TP-PCR) of both DNA strands followed by direct sequencing, Musova et al. identified interruptions within expanded DM1 CTG repeats in almost 5% (3 of 63) of Czech DM1 families and in 2 of 2 intermediate alleles [13]. None of 261 normal Czech alleles tested carried interruptions. The expanded alleles contained either regular runs of a (CCGCTG)n hexamer or showed a much higher complexity; they were always located at the 3-prime end of the repeat. The number and location of the interruptions were very unstable within families and subject to substantial change during transmission. However, four of five transmissions of the interrupted expanded allele in one family were accompanied by repeat contraction, suggesting that the interruptions render the DMPK CTG repeat more stable or could even predispose it to contractions. Overall, the contribution of the interrupted alleles to the phenotype was uncertain. Musova et al. suggested that the occurrence of interruptions may be missed by routine testing using PCR or Southern blotting.

1.3.3 Genotype-Phenotype Correlations

1.3.3.1 Congenital DM

Clinically, congenital DM presents with infantile hypotonia, respiratory failure, learning disability, and cardiorespiratory complications. For congenital DM1, an estimated incidence of 2.1–28.6 per 100,000 live births has been reported based on accumulated studies [6]. Onset of typically symptoms present at birth. The majority of congenital DM cases show a maternal transmission pattern, due to a higher probability for expanded CTG repeats in mothers compared with fathers [3, 6, 8]. The mean length of maternal trinucleotide CTG repeats is greater in congenital DM cases compared with adult-onset DM with CTG expansions in excess of 1000 repeats [32]. This is in contrast with paternal trinucleotide repeats, which are smaller and/or show no symptoms at childbirth [6]. Maternal DM can go undetected for most of adult life, with a diagnosis made only after the birth of a neonate with congenital DM [5, 6, 24].

Evidence of congenital DM1 may be detected in utero, with features of clubfoot, polyhydramnios (common in mostly severe cases with swallowing problems in infancy), cardiomyopathy (severe cases), and reduced fetal movement. The mother may experience prolonged labor, likely due to abnormalities in the uterine muscle [3, 5]. Preterm (<34 weeks) and prolonged labor is likely in DM1-affected women, with a resultant increase in the rate of cesarean delivery [6]. Other complications of pregnancy have been reported in DM1. The occurrence of preterm labor was attributed in part to the effects of congenital DM on affected fetuses. Other pregnancy complications have been reported in both DM1 and DM2. In one study of pregnancies in women, ectopic pregnancy (4%), placenta previa (9%), and postpartum hemorrhage (rare) were reported. Uterine atony due to myotonia and muscle weakness may account for the postpartum hemorrhage observed in this cohort, although it is a rare occurrence in DM1-affected individuals [6].

Facial diplegia (bilateral facial paralysis) with a typical V-shaped upper lip, marked hypotonia, poor feeding, respiratory failure, and joint contractures (mainly in the legs) are all typical features of congenital DM1. Muscle weakness and facial diplegia persist into early childhood with a slow and progressive increase in motor function.

Feeding difficulties may necessitate nasogastric feeding and intensive management. Death may occur in the neonatal period due to respiratory abnormalities or in infancy due to abnormalities in cardiac function and feeding [4, 6]. Affected neonates and infants may have to be managed with a feeding tube and mechanical ventilation, in a significant number of cases [6].

Abnormalities of the foot as well as mental and behavioral disorders are common clinical presentations in early childhood (aged 3–5 years). Marked developmental delays in mental and motor development occur in half or more children at this stage. Survival into early childhood is fraught with marked morbidities in the cardiovascular and respiratory systems with a high likelihood of deaths.

The severity of complications in congenital DM1 shows no association with the range of abnormalities that may be observed in teens presenting with the classic, non-congenital DM phenotype. In these cases, abnormalities in muscular and cardiac function present with symptoms and signs of myotonia, muscle weakness (mainly in the lower parts of the body), and cardiac deficits.

Childhood-Onset/Juvenile DM1 In childhood DM1, the CTG-repeat lengths are usually greater (>800), with muscle weakness and physical disability developing later in life, usually before age 10 years [1, 5, 6, 32]. Cases in which the repeat lengths were lower than 800 have been reported [6]. In addition to musculoskeletal abnormalities, behavioral, cardiovascular, and mental complications are typical in childhood DM. Children with cardiovascular complications can go undiagnosed. Behavioral problems can manifest as attentional deficit, anxiety, executive dysfunction, low IQ, and mood disorder.

Adult-Onset Classic DM1 Individuals with classic DM1 have CTG-repeat sizes of 50–1000. Classic DM develops between the first and fourth decades of life, but in the adult-onset classic type of DM, muscle weakness develops later in life [1, 6]. Cataracts, cardiac arrhythmia, excessive daytime sleepiness, and myotonia are presenting features in individuals with classic DM1. Affected individuals have a lower average life expectancy.

Late-Onset/Asymptomatic DM1 Individuals with mild DM also display a CTG-repeat size of 50–150. The onset of DM usually occurs in later years (20–70 years), and individuals have a normal life expectancy. Features common to DM are typical of individuals with mild DM, including cataracts, mild weakness, and myotonia [6]. In a multicenter study, a high sensitivity for detection and reporting of expanded repeats was observed [36]. Also, a high specificity to within two repeats of the consensus was reported in allelic examinations [36]. The ability of genetic testing to accurately define the presence or absence of DM is central to DM management, as a cure is yet to be developed.

More than half of asymptomatic DM cases go undetected throughout childhood, only to have an arrhythmia induced in adolescence due to participation in sports and physical activities. Children of individuals with asymptomatic DM have a high risk of intergenerational expansion or activation, with larger repeat sizes and probability of having symptoms.

Premutation DM1 Generally, the presence of 5–37 repeats is considered normal.

Individuals with premutation DM1 display repeat sizes ranging between 38 and 49. Symptoms begin to manifest in individuals with greater than 50 repeats. Individuals with CTG expansions in the premutation range have been reported to be asymptomatic but are at increased risk of having their children inherit further expanded repeats and thus having symptomatic disease.

1.3.4 Population Genetics and Epidemiology

In general, the prevalence of DM1 ranges from approximately 1:100,000 in certain parts of Japan to 1:10,000 in Iceland; the global estimated prevalence of DM1 is reported to be 1:20,000 [37]. Prevalence may increase in specific regions, such as Quebec, and this is thought to be as a result of founder effects [38, 39]

1.4 DM2

After the discovery of DM1 gene defect, DNA testing identified a group of patients lacking this defect but showing dominantly inherited myotonia, proximal greater than distal weakness, and cataracts. In Europe, this disease was termed proximal myotonic myopathy (PROMM, OMIM 602668) [40, 41] or proximal myotonic dystrophy (PDM) [42], while in the United States, it was termed myotonic dystrophy with no CTG-repeat expansion or myotonic dystrophy type 2 (DM2) [43, 44, 45]. Subsequently, it was demonstrated that many of the families identified as having PROMM, PDM, or DM2 had the same disease that results from an unstable tetranucleotide CCTG-repeat expansion in intron 1 of the nucleic acid-binding protein (CNBP) gene (previously known as zinc-finger 9 gene, ZNF9; OMIM 116955) on chromosome 3q21 [46].

1.4.1 Mapping

The locus for DM2 is located on the 3q 21.3region. In one study of nine German families, linkage analysis based on DNA markers (D3S1541 and D3S1589) showed a close genetic link or allelism between PROMM and DM2, both located on the long arm of chromosome 3 [40].

In study of a five-generation family with myotonic dystrophy [44, 45], Ranum et al. found that the disease locus, DM2, mapped to a 10-cM region of 3q. In addition to excluding the DM1 locus on chromosome 19 in the large family reported by Ranum et al. [44], Day et al. subsequently excluded the chromosomal regions containing the genes for muscle sodium and chloride channels that are involved in other myotonic disorders [45].

Another report described a Norwegian PROMM family in which the proband was clinically diagnosed with myotonic dystrophy but lacked the pathognomonic (CTG)n expansion [41]. Haplotype analysis suggested exclusion of the DM2 locus as well, perhaps indicating further genetic heterogeneity. Interestingly, all family members, affected and unaffected, were heterozygous for the arg894-to-ter (R894X) mutation in the CLCN1 gene [47]. The authors noted that Mastaglia et al.[48] had reported the R894X mutation in only one of two children with PROMM, indicating that it was not the disease-causing mutation in that family: they had termed it an

incidental finding. Furthermore, Sun et al. suggested that their findings, combined with those of Mastaglia et al., likely reflected a fairly high carrier frequency in the population, and they presented preliminary data indicating an R894X allele frequency of 0.87% (4/460) in northern Scandinavia [41].

1.4.2 Molecular Genetics

DM2 is caused by expansion of a tetranucleotide CCTG repeat in intron 1 of the CNBP gene on chromosome 3q21 [46]. Patients with DM2 exhibit a wide range of phenotypes that include myotonia, muscle weakness, cardiac anomalies, cataracts, diabetes mellitus, and testicular failure [49]. In a normal allele, the repeat shows a complex motif with an overall configuration of (TG)n(TCTG)n(CCTG)n. The number of CCTG tracts is less than 30, with repeat interruptions of GCTG and/or TCTG motifs [46], and is stably transmitted from one generation to the next. However, in the expanded allele, only the CCTG tract elongates, and the GCTG and TCTG interruptions disappear from the repeat tract. The sizes of expanded alleles are extremely variable, ranging from 75 to 11,000 repeats, with a mean of 5000 repeats. The expanded DM2 alleles show marked somatic instability, with significant increases in length over time [46]. Over 25% of affected individuals have two or more CCTG expansion sizes that can be detected in peripheral blood. Because of this somatic heterogeneity of the repeat size, it can be difficult to establish a pathogenic threshold, and thus affected individuals with the shortest identified CCTG-repeat expansion on one allele (approximately 75 CCTG repeats or 300 bp) would also have an allele with an extremely sizeable CCTG expansion with over 11,000 CCTG repeats (or 44,000 bp); any or both of the expanded alleles could be pathogenic. In DM2, the CCTG expansion usually contracts in the next generation, with no significant differences determined by the gender of the transmitting parent. This may explain the missing of congenital form and the lack of genetic anticipation in DM2.

Three classes of large non-DM2 alleles have been identified [50]. They include short interrupted alleles of up to 24 repeats in CCTG (with up to 2 interruptions), long interrupted alleles of up to 32 repeats in CCTG (with up to 4 interruptions), and uninterrupted alleles of 23–33 repeats in CCTG (with lengths of 92–132 base pairs). The instability common to these large, non-DM2 repeat alleles was higher in the uninterrupted alleles compared to interrupted alleles [50].

Analyses based on single-nucleotide polymorphisms showed a similar haplotype for expanded DM2 repeats and the three classes of large non-DM2 repeat alleles. Thus, a premutation allele pool from the unstable interrupted CCTG alleles may explain the full mutations seen in individuals with DM2. Large non-DM2 classes are more common among African-Americans (8.5%) than European-Caucasians (<2%) [50].

The DM2 CCTG repeat was originally reported to be derived from the *Alu* element insertion, similar to two other repeat expansion disorders, Friedreich's ataxia and spinocerebellar ataxia type 10, posing challenges regarding the mechanisms underlying development of *Alu*-mediated repeats into large, highly unstable expansions common to all three disorders [51].

Although the mechanisms responsible for this unique instability are mostly unknown, the uninterrupted CCTG repeat tends to form a stable hairpin/dumbbell DNA structure with expansion due to an error in the recombination-repair mechanism [52, 53] DM2 CCTG*CAGG repeats are crossover hotspots that are more prone to expansions in comparison with the DM1 CTG*CAG repeats in *Escherichia coli* [52, 56].

1.4.3 Genotype-Phenotype Correlations

Different from DM1, no significant correlation exists between CCTG repeat size and age of onset of muscle weakness or other indices of disease severity (such as age at cataract extraction). The fact that phenotype in individuals with CCTG-repeat expansions in both *CNBP* alleles is as severe as those seen in their heterozygous sibs and parents goes to underscore that CCTG repeat number does not modify the clinical course [54].

In DM2, there is no clear correlation between repeat size and the age of the individual at the time that repeat size is measured, demonstrating that repeat length increases with age [46, 49, 55].

Among families that participated in the original characterization of DM2, the severity of clinical features were reported to have increased with successive generations [49, 55]. Data suggested that this was caused by the phenomenon earlier described phenomenon of anticipation (where individuals in successive generations tend to present at an earlier age and/or with more severe phenotype or clinical features) rather than bias constituted ascertainment (inadvertent inclusion of more severely affected younger-generation family members in the study).

However, molecular genetic testing for the *CNBP* gene showed that there was no obvious congenital form of DM2 analogous to the congenital form of DM1, which established the role of anticipation in that disease. Furthermore, the lack of correlation between disease severity and CCTG repeat length underscores the observation that intergenerational changes in repeat length in successive generations are unlikely to be associated with a definitive increase in disease severity [56].

1.4.4 Population Genetics and Epidemiology

Myotonic dystrophy is the most common adult form of muscular dystrophy, with an estimated incidence of approximately 1 in 8000 in the general population. The exact proportion of myotonic dystrophy represented by DM1 and the milder version DM2 are as yet unknown. Also, the varying range of severity of clinical features obscures the incidence of DM2. An incidence similar to DM1 has been reported in Europe, although it is likely lower in the United States [6].

Prevalence apparently differs in different populations, but comprehensive demographic studies in this regard are limited. The prevalence of DM2 has been reported

to be higher in Germany and Poland and in individuals of either German or Polish descent [57] and is reported to be 1:1830 in Finland where the incidence of DM2 is markedly higher than that of DM1 [58]. There have been reports of cases of DM2 in Afghanistan and Sri Lanka, but the condition has not been observed in China, or sub-Saharan Africa. The majority of DM2 mutations have been identified in Caucasians of European descent that are known to have descended from a single common founder and share an identical haplotype [59, 60, 61]. Nevertheless, a previous study has identified the first Japanese DM2 pedigree harboring a distinctive haplotype different from that shared by Caucasians, suggesting the occurrence of DM2 in non-Caucasian populations as well, and that this likely has separate founders [62, 63]. Thus, it would be beneficial to examine whether there is phenotypic haplotypic comparability among DM2 patients from different ethnicities compared with the predominantly European patients that share a common haplotype.

1.5 Genetic Basis for Diagnosis

DM1 and DM2 are clinically and genetically similar but distinct disorders requiring different diagnostic and treatment strategies [3], and thus diagnosis is based on suggestive clinical features and testing, which comprises genetic and nongenetic tests. As is the case with all genetic disease, the key to diagnosis is confirming the presence of the causative mutation by genetic testing. Molecular genetic testing is the gold standard for establishing a diagnosis of DM.

The clinical diagnosis of DM1 can be made where characteristic clinical features such as muscle weakness, demonstrable progressive distal and bulbar dystrophy with myotonia, frontal balding, and cataracts in addition to a positive family history are present. However, clinical diagnosis can be difficult in mild cases, but a high index of suspicion should be maintained in atypical cases of DM, where muscle weakness is absent or a family history is lacking [6]. Clinical diagnosis can be further confirmed by demonstration of depressed serum IgG and elevated CPK as well as other ancillary tests.

For DM2, disease onset is characteristically in adulthood with generally milder symptoms than DM1, and the phenotype clinical manifestations are variable, with early-onset cataracts (before 50 years of age), proximal weakness, varying grip myotonia, as well as demonstrable autosomal dominant inheritance.

1.5.1 Genetic Testing

The major indications for genetic testing for DM1 are as recommended by the International Myotonic Dystrophy Consortium (IDMC) [64].

Genetic testing for DM can be confirmatory/symptomatic, preclinical/asymptomatic, prenatal testing, and preimplantation testing [65]. The procedure must be accompanied by genetic counseling.

1.5.2 Molecular Genetic Analysis

This is the gold standard for the diagnosis of DM [6]. Targeted analysis for pathogenic variants for the presence of expanded repeats for CTG in the DMPK gene (DM1) confirms a diagnosis of DM1. The number of DMPK CTG trinucleotide repeats can be quantified by PCR analysis for expanded alleles with about 100–150 CTG repeats. Southern blot analysis is used for detection of larger CTG expansions. The absence of the CTG-repeat expansion warrants tests for expanded repeats for CCTG in the CNBP gene, a characteristic of DM2.

However, it is requisite to identify the presence of CNBP, which is the only gene in which mutation is known to cause DM2. The CNBP intron 1 carries a complex repeat motif, (TG)n(TCTG)n(CCTG)n, and expansion of the CCTG repeat is causative of DM2 [46]. Because the extremely large size of the expansions in DM2 and marked somatic instability render Southern blot analysis and its interpretation difficult, a repeat-primed PCR assay was developed by Day et al. [49] and was found to increase molecular detection rate of DM2 to 99%. In comparison to laborious and time-consuming Southern blot, this simple PCR assay cannot determine the size of the expansion but can readily and speedily detect the mutational status (the existence of expansion). Recently, a simultaneous repeat assay for both DMPK CTG and CNBP CCTG expansion has also been developed [66].

1.5.3 Prenatal Testing

Individuals at risk of DM should be tested and advised of the options available to them. They should be allowed to make an informed decision concerning the outcome of prenatal tests [1, 10].

1.5.4 Preimplantation Genetic Diagnosis

Where available and in cases where the pathologic expanded repeats have been confirmed, evaluation of the genetic makeup of the fetus at implantation could further assist the parents in making an informed decision [1, 10].

1.6 Conclusion

DM is the most common adult muscular dystrophy, characterized by autosomal dominant progressive myopathy, myotonia, and multiorgan involvement. To date, two distinct forms caused by similar mutations in two different genes have been

identified: DM1 and DM2. Aberrant transcription and mRNA processing of multiple genes due to RNA-mediated toxic gain of function have been suggested to cause the complex phenotype in DM1 and DM2. Although the size of the respective repeated DNA sequence was thought to correlate with disease severity and age of onset, the size of the repeats alone does not explain all the differences in DM phenotypes. This shows the importance of considering other factors to regulate disease phenotype in DM.

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Chapter 2
Molecular Mechanisms of Myotonic Dystrophy: RNA-Mediated Pathogenesis and RNA-Binding Proteins

Yoshihiro Kino, Jun-ichi Satoh, and Shoichi Ishiura

Abstract Since the identification of a repeat expansion as a causative mutation of myotonic dystrophy (dystrophia myotonica, DM) type 1 (DM1), several pathomechanisms have been proposed. Among them, an RNA-mediated mechanism is thought to play a central role in the pathogenesis of DM. In both DM1 and DM type 2 (DM2), mutated alleles produce transcripts containing an expanded CUG (DM1) or CCUG (DM2) tract. These aberrant RNAs accumulate in nuclei and form ribonuclear inclusions or nuclear RNA foci, a pathological hallmark of DM. These transcripts sequester muscleblind-like (MBNL) proteins, which regulate alternative splicing and other RNA processing. Loss of function of MBNL proteins in mouse models recapitulates many aspects of DM. In this chapter, the pathomechanisms of DM1 and DM2 are discussed, with an emphasis on the roles of RNA-binding proteins together with recent findings in this field.

Keywords RNA foci · MBNL proteins · CELF proteins · Alternative splicing · RAN translation · RNA gelation

2.1 Toxic Repeat RNAs as the Primary Cause of Myotonic Dystrophy

2.1.1 Does Repeat Expansion Have Cis-regulatory Effects on DMPK *and/or Its Flanking Genes?*

Myotonic dystrophy (dystrophia myotonica, DM) type 1 (DM1) is caused by an expansion of a CTG repeat located in the 3′ untranslated region (UTR) of the dystrophia myotonica protein kinase *(DMPK)* gene [1–3]. In general, longer CTG

Y. Kino (✉) · J.-i. Satoh
Department of Bioinformatics and Molecular Neuropathology,
Meiji Pharmaceuticals University, Tokyo, Japan
e-mail: kino@my-pharm.ac.jp

S. Ishiura
Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

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expansions are associated with more severe phenotypes and earlier disease onset [4]. The identification of the causal mutation in patients with DM1 has prompted several hypotheses regarding the molecular mechanisms underlying DM. First, the loss of DMPK function due to a cis-regulatory effect of the repeat expansion was considered. As DM1 is an autosomal dominant disease, haploinsufficiency of DMPK due to the CTG repeat expansion was postulated. According to this hypothesis, the CTG expansion affects the local chromatin structure and/or methylation status around the *DMPK* locus and thereby hinders *DMPK* transcription. While *Dmpk* heterozygote knockout mice show cardiac conduction deficits [5], they fail to recapitulate the multisystemic symptoms of DM1. Homozygote *Dmpk* knockout mice were initially reported to show mild skeletal myopathy and cardiac conduction abnormalities [5–7]. However, a more recent study reevaluated homozygote *Dmpk* knockout mice and found normal cardiac conduction, muscle contraction, growth, and survival in these mice [8].

Second, the CTG repeat expansion in *DMPK* could affect the expression of nearby genes. Sine oculis homeobox homolog 5 (*SIX5*) is located near the downstream region of the 3′UTR of *DMPK* and is downregulated in DM1 cells [9, 10]. Remarkably, heterozygous *Six5* knockout mice show cataracts, without any skeletal muscle symptoms [11]. As *Six5* is essential for spermatogenesis, its reduced function might mediate male reproductive deficits in DM1 [12]. Even if this is the case, additional mechanisms are needed to explain other symptoms of DM1.

2.1.2 RNA-Dependent Mechanism of DM

RNA foci, characterized by abnormal RNA accumulation, have been identified in the nuclei of DM1 cells by in situ hybridization [13]. As DM1 mutations are located within a noncoding region (3′UTR), they do not alter the protein sequence of DMPK, but they could have adverse effects at the RNA level. Accordingly, research has focused on the roles of RNA-binding proteins. CEFL1, also known as CUGBP1, binds to a short CUG repeat [14]. Aberrant RNA processing in DM1 was first recognized by the finding of missplicing of cardiac troponin T (cTNT, encoded by *TNNT2*), which is regulated by CELF1 [15]. Subsequently, missplicing of insulin receptor (*INSR*) was identified in DM1 cells, which is again regulated by CELF1 [16]. Insulin resistance is frequently observed in DM1, and the splicing defects of *INSR* are a plausible cause of this phenotype. Moreover, aberrant splicing of the skeletal muscle chloride channel *CLCN1* was identified [17, 18]. Loss of function of *CLCN1* is sufficient to cause myotonia, as its genetic mutations cause myotonia congenita. Thus, splicing abnormalities have become a cardinal feature of DM1.

2.1.3 Mouse Models That Recapitulate DM Phenotypes

Another important clue came from transgenic HSA^{LR} mice [19]. These HSA^{LR} mice harbor a long CTG repeat tract embedded in the 3′UTR of human skeletal actin and express mutant RNA in the skeletal muscle. These mice manifested myotonia and

abnormal muscle histology. Importantly, phenotypic severity in these mice is correlated with the expression level of CUG repeat RNA, suggesting a trans-dominant effect of expanded RNA. These findings provided in vivo proof of concept for an "RNA gain of function." Until now, *HSA*^{LR} mice have been used for preclinical studies to test therapeutic interventions as well as for the identification of molecular abnormalities derived from CTG expansion. In addition to *HSA*^{LR} mice, several other transgenic mouse models have been established. DM300 mice carrying a large genomic fragment of the human *DMPK* gene with at least 300 CTG repeats recapitulated multisystemic phenotypes, including myotonia, abnormal muscle histology, altered tau isoform expression in the brain, and glucose intolerance [20, 21]. This mouse strain showed intergenerational CTG instability and led to the generation of animals with much larger CTG expansions (DMSXL), which carry 1000–1800 CTG repeats and manifest more severe phenotypes [22]. Conditional transgenic models of the *DMPK* 3′ region allow the tissue-specific expression of the *DMPK* 3′UTR with a 960 CUG repeat (with interruptions) that can be induced by drug administration. Heart and skeletal muscle-specific expression of the transgene resulted in DM-like abnormalities and splicing misregulation in respective tissues [23, 24]. These results support the hypothesis that DM1 is caused by the expression of repeat-containing RNAs, rather than the loss of function of *DMPK* or flanking genes.

2.1.4 MBNL Protein Sequestration by CUG Repeats

Muscleblind-like 1 (MBNL1) was identified in a biochemical study as a protein that binds to long CUG repeats [25]. Importantly, MBNL1, but not CELF1, colocalized with RNA foci in DM cells [26, 27]. Moreover, MBNL1 regulates alternative splicing of *TNNT2* and *INSR* in the opposite direction to CELF1 [28]. Most strikingly, a knockout model of *Mbnl1* (*Mbnl1*$^{\Delta E3/\Delta E3}$) showed some DM-like symptoms, such as myotonia, abnormal muscle histology, and cataracts [29]. These mice showed splicing misregulation of a number of genes, including *Clcn1*, *TnnT2*, and *Tnnt3*. Thus, MBNL1 has become a strong candidate protein that directly links CTG expansion to splicing misregulation observed in DM1. Further supporting this model, the overexpression of MBNL1 using an adeno-associated viral vector improved the phenotypes of the *HSA*^{LR} mice [30]. Similarly, the phenotypes of *HSA*^{LR} mice were ameliorated by crosses with *MBNL1* transgenic mice [31]. Thus, MBNL1 has become a central player in the pathogenesis of DM.

2.1.5 Additional Support for RNA-Mediated Pathogenesis of DM: DM Type 2 (DM2)

Another important clue supporting an RNA-mediated pathogenesis came from analyses of DM2. Some patients with heritable DM-like symptoms lack DMPK mutations. These patients were classified as DM2 or proximal myotonic myopathy

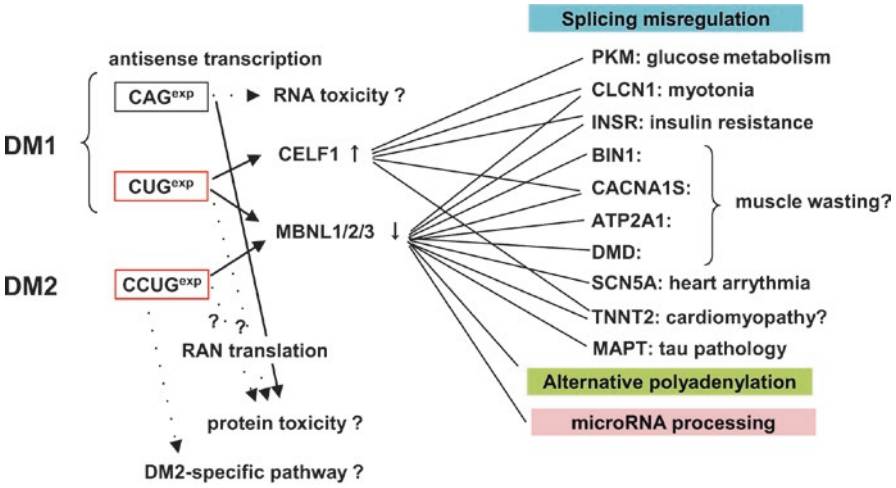

Fig. 2.1 RNA-mediated pathomechanisms of myotonic dystrophy

(PROMM). A CCTG repeat expansion in the first intron of the cellular nucleic acid-binding protein (*CNBP*) gene (previously known as zinc finger 9, *ZNF9*) was discovered as the causative mutation of DM2 [32]. Importantly, *DMPK* and *CNBP* are located on different chromosomes (chromosome 19 and 3, respectively). Thus, it is unlikely that the cis-regulatory effects of the expanded CTG repeat in *DMPK* account for the common symptoms associated with DM1 and DM2. The function of genes responsible for DM1 and DM2 appear to be different. While DMPK is a kinase that regulates mitochondria clustering and nuclear envelope integrity [33, 34], CNBP is an RNA-binding protein involved in the regulation of translation [35–37]. Thus, it seems unlikely that the losses of function of DMPK and CNBP have similar effects. Rather, the expanded repeat tracts themselves have become the most plausible components of the pathology of both DM1 and DM2. Consistently, RNA foci were also detected in DM2 patients and colocalized with MBNL1 [38]. Furthermore, splicing misregulation of *CLCN1* and *INSR* was also detected in patients with DM2 [18, 39]. These observations strongly suggested that repeat RNA-mediated mechanisms are shared in DM1 and DM2 (summarized in Fig. 2.1). Hereafter, we will focus on the details of abnormal RNA metabolisms observed in DM, with a focus on the roles of RNA-binding proteins.

2.2 Muscleblind-Like (MBNL) Proteins

2.2.1 Mammalian MBNL Family Proteins

MBNL1, the most well-characterized member of the MBNL family, was first identified by affinity chromatography to purify proteins that bind to double-stranded CUG repeat RNAs [25]. MBNL family proteins are evolutionarily conserved and found

not only in vertebrates but also in insects and other invertebrates [40]. Its fly ortholog muscleblind is involved in the development of muscle and eyes [41, 42]. There are three mammalian MBNL paralogs, MBNL1, MBNL2, and MBNL3, with distinct expression patterns [26, 43]. MBNL1 and MBNL2 are widely expressed in multiple tissues, including the skeletal muscle, heart, spleen, kidney, and liver, and MBNL1 is highly expressed in the muscle and heart. In contrast, MBNL3 expression is restricted to the placenta and undifferentiated muscle cells. All of these proteins share four C3H-type zinc finger (ZnF) motifs (ZnF1-ZnF4) and show extensive splicing variation. MBNL1 binds directly to both CUG and CCUG repeats in a length-dependent manner [44]. Additionally, all three proteins are colocalized with CUG and CCUG repeat foci when expressed in DM1 and DM2 cells, respectively [26].

2.2.2 Knockout Mouse Models of MBNL Proteins

Various knockout mouse models of MBNL proteins have been established. As described above, *Mbnl1*$^{\Delta E3/\Delta E3}$ mice manifest myotonia, cataracts, and histological abnormalities of skeletal muscle [29]. These mice also show splicing misregulation that recapitulates the splicing patterns of patients with DM. Importantly, a comparison of splicing regulation in *Mbnl1*$^{\Delta E3/\Delta E3}$ and *HSA*^{LR} mice suggests that more than 80% of splicing misregulation of *HSA*^{LR} mice can be explained by a loss of function of *Mbnl1* [45]. Three *Mbnl2* knockout mice models have been reported thus far. Among them, two models are based on the gene trap strategy. A gene trap insertion in intron 4, which leads to a reduction in the *Mbnl2* expression level to less than 10% of the levels in wild-type mice, resulted in normal phenotypes [27]. In contrast, *Mbnl2* gene trap mice with an insertion at intron 2 showed myopathy and myotonia together with *Clcn1* misplicing [46]. Subsequently, an *Mbnl2*$^{\Delta E2/\Delta E2}$ model lacking exon 2 that does not produce full-length Mbnl2 was generated [47]. This model did not show muscle pathology or myotonia, but showed brain phenotypes including abnormalities in sleep regulation, learning and memory, and seizure susceptibility, together with splicing misregulation in the brain. Misregulated splicing events in these mice were also identified in DM1 patients [47]. Regarding MBNL3, one knockout mouse model (*Mbnl3*$^{\Delta E2}$) has been developed. These mice did not exhibit overt muscle or central nervous system (CNS) phenotypes, but showed late-onset and age-associated impairment of muscle regeneration after muscle injury [48]. This phenotype suggests that repeat RNA-mediated functional impairment of MBNL3 may contribute to age-associated muscle wasting in DM. Interestingly, an analysis of this model revealed that *Mbnl3* deficiency caused some age-associated pathologies, including glucose intolerance with elevated insulin levels, cardiac systole deficits, left ventricle hypertrophy, and cataracts [49].

Since MBNL proteins have a similar RNA-binding specificity, depletion of one MBNL protein can be partially compensated by other MBNL proteins, potentially masking the effects of the depletion. The double knockout of *Mbnl1* and *Mbnl2* (*Mbnl1*$^{\Delta E3/\Delta E3}$/*Mbnl2*$^{\Delta E2/\Delta E2}$) was embryonically lethal in mice, indicating an essential role of these proteins during development [50]. *Mbnl1*$^{\Delta E3/\Delta E3}$ and *Mbnl2*$^{\Delta E2/+}$ mice were viable and showed more severe phenotypes than those of *Mbnl1*$^{\Delta E3/\Delta E3}$

single-knockout mice, with a reduced life span, heart conduction block, myotonia, and progressive skeletal muscle weakness [50]. A double-knockout model of *Mbnl1* and *Mbnl3* (*Mbnl1*$^{\Delta E3/\Delta E3}$/*Mbnl3*$^{\Delta E2}$) showed lower force production than that of wild-type mice and *Mbnl1*$^{\Delta E3/\Delta E3}$ mice. In addition, the *Mbnl1*$^{\Delta E3/\Delta E3}$/*Mbnl3*$^{\Delta E2}$ double-knockout mice also showed more severe myotonia due to the defective translation of *Clcn1* mRNA together with its splicing misregulation [51]. These results demonstrate that the simultaneous depletion of multiple MBNL proteins by CUG/CCUG repeats is a critical event in the pathogenesis of DM.

2.2.3 RNA-Binding Properties of MBNL1

RNA-binding specificity of MBNL1 is a key determinant of the molecular pathogenesis of DM as it not only provides a biochemical basis for RNA-mediated pathogenesis but is also essential for developing therapeutic strategies targeting pathological MBNL1-RNA interactions. While it was initially identified as a protein that binds to CUG repeats, MBNL1 can bind to several other sequences that contain motifs similar to CUG. A yeast three-hybrid system, in which protein-RNA interactions can be detected in yeast cells, indicated that MBNL1 binds to CHG and CHHG repeats (where H corresponds to A, C, or U) [44]. Expanded CUG and CCUG repeats are thought to adopt a hairpin structure, in which GC nucleotides form base pairs with U-U or U-C mismatches. Additionally, MBNL1 prefers mismatch-containing RNAs. For example, while MBNL1 binds to each of $(CUG)_{16}$ and $(CAG)_{16}$ repeats individually, it does not bind to $(CUG)_{16}$ + $(CAG)_{16}$, which forms a long stem-loop structure without mismatches [44]. A similar conclusion was obtained in another study, which also revealed that MBNL1 prefers pyrimidine mismatches over purine or purine-pyrimidine mismatches [52]. Importantly, the binding sites of MBNL1 in its target RNAs adopt similar stem-loop hairpin structures in *TNNT2* and *TNNT3* [52, 53]. MBNL1 binds to CUG/CCUG repeat RNA with an affinity similar to that of pre-mRNA targets [52]. Thus, functional sequestration of MBNL1 is mainly explained by the number of CUG/CCUG units, becoming greater when expanded. More recently, it has also been suggested that the binding of MBNL1 alters the structure of bound RNA from double-stranded to single-stranded [54].

Based on an analysis of MBNL1-binding sites in *TNNT2*, a YGCU(U/G)Y motif has been proposed as a consensus sequence recognized by MBNL1 [28]. The mutation or deletion of these motifs disrupted splicing regulation by MBNL1 [28, 55]. Subsequently, the crystal structure of MBNL1 ZnF motifs bound to a CGCUGU motif was solved [56]. This analysis provided direct evidence that the ZnF3/4 domain of MBNL1 bound to two GC dinucleotides, or GC steps, where ZnF3 bound to one GC motif and ZnF4 bound to another one in an antiparallel orientation. It was also demonstrated that ZnF3/4 prefers two GCU motifs separated by a sufficient length of linker that can form a looped RNA structure by binding to MBNL1 [56]. Systemic evolution of ligands by exponential enrichment (SELEX) is a method to enrich RNA/DNA sequences that specifically bind to a target molecule through iterative rounds of selection and RNA/DNA amplification. Using this strategy,

MBNL1 was shown to bind to GC motifs embedded in pyrimidines or YGCY motifs (where Y corresponds to U or C) [57]. High-throughput analyses using microarray or next-generation sequencing approaches have also contributed to the specification of motifs bound by MBNL1. These analyses are based on two major strategies: (1) analyses of motif enrichment around exons whose splicing pattern is altered by MBNL1 depletion and (2) direct reading of the RNA sequence bound by MBNL1 [45, 58–60]. Overall, these approaches provide an unbiased picture of the preference of RNA-binding proteins and revealed that consensus motifs of MBNL1 can be essentially summarized as YGCY, consistent with the abovementioned biochemical studies. In addition, MBNL2 and MBNL3 have been shown to recognize similar motifs [47, 48]. Accordingly, MBNL proteins prefer RNA sequences that contain multiple YGCY motifs, which include pathological CUG and CCUG repeats. For more details regarding RNA binding, see Konieczny et al. [61].

2.2.4 Splicing Misregulation Observed in DM

A number of genes are regulated by MBNL proteins. Using a splicing-sensitive microarray, more than 200 splicing events were found to be altered in *Mbnl1* knockout mice [45]. As mentioned above, there is large overlap in splicing changes between HSA^{LR} and $Mbnl1^{\Delta E3/\Delta E3}$ mice [45]. An RNA sequencing (RNA-seq) analysis of various tissues from $Mbnl1^{\Delta E3/\Delta E3}$ and control mice revealed 912 splicing events that are dependent on *Mbnl1* [59]. Thus, at least several hundred splicing events are regulated by MBNL1. An analysis of human patients of DM1 and DM2 using a splicing-sensitive microarray revealed similar overall gene expression patterns [62]. Among 55 exons that are affected in DM1 and regulated during development, 29 showed consistent splicing changes in $Mbnl1^{\Delta E3/\Delta E3}$ mice [62]. Thus, the loss of MBNL1 can account for a large fraction, if not all, of splicing misregulation in patients with DM.

Remarkably, the vast majority of misregulated splicing events (and alternative polyadenylation) in DM are those regulated during development. In both DM1 and DM2, expression levels of embryonic isoforms are increased and are correlated with disease severity. For many genes, RNA processing is coordinately regulated by a set of RNA-binding proteins during tissue development, yielding gene products that can fulfill the functional demands of cells. MBNL and CELF represent such regulators of the developmental program and act antagonistically, especially in the skeletal muscle and heart. Hereafter, we examine representative splicing events that are misregulated in DM. Importantly, these splicing events are developmentally regulated and their embryonic isoforms are elevated in DM tissues.

2.2.4.1 Skeletal Muscle Chloride Channel *CLCN1* (*CLC-1*)

CLCN1 encodes a skeletal muscle chloride channel whose genetic mutations cause non-syndromic myotonia. Retention of intron 2 and increased inclusion of exons 6b and 7a are observed in DM, leading to a functional reduction of the CLCN1 [17, 18].

Alternative splicing of *CLCN1* exon 7a is regulated by MBNL1, whereas intron 2 retention is regulated by CELF1. The misregulation of this gene exemplifies "causal" missplicing events that can directly explain a symptom. This was clearly demonstrated by the treatment of HSA^{LR} and $Mbnl1^{\Delta E3/\Delta E3}$ mice with antisense oligonucleotides to skip exon 7a of murine *Clcn1* that not only corrected the missplicing but also eliminated myotonia [63].

2.2.4.2 Genes Involved in Skeletal Muscle Structures

Although genes are misregulated in the DM skeletal muscle, it has been difficult to specify those that are responsible for muscle weakness and atrophy in DM. It is likely, however, that a cumulative effect of the simultaneous misregulation of multiple genes causes these muscular phenotypes. Nevertheless, there are several genes whose splicing patterns affect the function or structure of muscle.

Bridging integrator 1 (*BIN1*) is required for the biogenesis of muscle T-tubules, a membrane structure essential for excitation-contraction coupling. Importantly, *BIN1* is a causative gene of centronuclear myopathy, which shares some histopathological features of muscle with congenital DM1. *BIN1* exon 11 is regulated by MBNL1 and MBNL2, and its skipping is sufficient to induce muscle weakness [50, 64]. *CACNA1S* encodes $Ca_v1.1$, a calcium channel involved in excitation-contraction coupling, and its alternative exon 29 is misregulated in DM1 and DM2 [65]. The degree of exon 29 skipping is regulated by MBNL1 and CELF1 and is correlated with muscle strength in DM as well as the conductance and calcium sensitivity of $Ca_v1.1$ in mice. Remarkably, the muscle histology of HSA^{LR} mice was aggravated by the forced skipping of *Cacna1s* exon 29 [65]. *ATP2A1* (also known as *SERCA1)* is associated with Brody myopathy, characterized by exercise-induced impairment of skeletal muscle relaxation. Exon 22 of *ATP2A1* shows one of largest splicing pattern changes in DM1 and is regulated by MBNL1 [55, 62, 66]. Skipping of this exon, the predominant pattern in DM, alters the protein C-terminus and ATPase activity [67]. The *DMD* gene encodes dystrophin, a very large subsarcolemmal protein that provides a scaffold for force transmission during muscle contraction; its mutations are causal factors in Duchenne and Becker muscular dystrophy. Like *ATP2A1*, alternative splicing of *DMD* exon 78 alters the C-terminus of the protein and its function. Exon 78 splicing is regulated by MBNL1/2 and its skipping is increased in DM1 [68, 69]. Forced skipping of *DMD* exon 78 was sufficient to cause abnormalities in muscle structures [69].

2.2.4.3 Genes Involved in Abnormal Metabolisms

Exon 11 of insulin receptor (*INSR*) is included in the lower-signaling isoform, which is increased in DM muscle [16]. Inclusion of this exon is repressed by MBNL1 and promoted by CELF1. Glucose uptake is reduced in DM1 myoblasts. Misregulation of *INSR* can contribute to the insulin resistance observed in DM1 and DM2. The pyruvate kinase gene *PKM* has two isoforms, PKM1 and PKM2, which are

developmentally regulated. The embryonic isoform PKM2 is selectively increased in the type 1 fiber of DM1, which is mediated by CELF1, but not MBNL1 [70]. PKM2 is essential for aerobic glycolysis and is involved in the Warburg effect in cancer. Forced induction of the PKM2 isoform leads to an alteration of glucose metabolism in cultured myotubes and enhanced energy consumption in mice [70]. These PKM2-induced changes may disrupt metabolic homeostasis or may contribute to muscle wasting in DM1; these changes also point to an important role of CELF1.

2.2.4.4 Genes Involved in Cardiac Dysfunction

Cardiac defects are frequently observed in patients with DM and the second most common cause of death related to the disease [71, 72]. *TNNT2* encodes cardiac troponin T (cTNT), a subunit of the troponin complex that regulates muscle contraction in response to calcium ions. Mutations in *TNNT2* cause cardiomyopathy [73]. Exon 5 of *TNNT2* is an embryonic exon whose inclusion is increased in hearts in DM1 and DM2. Splicing of this exon is repressed by MBNL1 and promoted by CELF1 [28]. *SCN5A* encodes the voltage-gated sodium channel $Na_v1.5$ and plays a key role in the excitability of cardiomyocytes. Mutations in *SCN5A* cause several arrhythmic disorders. Mutually exclusive exons 6A and 6B are developmentally regulated by MBNL1, but not CELF1, and are misregulated in both DM1 and DM2 [60]. Forced inclusion of the fetal exon 6A in mice, mimicking DM hearts, caused heart conduction defects and arrhythmias [60].

2.2.4.5 Genes Involved in Brain Abnormalities

Several genes are misspliced in DM1 brains, including *NMDAR1* (*GRIN1*), *MAPT*, and *APP* [74, 75]. Among them, *MAPT* missplicing is notable because DM1 is known as a tauopathy [76]. While $Mbnl1^{\Delta E3/\Delta E3}$ mice show modest changes in splicing in the brain, an analysis of $Mbnl2^{\Delta E2/\Delta E2}$ mice suggested that *Mbnl2* is a major regulator of developmental transition of splicing patterns in the brain [47]. Subsequent analyses of brain-specific double-knockout mice lacking *Mbnl1* and *Mbnl2* exhibited larger changes in splicing regulation compared to those in $Mbnl2^{\Delta E2/\Delta E2}$ mice and showed misregulation of *Grin1* exon 5 and *Mapt* exons 2, 3, and 10, as observed in DM1 [77]. Thus, it is likely that both MBNL1 and MBNL2 are involved in the splicing regulation of CNS.

2.2.4.6 Is Spliceopathy in DM a Primary Cause or a Consequence?

It is important to note that changes in splicing patterns can be observed in other muscular dystrophy models as secondary changes to muscle regeneration [78, 79]. Thus, it is not clear whether the splicing misregulation in DM is a cause or consequence of the disease. In the case of *Clcn1* missplicing in HSA^{LR} mice, treatment with an antisense oligonucleotide targeting *Clcn1* exon 7a not only corrected exon 7a splicing

but also ameliorated myotonia [63]. The results of this and other aforementioned studies using forced expression of certain splice isoforms strongly suggest that at least some splicing abnormalities are the primary cause of symptoms.

2.2.5 *RNA Processing Events Other Than Splicing Regulation Are Altered in DM*

Although DM is recognized as a spliceopathy, not all disease phenotypes can be attributed to splicing misregulation. Indeed, recent analyses have indicated that MBNL and CELF are involved in RNA trafficking, mRNA decay, and alternative polyadenylation, in addition to splicing regulation [58, 59, 80–82]. At present, the relevance of these functions of MBNL and CELF in the pathogenesis of DM is less clear compared to the roles of splicing regulation.

MBNL1 regulates the microRNA processing of miR-1, which is reduced in the hearts of DM1 and DM2 [83]. A reduction of this microRNA leads to the increased expression of its targets, GJA1 (connexin 43) and CACNA1C ($Ca_v1.2$), and may contribute to cardiac dysfunction in DM. Recently, the downregulation of a glutamate transporter, GLT1, has been found in the cerebellum of DMSXL mice and DM1 [84]. GLT1 downregulation in astrocytes increases glutamate toxicity to neurons. Depletion of *Mbnl1*, but not *Mbnl2*, can cause GLT downregulation, possibly via alternative polyadenylation. Intriguingly, ceftriaxone, an antibiotic that promotes GLT1 expression, improved Purkinje cell firing and motor coordination abnormalities in DMSXL mice [84].

2.3 RNA-Binding Proteins Implicated in the Pathology of DM Other Than MBNL

2.3.1 *CUG-BP and ETR-3-Like Family (CELF) Proteins*

CELF1, also known as CUG-BP or CUGBP1, was identified as a protein that binds to CUG repeats. Subsequently, this protein was shown to prefer UG-rich motifs, rather than CUG repeats, and is not sequestered in RNA foci [85]. CELF1 regulates the alternative splicing of several pre-mRNAs that are misregulated in the muscle and heart of DM, including *TNNT2* and *INSR* [28]. Remarkably, the regulation of *TNNT2* and *INSR* by CELF1 is in the opposite direction compared to the regulation by MBNL1 [28]. These two proteins recognize distinct motifs around the target exons. The protein level of CELF1 is elevated in the skeletal muscle, heart tissues, and myoblasts in DM1, but not in DM2 [39, 86]. Protein kinase C (PKC) phosphorylates CELF1 and this results in protein stabilization and upregulation [87]. Importantly, inhibition of PKC by small molecules corrects CELF protein levels and improves cardiac defects as well as CELF1-dependent splicing in heart-specific

DM1 mouse models [88]. In addition to splicing regulation, CELF1 has multiple roles in RNA processing, including translation and mRNA decay [58, 89, 90].

Until now, several strains of *CELF1* transgenic mice have been reported. The first line (TR) exhibited growth retardation, delayed myogenesis, and histological abnormalities [91]. The second line (MCKCUG-BP1) showed abnormalities in muscle histology and splicing [92]. Both lines also showed high mortality, indicating the toxicity of *CELF1* overexpression. Additionally, tissue-specific transgenic mice of *CELF1* have been established. *CELF1* overexpression in the skeletal muscle caused myopathy and splicing misregulation. Similarly, *CELF1* overexpression in the heart caused cardiomyopathy. Thus, elevated levels of *CELF1* can account for key features of DM1.

CELF1 is the founding member of the CELF family, which comprises six proteins [93, 94]. CELF1 is widely expressed, including in the skeletal muscle, heart, brain, and many other tissues. Similarly, CELF2, also known as CUGBP2, ETR-3, or NAPOR, is expressed in a wide variety of tissues. In contrast, other CELF proteins, i.e., CELF3, CELF4, CELF5, and CELF6, are expressed mainly in the CNS. The contributions of CELF proteins other than CELF1 to the pathogenesis of DM are unclear. Like CELF1, the expression of CUG repeat RNA leads to the upregulation of CELF2 [23]. CELF2 regulates the alternative splicing of *NMDAR1* exon 5 and *MAPT* exons 2 and 10 [95, 96].

2.3.2 Heterogenous Ribonucleoprotein H (hnRNP H)

In addition to MBNL and CELF, several other proteins have been implicated in DM. hnRNP H is a regulator of alternative splicing and its expression level is elevated in DM1 myoblasts [97]. In addition, this protein is also detected in CUG repeat RNA foci. However, it exhibits only partial sequestration, unlike MBNL1/2. Co-expression of hnRNP H and either MBNL1 or MBNL2 enhanced colocalization of hnRNP H and RNA foci. Like CELF1, hnRNP H regulates *INSR* splicing in the opposite direction compared to MBNL1/2 [97]. However, the overall contribution of hnRNP H in the splicing misregulation in DM has been elusive. This protein is also involved in the nuclear export of CUG-containing RNA [98].

2.3.3 Staufen1 (Stau1)

Stau1 is a double-stranded RNA-binding protein elevated in the skeletal muscle of patients with DM1 and HSA^{LR} mice [99]. Like CELF1, Stau1 is not colocalized with RNA foci. Interestingly, Stau1 regulates the alternative splicing of *INSR* in the same direction as MBNL1. Furthermore, Stau1 regulates additional DM-affected splicing events. Some of these events are regulated by Stau1 in the normal direction, while others are regulated in the DM-like direction [100]. Stau1 is also involved in the deficit of stress granule formation observed in DM1, and the knockdown of Stau1

rescues this phenotype [101]. Thus, Stau1 might act as a disease modifier of DM1, though its overall effects in vivo are still unclear.

2.3.4 DEAD Box Helicases, DDX6 and DDX5/p68

The overexpression of DDX6, a DEAD box helicase, reduced in nuclear RNA foci, but increased cytoplasmic RNA foci, with partial rescue of missplicing in DM1 cells. In contrast, the depletion of DDX6 increased the number of CUG foci. Interestingly, these effects were not observed for a CCUG repeat [102]. Another DEAD box helicase, DDX5 (p68), enhances the binding of MBNL1 to expanded CUG repeats as well as to a target RNA of MBNL1, while the knockdown of DDX5 together with its paralog p72 leads to a decrease in RNA foci [103]. Another study indicated that DDX5 is downregulated in human DM1 and HSA^{LR} mice and the overexpression of DDX5 in the muscle of HSA^{LR} mice improves the muscle pathology [104]. In contrast to the results of [102], DDX5 destabilized expanded CUG or CCUG repeats and reduced the number of CUG foci [104]. Thus, RNA helicases may constitute a class of modifiers that regulate expanded repeat RNAs, possibly via their unwinding activity, altering the secondary structure of CUG or CCUG repeats as well as accessibility to MBNL1 [102].

2.4 Antisense Transcription and RAN Translation

2.4.1 Antisense Transcription

Antisense transcription starting from the *SIX5* locus has been suggested previously [105]. Later, antisense transcripts of *DMPK* were detected in several tissues of DMSXL mice, including the heart, skeletal muscle, and brain, though at much lower levels compared to those of the sense transcript. Interestingly, CUG and CAG repeat RNAs form discrete foci that are not colocalized with each other [22]. These foci could be observed even at prenatal stages in both DMSXL mice and human DM1 fetuses and colocalized with MBNL [106]. This indicates that MBNL sequestration begins at the prenatal stage, which may contribute to congenital DM. Based on an analysis of the transcription start sites and polyadenylation sites, there are multiple *DMPK* antisense transcripts with and without CAG repeats [107].

2.4.2 Repeat-Associated Non-ATG (RAN) Translation

Repeat-associated non-ATG (RAN) translation is an unusual translation mechanism independent of initiation codons that occurs from a repeat tract with secondary structures [108]. Ranum and colleagues found that an apparently noncoding CAG

repeat located downstream of multiple stop codons in all frames produces homopolymeric proteins containing polyglutamine (from CAG codons), polyalanine (from GCA codons), or polyserine (from AGC codons). These repetitive protein products are called RAN proteins. Thus, repeat RNA tracts allow translation initiation from within or around the repeat tract in all three reading frames. RAN translation is dependent on the secondary structure of the repeat tract, as a CAA repeat tract that does not adopt a hairpin structure does not produce RAN proteins [108]. In the brain of human spinocerebellar ataxia type 8 and its mouse model, RAN protein containing the polyalanine tract was detected by an antibody specific to the peptides predicted to appear in the downstream region of the polyalanine tract. Moreover, using the same strategy, a polyglutamine-containing RAN protein derived from a CAG-containing antisense transcript of *DMPK* was detected in the cardiomyocytes of the DM model mice as well as human DM1 myoblasts [109]. Subsequently, RAN translation was verified in some other repeat expansion-related diseases and may be a general phenomenon associated with repeat expansions [110]. Further studies are needed to clarify the details of RAN proteins expressed in DM1 and DM2 as well as their contribution to the disease pathogenesis.

2.5 Differences Between DM1 and DM2

Although DM1 and DM2 share many pathophysiological characteristics, there are also differences between these diseases. The congenital form is only observed in DM1. While the size of the CTG expansion is correlated with the severity and the age of onset in DM1 [4], the size of the CCTG expansion (75–11,000) does not predict the clinical course of DM2 [111]. In general, DM2 manifests milder phenotypes compared to those of DM1. The CTG repeat length tends to increase when transmitted from the affected mother, often resulting in more severe phenotypes in the next generation. This intergenerational acceleration of disease phenotypes, known as anticipation, is not observed in DM2. In the case of DM2, the mutant allele usually contracts in the next generation [111], and this contraction may partly explain the absence of the congenital form or genetic anticipation in DM2. In the muscle pathology, DM1 shows type I atrophy, whereas DM2 shows predominant type II fiber atrophy. Regarding symptoms, muscle pain is frequent in DM2 but absent in DM1. There are several other clinical differences between these two diseases as summarized in Meola et al. [112].

These differences may be explained by the following factors. First, the spatiotemporal expression patterns of DMPK and CNBP are different. While DMPK is highly expressed in the skeletal muscle and heart [113], CNBP is expressed in a wide variety of tissues [37]. Second, the consequences of the functional losses of DMPK and CNBP might be different. As described above, the loss of DMPK function might explain limited phenotypes of DM1. In the case of DM2, surprisingly, CNBP heterozygous knockout mice show some DM-like phenotypes, including myotonia, muscle wasting, ocular cataracts, and cardiac conduction defects [114]. While initial reports of DM2 have suggested that CNBP is not reduced in DM2

[115], some recent reports have indicated that CNBP is reduced in patients with DM2, but not DM1 [86, 116]. Finally, CUG and CCUG repeats might have distinct properties. For example, while both repeats sequester MBNL proteins, localization of only CUG repeat foci can be affected by DDX6 [102]. In addition, minute localization of nuclear RNA foci differs. While CUG foci in DM1 cells are associated with splicing speckles, CCUG foci in DM2 cells are not [117]. In addition, CUG repeats in DM1 are retained in the mature transcript, whereas CCUG repeats in DM2 are spliced as introns and the flanking regions of the repeat are eliminated [115]. Some CUG- or CCUG-specific interactors may mediate differences between DM1 and DM2.

2.6 Signaling Pathways Altered in DM1

Alterations in some signaling pathways have been detected in DM1. GSK3beta is a kinase involved in the phosphorylation of CELF1 in a cyclin D3-dependent manner. The activity and stability of GSK3beta is increased, while cyclin D3 is elevated in the skeletal muscle of HSA^{LR} mice and patients with DM1 [118]. Interestingly, TDZD-8, a specific inhibitor of GSK3beta, improved muscle strength as well as the histology of HSA^{LR} mice, and these phenotypes were accompanied by the normalization of the number of satellite cells [118].

AMPK/mTORC1 pathways are involved in multiple aspects of cellular metabolism, including energy metabolism, biogenesis, protein synthesis, and autophagy. Recently, AMPK/mTORC1 pathways were found to be affected in HSA^{LR} mice and DM1 muscle cells [119], where the activation of AMPK signaling in muscle was impaired under starvation, while mTOR signaling remained active. Interestingly, treatment with an AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), improved muscle relaxation after tetanic stimulation with the partial correction of *Clcn1* missplicing and reduction in RNA foci. On the other hand, an mTORC1 inhibitor, rapamycin, also improved muscle relaxation, but without the correction of splicing. Although the molecular pathways that affect AMPK/mTORC1 signaling in DM1 are still unknown, these results may provide novel therapeutic targets [119].

2.7 RNA Foci

Ribonuclear inclusions are a pathological hallmark of both DM1 and DM2 and sequester MBNL proteins. In the presence of RNA foci, the level of nucleoplasmic MBNL1 is reduced by more than 78% in DM1 and DM2 myonuclei [27]. Thus, it is reasonable to postulate that RNA foci are the sites of MBNL functional sequestration. Still unclear is whether sequestration of MBNL proteins by CUG/CCUG repeat occurred outside of RNA foci and, if so, to what extent it contributes to the overall impairment of MBNL activity.

2.7.1 RNA Foci and MBNL1

RNA foci are dynamic and reversible structures, in which MBNL1 is mobile [120, 121]. A previous study revealed that the RNAi-mediated depletion of MBNL1 in DM1 fibroblasts reduces the number of RNA foci, strongly suggesting that MBNL proteins are involved in the formation or maintenance of RNA foci [122]. In line with this notion, we recently reported that the overexpression of MBNL1 induces the formation of RNA foci in cultured cells [40]. Therefore, the "sequestration of MBNL1" is not simply a passive event. Rather, MBNL1 may have an active role in the formation of foci. Long noncoding CAG repeats can produce RAN proteins (polyglutamine, polyalanine, and polyserine). Importantly, the overexpression of MBNL1 could suppress the expression of these RAN proteins by preventing the CAG repeat RNA from being exported to the cytoplasm and translated. Thus, it was proposed that MBNL-RNA interactions have two distinct aspects. First, as previously supposed, the accumulation of MBNL proteins in nuclear RNA foci hinders the regulation of their natural target transcripts. Second, the interaction may have a protective role against the production of RAN proteins, such as polyglutamine, which are aggregation-prone and often cytotoxic [40]. Although RAN proteins have been detected in DM1 tissues [108], their toxicity and phenotypic effects are still unclear. Nevertheless, it should be noted that therapeutic attempts to block the MBNL1-CUG/CCUG interaction and/or disperse RNA foci have a potential risk, i.e., the released mutant RNA can be subjected to RAN translation and produce potentially toxic proteins.

RNAs containing expanded repeats are retained in the nucleus for unknown reasons. Although cytoplasmic RNA foci are found in some conditions, they seem to have less toxicity than that of nuclear foci [102, 123]. RNA foci of CUG repeats are closely associated with intranuclear structures known as splicing speckles, which are enriched in splicing factors, such as SC35 [117, 124]. Normal DMPK transcripts enter into splicing speckles, while mutant DMPK transcripts do not enter speckles, but accumulate as RNA foci at their periphery of them, and this is mediated by MBNL1 [124].

2.7.2 An Emerging Aspect of RNA Foci Formation

Recently, it has been reported that CUG or CAG repeat RNA alone can form gel or droplets with liquid-like properties in vitro [125]. RNA gels can form, without the involvement of protein components. This RNA gelation is mediated by intermolecular multivalent interactions of repeat tracts in a length-dependent manner and is blocked by antisense oligonucleotides that recognize the repeat tract. Moreover, RNA gelation can be disrupted by reagents containing monovalent cations, such as ammonium acetate and sodium chloride, and the nucleic acid intercalator doxorubicin. Strikingly, these compounds also reverse the formation of nuclear RNA foci in cells [125]. Furthermore, the dynamic nature of RNA foci is dependent on ATP, suggesting a role of RNA helicases or other enzymes in the formation or

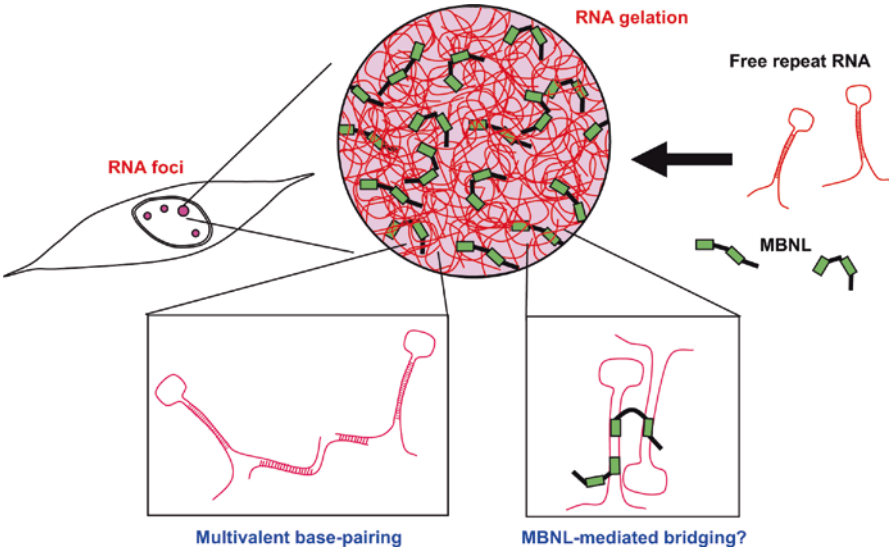

Fig. 2.2 RNA foci formation

maintenance of RNA foci. Taken together with previous results, it is possible that repeat RNA gelation can occur by itself in vitro, but some cellular factors, such as MBNL1, are required in cells to enhance the local RNA concentration and/or to provide RNA structures that are suitable for efficient gelation. MBNL proteins might act as RNA chaperones to alter CUG/CCUG RNA structures or bridge multiple repeat RNA molecules using ZnF1/2 and ZnF3/4 separately to facilitate RNA gelation [54, 126] (Fig. 2.2). In addition, DDX6 and DDX5/p68 are candidate ATP-dependent regulators of the formation of RNA foci, as mentioned above.

In addition to CAG and CUG repeats, GGGGCC repeats, whose expansion causes amyotrophic lateral sclerosis, also form RNA gels [125]. Thus, RNA gel formation may underlie a broad range of repeat expansion-related diseases. Furthermore, liquid-like droplet formation is a common mechanism of intracellular granular structures, including stress granules and the nucleolus, which are often mediated by low-complexity domains of RNA-binding proteins. These granular structures are collectively called "membrane-less organelles" or "droplet organelles" and are under intense investigation [127]. RNA foci in DM and other diseases might represent a pathological droplet organelle aberrantly induced by expanded repeat RNAs.

2.8 Concluding Remarks

Great advances have been made in our understanding of the pathomechanisms of DM since the identification of disease mutations underlying DM1 and DM2. Establishment of the RNA-mediated pathogenesis of DM has been a significant step

toward the development of therapeutic interventions for this disease. First, transcripts containing expanded CUG/CCUG repeats have become promising therapeutic targets [128, 129]. Indeed, antisense oligonucleotides targeting mutant transcripts have entered human clinical trials for patients with DM1. Second, RNA foci and/or splicing patterns might be used as biomarkers of disease status or for treatment [62, 130]. Nevertheless, there are several open questions. Although DM1 and DM2 are multisystemic diseases, current researches have mainly focused on the skeletal muscle and heart. The molecular mechanisms underlying other symptoms, such as cataracts and CNS abnormalities, are still unclear. Although previous studies have provided solid evidence that MBNL proteins (and CELF1 in DM1) mediate a substantial part of the effects of toxic RNA, the extent to which these proteins account for the constellation of DM symptoms is unclear. Although the single or simultaneous depletion of MBNL proteins, overexpression of CELF1, or heterozygosity of CNBP in mice can each recapitulate some DM-like phenotypes, it is unclear whether the functions of these proteins are indeed altered in patients with DM to the degrees observed in these mouse models. Careful comparisons of DM1 and DM2 would provide insights into the common molecular pathways as well as those that are specific to DM1 and DM2. The identification of novel aspects of DM, such as RAN translation and RNA gelation, would broaden our understanding of the molecular pathogenesis of DM. Finally, additional clues regarding the disease mechanisms will come from therapeutic approaches targeting specific aspects of DM phenotypes.

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Chapter 3
Clinical Features of Skeletal Muscle and Their Underlying Molecular Mechanism

Masanori P. Takahashi

Abstract The cardinal features of myotonic dystrophy are muscle stiffness (myotonia) and muscle wasting. RNA gain-of-function has been established as an underlying disease mechanism. Myotonia has been shown to be the result of the missplicing of chloride channel mRNA caused by changes to RNA-binding proteins, including MBNL and CELF. Many types of RNA that play essential roles in skeletal muscle function have been identified as possibly being implicated in muscle wasting. Recently, other mechanisms, such as repeat-associated non-ATG translation, have been proposed, and their contribution to muscle phenotypes is currently the subject of study.

Keywords Myotonia · Muscle atrophy · Splicing · Chloride channel · Cytoskeletal protein · Ca^{2+} Handling protein

3.1 Introduction

As its name indicates, the cardinal features of myotonic dystrophy (or dystrophia myotonica, DM) are muscle stiffness (myotonia) and muscle wasting. Our understanding of the pathophysiology of myotonia in DM has benefited from the identification of causative genes for non-dystrophic myotonias, such as myotonia congenita, and from physiological studies of domesticated animals exhibiting myotonia. An important breakthrough resulted from an animal model that expressed repeat expansion in a different genetic context and the identification of the DM2 gene. This established the concepts of an RNA disease and RNA gain-of-function. Since then, abnormalities have been revealed in many types of RNA that play key roles in skeletal muscle function, and it is likely that the RNA gain-of-function mechanism plays a major role in the pathophysiology of DM. Recently, several new mechanisms have been proposed; their contributions to the pathomechanism for DM are currently the subject of extensive investigation.

M. P. Takahashi
Clinical Neurophysiology, Department of Functional Diagnostic Science,
Osaka University Graduate School of Medicine, Suita, Osaka, Japan
e-mail: mtakahas@sahs.med.osaka-u.ac.jp

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The clinical phenotype of DM2 differs somewhat from that of DM1, and there have been reports that have emphasized differences between DM1 and DM2 in their underlying molecular pathomechanisms. However, this chapter does not discuss these differences; instead, its aim is to provide a global view of skeletal muscle pathophysiology in DM in general.

3.2 Myotonia

Myotonia, defined as the delayed relaxation of muscle, is a key muscle feature of DM, which often leads to the correct diagnosis. Patients describe myotonia as "stiffness" or "locking" of muscles, but they sometimes do not recognize this as a symptom of disease. This contrasts with other hereditary myotonic disorders such as myotonia congenita (MC). Thus it should be noted that myotonia can be easily missed by inexperienced physicians. Myotonia is apparent in classical adult DM patients but not in congenital DM patients at birth.

3.2.1 Clinical Presentation of Myotonia

Myotonia can occur in any skeletal muscle, but the most common complaint of DM1 patients is stiffness of the fingers, tongue, and legs. This can often be prominent when starting actions, such as during the first few words of speech or for the first few steps after rising from a sitting position. It ameliorates over several seconds with repeated contraction of the muscle, known as the "warm-up" phenomenon. Myotonia in the eyelid muscle is commonly observed in paramyotonia congenita (PMC), another inheritable myotonic disorder, but rarely in DM. Although DM patients complain that myotonia worsens with cooling of the muscles, this phenomenon is less prominent than for patients with PMC. The differences in the clinical features of DM, MC, and PMC are summarized in Table 3.1.

At the bedside, myotonic phenomena can be observed as grip myotonia (difficulty relaxing fingers after gripping strenuously) or as percussion myotonia (involuntary

Table 3.1 Clinical features of myotonic dystrophy and other myotonic disorders

	Myotonic dystrophy	Myotonia congenita	Paramyotonia congenita
Inheritance	AD with anticipation	AD/AR	AD
Causative gene	Type 1 *DMPK* Type 2 *CNBP*	*CLCN1*	*SCN4A*
Onset of myotonia	Juvenile–adult	Infancy–early childhood	Infancy–early childhood
Warm-up	+	+	+/−
Paramyotonia	−	−	+
Temporary weakness	−	−	+

AD autosomal dominant, *AR* autosomal recessive

muscle contraction elicited by tapping a muscle such as the thenar, hypothenar, or tongue muscle with a reflex hammer).

In congenital DM (cDM), as mentioned earlier, myotonia is not observed at birth but first starts appearing around 4 years of age in early cases. In classical DM, it is most apparent when patients are in their 20s and 30s but becomes less obvious with the advance of the disease. In advanced cases with prominent muscle wasting, grip or percussion myotonia becomes difficult to evoke at the bedside.

3.2.2 Clinical Electrophysiology of Myotonia

The electrophysiological basis of myotonia is the hyperexcitability of muscle fibers. Needle electromyography (EMG) recording typically shows increased insertion activity and spontaneous repetitive discharge (myotonic discharge), as well as myopathic changes due to the dystrophic nature of the disease. The repeated increase and decrease in amplitude and frequency of myotonic discharges results in a characteristic sound, described as being like a "*revving* engine" or "dive-bomber," which can be heard with an EMG audio speaker. In DM1, myotonic discharges are prominent in the hand muscles, forearm extensor, and anterior tibial muscles but less prominent in the proximal muscles [1].

Myotonic discharge is also observed in non-dystrophic myotonic syndromes such as PMC or MC, but the character of the myotonic discharges in these disorders seems to differ from that in DM1, where the myotonic discharges tend to last longer and their changes in amplitude and frequency are more prominent [2, 3].

Fournier et al. showed that a combination of electrophysiological tests is useful for estimating the underlying genetic defect of the channel for non-dystrophic myotonic syndromes and periodic paralysis [4, 5] and proposed an electrophysiological classification, from I to V, based on the pattern of CMAP change after prolonged exercise or repeated short exercise. In this classification, patients with myotonia were separated into three groups (patterns I–III). Patients with MC due to chloride channel mutations mostly displayed pattern II. PMC and other myotonias due to sodium channel mutations showed patterns I and III, respectively. Like the patients with MC due to chloride channel defects, DM patients displayed pattern II [5], which is consistent with chloride channel dysfunction; this is discussed below.

3.2.3 The Molecular Mechanism of Myotonia

3.2.3.1 Physiology of Muscle Excitability and Mutations of Ion Channel Genes

The excitability of skeletal muscle membrane is regulated by voltage-gated ion channels, including those for sodium, potassium, and chloride. Genetic defects of these channels are responsible for inheritable disorders that exhibit abnormal muscle

excitability, myotonia, and/or paralytic attack [6]. As mentioned previously, MC is due to mutations of the chloride channel, whereas PMC and other myotonias are due to mutations of the sodium channel. Myotonias associated with sodium channel mutations show autosomal dominant inheritance; when the mutant channel is tested *in vitro* for heterologous expression, gain-of-function defects are observed, as either disrupted inactivation or enhanced activation. MC shows both dominant inheritance (Thomsen's disease) and recessive inheritance (Becker's disease), and the mutant chloride channel shows a loss-of-function defect, with reduced or no current.

In neurons or cardiac muscle cells, the resting conductance is based almost entirely on potassium conductance, whereas two-thirds of the resting conductance in skeletal muscle is derived from chloride conductance [7]. It has been shown that a large reduction of chloride conductance is required for myotonia to occur [8]. Dominantly inherited mutations associated with Thomsen's disease often show a dominant negative effect in heteromeric chloride channels, resulting in a sufficiently large reduction of conductance to cause myotonia.

3.2.3.2 The Molecular Mechanism of Myotonia in Myotonic Dystrophy

Since the discovery of the causative gene for DM, the underlying mechanism of myotonia has been controversial. For instance, there have been reports of an increase in small-conductance calcium-activated potassium channels (SK3) in DM1 muscle [9, 10] or modulation of skeletal muscle sodium channels by DMPK activity [11, 12]. A reduction in chloride conductance in DM muscle has also been reported, but this was inconclusive [13, 14].

A mouse model HSA^{LR} has contributed greatly to the understanding of the disease mechanism for DM1, including myotonia [15]. This model, which expressed the human skeletal actin (*HSA*) transgene with (CTG)250 inserted in the 3′ untranslated region, showed the cardinal features of DM: myotonia and myopathy. This finding and the discovery of another type of DM caused by an expansion of CCTG tetranucleotide repeats located on an intron of the *CNBP* (formerly *ZNF9*) gene [16] clearly demonstrated that transcripts with expanded tri- or tetranucleotide repeats were responsible for the pathogenesis of DM and established the concept of an RNA-mediated disease (described in detail in Chap. 2 and in other literature reviews) [17].

An analysis of HSA^{LR} revealed that reduced chloride conductance was responsible for the myotonia [18]. Two-electrode recordings from the acutely dissected intercostal muscles of HSA^{LR} mice showed reduced threshold currents and increased latency of the action potential, suggesting decreased membrane conductance (Fig. 3.1 left). Further experiments that substituted chloride ions in a bath solution demonstrated that the decreased conductance was due to a decrease in the chloride current and that the chloride conductance was nearly absent in the skeletal muscle of the model mice [18]. The reduced immunohistochemical expression of the skeletal muscle chloride channel protein (Fig. 3.1 right) and the aberrant splicing of its RNA were observed in both the mice model and DM patients.

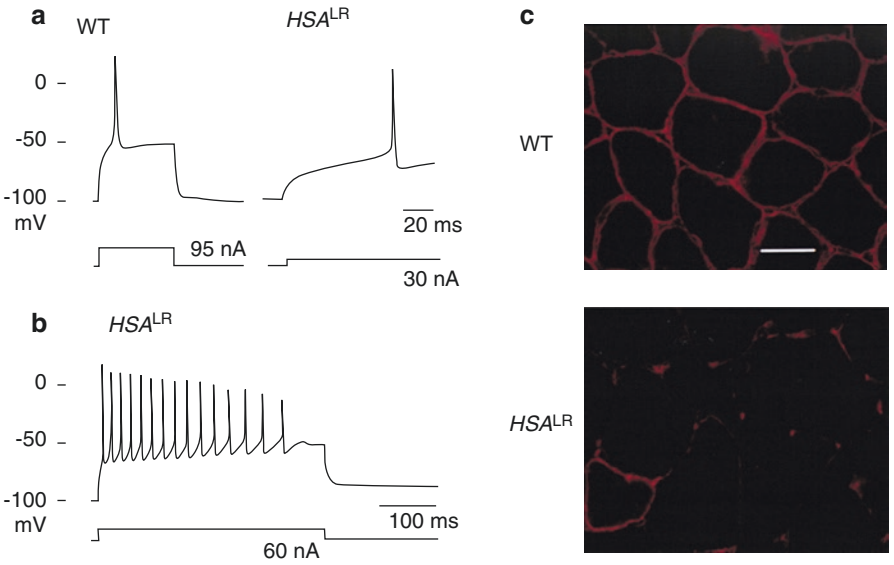

Fig. 3.1 Increased membrane excitability and reduced ClC-1 protein in *HSA*^{LR} mice. (**a**) Threshold current intensity is reduced and action potential latency is prolonged in *HSA*^{LR} fibers. (**b**) Repetitive discharges are elicited in *HSA*^{LR} fibers by a current intensity of 2× threshold, whereas WT fibers discharge only once (data not shown). (**c**) With immunofluorescence study, a normal circumferential rim of ClC-1 is seen at the surface membrane in controls (Top). ClC-1 is absent or reduced in most fibers in *HSA*^{LR} mice. Adapted with permission from Figures 1 and 3 of [18]

Changes in two RNA-binding proteins, MBNL (muscleblind-like) family and CELF1 (CUG-binding protein Elav-like family member 1), which can bind CUG repeats, have been shown to be responsible for the abnormal splicing of the chloride channel [19, 20]. The RNA with expanded repeats is abnormally retained in the nuclei as foci, and the normal function of the RNA-binding proteins is perturbed. The reduction of MBNL due to its sequestration to nuclear foci [21] and the upregulation of CELF1 by phosphorylation [22] have been reported to be underlying mechanisms of misregulated splicing in DM1 muscle, including the chloride channel and transcripts of many important genes; this is discussed below.

Although it may have been established that reduced chloride conductance is responsible for myotonia in DM, its myotonic discharges recorded clinically with needle EMG, as described earlier, seem to differ from those in MC, which also shows a loss of chloride conductance. This may be due to differences between fibers, or within segments of a fiber, in the reduction of the chloride channel protein in DM; however, it is possible that additional changes in other ion channels may be responsible for the differences in myotonic discharge. For example, the increase in small-conductance calcium-activated potassium channels (SK3) in DM1 muscle has been confirmed with mRNA and histological analysis [23, 24]. This increase could be related to the prolonged myotonic discharge characteristic of DM1, because it is known that an increase in SK3 expression is associated with

hyperexcitability of denervated muscles [25]. It should also be noted that SK3 channels decrease during normal skeletal muscle development. Thus, the increased SK3 expression may be caused by the impairment of maturation at a later stage of muscle differentiation.

The reduction of chloride conductance appears to have been established as the main cause of myotonia, but further studies, such as analyses of the gating of the sodium channel, are needed to understand the pathophysiological basis of the characteristic myotonic discharge in myotonic dystrophy.

3.3 Muscle Wasting

Muscle wasting is one of the most debilitating symptoms of DM, affecting patients' employment, social activities, and many aspects of their daily life. Muscle wasting is not limited to muscles for motor activity, but also affects muscles for eye movement, speech, chewing, swallowing, and respiration. Thus, muscle wasting can be serious and life-threatening.

Recent progress in research into myotonia, the other cardinal feature of DM, has highlighted the loss of chloride conductance as the basis of its pathophysiology; however, the pathophysiology of muscle wasting seems to be more complex.

3.3.1 Clinical Presentation of Muscle Wasting in Myotonic Dystrophy

The distribution of muscle wasting in DM1 is often described as distal dominant, but, strictly speaking, this is incorrect. Compared to Duchenne muscular dystrophy or other muscular disorders, which preferentially affect the proximal muscles, the pattern of muscle involvement in DM1 is somewhat distal dominant. In limb muscles, a decrease in grip power and steppage gait (drop foot) are common early features of DM1. However, the distribution in DM1 is not truly distal dominant when compared to disorders such as Charcot–Marie–Tooth disease. For example, although grip power is reduced due to the weakness of the flexor muscles in the forearms, weakness of the intrinsic hand muscles occurs later. Weakness and atrophy of the anterior neck muscle (the sternocleidomastoideus) is also common. Thus, it is appropriate to describe the pattern of muscle involvement in DM1 as having its own character. The distribution pattern of the affected muscles in DM2 is different from that in DM1, with the proximal muscles preferentially affected [26], previously referred to as proximal myotonic myopathy (PROMM).

The muscles preferentially affected in DM1 are the face, extraocular, jaw, palate, larynx, neck, forearm, and anterior tibial muscles; however, with the advancement of the disease, all muscles are eventually affected. The involvement of facial and jaw muscles is common, giving rise to the characteristic appearance referred

to as "hatchet face." In patients with congenital or juvenile-onset DM, the development of the craniofacial structure is also affected, and a high-arched palate and malocclusion of the teeth further exacerbate their problems with eating and speech.

Early involvement of the neck flexors, the sternocleidomastoideus muscle, is also a common clinical feature in DM1. In a study to design a questionnaire for screening patients with DM1, the patient's capability to lift their head was one of three questions about physical movements that showed excellent discrimination of DM1 [27]. Recently, it has been suggested that the early impairment of truncal muscles as well as the neck flexors affected walking capacity and balance in DM1 [28]. Interestingly, a recent report proposed that functional impairment with DM1 could be assessed by an ataxia rating scale, the Scale for the Assessment and Rating of Ataxia (SARA) [29].

Because muscle wasting progresses very slowly and the severity can vary between patients, the natural course of muscle weakness in DM is difficult to evaluate. However, significant efforts have recently been made to elucidate the time course of muscle wasting through the preparation of clinical studies or trials for therapeutic development. In a retrospective study of 204 DM1 patients, the rate of decline in muscle strength (MMT) and handgrip force were −0.111 units and −0.24 kg per year, respectively [30]. In a 5-year prospective study, the median changes in isometric muscle force relative to baseline were reported to be −6 to −18% [31].

3.3.2 Histopathological Features of Myotonic Dystrophy Muscle

The prevalence of genetic testing using blood samples has resulted in muscle biopsies for diagnostic purposes rarely being performed; however, a biopsy of DM muscle can show various abnormalities [32, 33]. Similar to other muscular dystrophies, myopathic changes, such as the variation in fiber size from small angulated fibers to hypertrophied fibers, and an increase in endomysial connective tissue are common, but necrosis and regeneration are less frequent. In DM1, type 1 fibers atrophy but predominate [34]; the opposite change occurs in DM2 [32]. This change observed in DM1 is postulated to be due to a transformation from type 2 to type 1 fibers. Characteristic architectural abnormalities in cytoplasm are ring fibers and sarcoplasmic masses.

The most characteristic abnormality in DM is change to the histopathology of nuclei [32]. An increase in the number of nuclei and centrally located nuclei (internal nuclei) are very common. The centrally located nuclei can be observed as chained nuclei in longitudinal sections. In small atrophied fibers, condensation of nuclei, pyknotic nuclear clump, is observed. As mentioned earlier, abnormally retained RNA with repeat expansion can be observed by fluorescent in situ hybridization (FISH) as small foci in the nucleus.

The histopathology of cDM muscle is quite different from that of adult DM, with immaturity the outstanding feature. Poorly differentiated small and round muscle fibers with large central nuclei, reminiscent of myotubes, are usually observed [35]. Thus, the pathology of cDM is very similar to that of myotubular (centronuclear) myopathy. In the later stages, as cDM patients survive into adulthood, the histopathological changes become similar to those in adult DM [35].

3.3.3 The Molecular Mechanism for Aberrant Splicing

The molecular mechanism that leads to muscle wasting is more complex than the mechanism for myotonia, in which the missplicing of chloride channels is a major contributor. More than 50 missplicing events have been identified through individual analysis or comprehensive approaches such as splicing array or RNA sequencing, including for many important molecules known to cause human diseases (Table 3.2). In a comprehensive analysis of mice models, *HSA*^{LR} and MBNL knockout was beneficial for identifying these abnormal splicing events in human DM patients. Importantly, most were persistent existence of the splicing isoforms, which should switch during normal development regulated by either MBNL or CELF1 or both.

3.3.3.1 Aberrant Splicing of Ca^{2+}-Handling Proteins

These missplicing events include those involving molecules crucial for the excitation–contraction (EC) coupling of muscle fibers. In skeletal muscle, ryanodine receptor 1 (RyR1) releases Ca^{2+} from the sarcoplasmic reticulum in response to plasma membrane depolarization, via a mechanical interaction with voltage-gated calcium channels (Cav 1.1). The released Ca^{2+} is subsequently pumped back into the sarcoplasmic reticulum by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), maintaining the normal resting cytosolic Ca^{2+} concentration.

There have been reports of the missplicing of all these Ca^{2+}-handling proteins (RyR1, SERCA1, SERCA2, and Cav 1.1) in DM muscle [36–38]. Functional consequences of each missplicing were investigated further, and the misspliced RyR1 splice variant (the juvenile isoform) was shown to exhibit reduced activity by binding and single-channel recordings and homologous expression in dyspedic myotubes [36]. However, it was later shown that the properties of EC coupling were affected by the missplicing and that depolarization-dependent Ca^{2+} release was enhanced in fibers that expressed the misspliced RyR1 isoform [39]. Misregulated alternative splicing of *CACNA1S*, the gene encoding the CaV1.1 L-type calcium channel (DHPRα1S), has been reported [37]. Interestingly, the severity of splicing defect CaV1.1 was shown to be correlated with muscle weakness across a broad spectrum of DM1 patients [37]. Furthermore, experiments in mice with an antisense oligonucleotide, which forced a splicing shift of CaV1.1, revealed altered gating of the L-type calcium channel and an increase in the peak electrically evoked Ca^{2+}

Table 3.2 Missplicing events described in skeletal muscle of DM

Gene	Function	Ref.
Cl^- ion transport		
CLCN1	Chloride channel	[18, 19]
Ca^{2+} homeostasis		
RYR1	Ryanodine receptor	[36]
ATP2A1	Calcium ATPase	[36]
ATP2A2	Calcium ATPase	[36]
CACNA1S	Calcium channel	[37]
Cytoskeletal component		
DMD	Dystrophin	[43]
DTNA	Dystrobrevin	[46]
TTN	Titin	[52]
LDB3 (ZASP)	Z-disc	[52, 68]
BIN1	T-tubule formation	[48]
CAPZB	Assembly of actin filament	[69]
TNNT3	Troponin T	[20]
PDLIM3 (ALP)	Z-disc	[52, 70]
PDLIM7	Z-disc	[44]
FHOD1 (FHOS)	Actin organization	[52]
NRAP	Myofibril assembly	[52]
ABLIM2	Z-disc	[44]
MYBPC1	Myosin-binding protein C	[44]
MYOM1	Z-disc	[71]
MYH14	Myosin heavy chain	[72]
ANK2	Membrane targeting	[69]
CLASP1	Microtubule-associated proteins	[73]
Signaling		
INSR	Insulin receptor	[74]
SOS1	Receptor signaling	[44]
ALPK3	Myogenesis	[44]
CAMK2B	Signaling	[44]
MAP4K4	Signaling	[44]
Transcription		
MBNL1	Alternative splicing	[52]
MBNL2	Alternative splicing	[52]
NFIX	Transcription factor	[57]
MEF2C	Transcription factor	[44]
NCOR2	Transcriptional silencing	[44]
FXR1	mRNA trafficking	[69]
SMYD1	Chromatin remodeling	[57]

(continued)

Table 3.2 (continued)

Gene	Function	Ref.
Others		
GFPT1 (GFAT1)	Protein glycosylation	[52]
IMPDH2	Nucleotide biosynthesis	[44]
MTMR1	Phosphatase activity	[51]
CAPN3	Intracellular protease	[52]
PHKA1	Glycogenesis	[44]
PHKA2	Glycogenesis	[44]
KIF13A	Endosome positioning	[44]
VPS39	Vesicle trafficking	[44]
ARFGAP2	Vesicle trafficking	[44]
COPZ2	Vesicle trafficking	[44]
TBC1D15	Intracellular trafficking	[44]
OPA1	Mitochondrial dynamics	[44]
UBE2D3	Ubiquitination	[44]
USP25	Deubiquitination	[44]
TXNL4A	PQBP1-binding protein	[44]
VEGFA	Angiogenesis	[44]
MAPT	Tau	[44]
MLF1	Oncoprotein	[44]
SPAG9	Cancer testis antigen	[57]

Adapted with permission from Table 3.1 of [75]

transient [37]. In addition, the functional differences of the misspliced variant of SERCA1 (SERCA1B) were reported to be reduced compared to those of its normal variant (SERCA1A), probably due to the different reaction to the inner microsomal Ca^{2+} concentration [40].

The specific functional changes caused by each missplicing event suggest there is an increase intracellular Ca^{2+}, which is believed to cause muscle degeneration in many muscular disorders. However, the combined abnormality of Ca^{2+} homeostasis in DM muscle fibers has not yet been fully elucidated. Relatively early studies suggested an increase in intracellular Ca^{2+} in cultured DM muscles [38, 41]. A recent study, albeit including only a small sample of patients, indicated that the amplitude of intracellular Ca^{2+} transients induced by the sustained membrane depolarization of cultured myotubes was higher in DM1 myotubes but lower in DM2 myotubes compared to that in controls [42]. Furthermore, the structure of transverse (T-)tubules, which are essential for EC coupling, can be altered by missplicing of BIN1, which could further exacerbate the abnormal Ca^{2+} homeostasis in DM muscle (see below).

3.3.3.2 Aberrant Splicing of Cytoskeletal Proteins

Genetic mutations of many cytoskeletal proteins in skeletal muscle are responsible for hereditary muscle disorders. The abnormal splicing of dystrophin, whose mutations are responsible for Duchenne and Becker muscular dystrophy, has been

reported in DM1 patients [43]. The extent of missplicing of the penultimate exon in *DMD* has been shown to correlate well with muscle strength [44]. The splicing switch of the penultimate dystrophin exon 78 produces alternative mRNA due to a frameshift; this encodes a hydrophobic C-terminus that replaces the usual hydrophilic terminus in adult tissues. More recently, it was shown that forced *Dmd* exon 78 skipping and the subsequent embryonic dystrophin re-expression in wild-type mice led to muscle fiber remodeling and ultrastructural abnormalities, including ringed fibers, sarcoplasmic masses, and Z-band disorganization, which are characteristic features of dystrophic DM1 skeletal muscles [45].

The missplicing of another protein, dystrobrevin, which forms the dystrophin-associated glycoprotein complex, has also been found in DM muscle in addition to that of dystrophin [46]. The aberrantly spliced alpha-dystrobrevin isoform was localized to the sarcolemma and showed increased binding with alpha-syntrophin [46]. No hereditary disorders due to defects in the dystrobrevin gene have been reported. However, as with dystrophin, the missplicing of dystrobrevin has been shown to be one of the missplicing events that correlated remarkably well with muscle strength [44].

Skeletal muscle fiber has a unique membrane structure, the T-tubule, which is a tubular invagination of the sarcolemma; this enables the simultaneous contraction of all fibers located either superficially or deep in the cell. A variety of proteins contribute to producing and maintaining the shape of the T-tubule system. For example, the protein BIN1 (amphiphysin 2) is responsible for forming the structure of the T-tubule and ensuring that the appropriate proteins (in particular, the L-type calcium channels) are located within the T-tubule membrane [47]. In DM muscles, the abnormal splicing of BIN1 results in the expression of an inactive form of BIN1 that lacks phosphatidylinositol 5-phosphate-binding and membrane-tubulating activities [48]. It has been reported that mutations in BIN1 are responsible for centronuclear myopathy, which shares, as mentioned earlier, some histopathological features with cDM, by interfering with the remodeling of T-tubules and/or endocytic membranes [49]. It has also been shown that reproducing BIN1 splicing alterations in mice resulted in T-tubule alterations and muscle weakness [48]. Notably, the missplicing of BIN1 was one of the missplicing events that correlated with muscle strength [44].

3.3.3.3 Other Abnormal Splicing Events

Many splicing events other than those for Ca^{2+}-handling and cytoskeletal proteins have been reported to be altered in DM (Table 3.2). The functional consequences of most of these missplicing events, and their role in DM pathophysiology, remain to be elucidated. However, some potentially important molecules have been identified.

As mentioned earlier, the histological features of DM, especially those of cDM, share some of those of centronuclear (myotubular) myopathy, for which the major causative genes include *DNM2*, *BIN1*, *TTN*, and *MTM1* [50]. Mutations of *MTM1* are responsible for the X-linked severe form of myotubular myopathy. The myotubularin-related 1 (*MTMR1*) gene, part of the same family as *MTM1*, was reported to be aberrantly spliced in cDM muscles [51].

The reduction of MBNL due to its sequestration into nuclear foci has been reported as an underlying mechanism for many missplicing events in DM1 muscle; in addition, the splicing of MBNL1 itself has been observed to be altered in DM muscle [52]. The alternative splicing of MBNL1 exon 7, reported to increase in DM1, has been shown to directly couple the activity of MBNL proteins and the nuclear localization of MBNL1 at the cellular level [53]. An autoregulatory feedback loop has been postulated: when the nuclear level or activity of MBNL proteins is low, MBNL isoforms containing exon 7 are predominantly produced; once the nuclear MBNL proteins reach a certain level, isoforms without exon 7 are predominantly produced [53]. Although the alternative splicing of MBNL would not be the main factor driving the sequestration, it may enhance the accumulation of MBNL1 in the nucleus and the interaction with repeat expansion RNA [53].

3.3.4 Pathomechanisms Other Than Aberrant Splicing

Although misregulated splicing caused by reduction of MBNL due to sequestration to nuclear foci and upregulation of CELF1 by phosphorylation seems to be the major contributing pathomechanism in DM muscle, other mechanisms have also been proposed.

3.3.4.1 Other Roles of MBNL

The functional role of MBNL is not limited to the regulation of alternative splicing. MBNL2 regulates the localization of integrin α3 protein [54], and it was reported that MBNL1 is essential for the accurate control of destabilization of a broad spectrum of mRNAs as well as of alternative splicing [55]. A comprehensive analysis has shown a global role for Mbnls in regulating the localization of mRNAs, as well as the Mbnl-dependent regulation of the translation and protein secretion of MBNL-bound mRNAs [56]. A comparison of two mouse models with *HSA*^{LR} and MBNL1 knockout revealed disrupted extracellular matrix (ECM) mRNA regulation, possibly mediated by MBNL2 [57]. It was postulated that the loss of regulation of ECM functions and the consequent effects on cell adhesion contributed to muscle defects in DM1, because mutations in some of these ECM genes cause muscular dystrophies or connective tissue diseases [57].

3.3.4.2 Translational Regulation by CELF1

As mentioned earlier, the protein level of CELF1 is elevated in DM1 due to its increased stability [58]. The phosphorylation of CELF1 by protein kinase C was reported to contribute to the increase in steady-state levels of CELF1 [22]. CELF1 was shown to regulate the translation of proteins crucial for skeletal muscle

development, with mice that overexpressed CELF1 showing developmental delay and muscular dystrophy [59]. In DM1 muscle, the translational activity of CELF1 was shown to decrease due to hypo-phosphorylation at S302 by a reduced level of cyclin D3-dependent kinase 4 [60]. It has also been shown that the reduction of cyclin D3 in DM1 muscle was associated with the phosphorylation of cyclin D3 at T283 by active GSK3β kinase [61]. Correction of the GSK3β/cyclin D3 pathway in the DM1 mouse model improved skeletal muscle strength and reduced myotonia [61]; thus, GSK3β has become a novel therapeutic target for treating DM1, and a clinical trial with a GSK3β inhibitor, AMO-02, is currently underway [62].

3.3.4.3 Antisense Transcription and Repeat-Associated Non-ATG (RAN) Translation

Accumulating evidence indicates that repeat expansion mutations are bidirectionally transcribed, producing RNAs with expanded repeats in both directions [63]. CTG expansion has been shown to alter the regional chromatin structure and gene expression at the DM1 locus [64]. The loss of the insulator function due to impaired CTCF binding between *DMPK* and *SIX5* by expanded repeat gave rise to an antisense transcript that emanated from the adjacent *SIX5* regulatory region downstream of the CTG repeat [63]. It has also been proposed that the loss of insulator function between *DMPK* and *SIX5* could result in higher *DMPK* expression late in embryogenesis and could contribute to the earlier disease phenotype in congenital DM1 [63].

Furthermore, repeat expansion mutations were shown to be capable of producing proteins in all three reading frames without an AUG initiation codon; this is referred to as "repeat-associated non-ATG (RAN) translation" [65, 66]. In DM1, polyGln-RAN proteins have been found at low frequency in myoblasts, skeletal muscle, and the heart; they were more common in blood [65]. The toxic role of these RAN proteins was extensively investigated in neurodegenerative diseases, including C9ORF72 amyotrophic lateral sclerosis/frontotemporal dementia and fragile X-associated tremor/ataxia syndrome [67]. However, the contribution of RAN proteins to the pathomechanism in DM muscle remains to be elucidated.

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Chapter 4
Cardiac Involvements in Myotonic Dystrophy

Hideki Itoh and Takuhisa Tamura

Abstract For the management of myotonic dystrophy, cardiac involvements should be treated as being important as same as the treatment of respiratory failure at the aspect of prognosis particularly in patients with myotonic dystrophy type 1 (DM1). The most critical cardiac involvement is cardiac conduction diseases including sick sinus syndrome and AV block to be cared with cardiac pacemakers. The progressive cardiac conduction disease has been reported to be associated with sudden death in DM1 patients, and physicians need to manage when cardiac devices should be implanted in DM1 patients on the results by noninvasive or invasive cardiac tests, e.g., electrocardiogram, 24-h Holter monitoring, or electrophysiological study. Ventricular tachyarrhythmias should be also monitored for DM1 patients, and implanted cardioverter defibrillator but not pacemakers may be suitable especially for DM1 patients with severe conduction diseases. Atrial fibrillation has been reported to be common and a clinical marker to be associated with sudden death in DM1 patients. We regularly need to care whether these arrhythmias appear on electrocardiograms. As shown in the recent guideline in American Heart Association, neurologists are required to care DM1 patients with cardiologists.

Keywords Myotonic dystrophy · Sudden death · Sick sinus syndrome · Atrioventricular block · Pacemaker · Heart failure · Defibrillator

H. Itoh (✉)
Department of Cardiovascular and Respiratory Medicine,
Shiga University of Medical Science, Otsu, Shiga, Japan
e-mail: itohhide@belle.shiga-med.ac.jp

T. Tamura
Department of Neurology, National Hospital Organization Higashisaitama
National Hospital, Saitama, Japan

M. P. Takahashi, T. Matsumura (eds.), *Myotonic Dystrophy*,
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Abbreviations

ACEI	Angiotensin-converting enzyme inhibitor
ARB	Angiotensin receptor blocker
AV	Atrioventricular
CRT	Cardiac resynchronization therapy
DM	Myotonic dystrophy
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMPK	Myotonin-protein kinase
ICD	Implantable cardioverter defibrillator
LVEF	Left ventricular ejection fraction
MMRGlu	Myocardial metabolic rate for glucose

4.1 Introduction

Myotonic dystrophy (DM) is a neuromuscular disease with autosomal dominant inheritance and presents multiorgan involvements. The prevalence of the disease is estimated 1–2 per 8000 on the diagnosis of clinical backgrounds [1, 2]. Two subtypes have been genetically identified: myotonic dystrophy type 1 (DM1, Steinert's disease) [3] and myotonic dystrophy type 2 (DM2) [4]. Cardiac involvement is a major cause of sudden death in DM1 as same as respiratory failure [5]. The noted cardiac involvements are variable such as AV blocks, intraventricular conduction block, atrial fibrillation, atrial flutter, ventricular tachyarrhythmias, or these combinations. Device therapy including pacemaker, implantable cardioverter defibrillator (ICD), or cardiac resynchronization therapy (CRT) is sometimes demanded for patients with severe conduction abnormality under considering that it remains unclear whether conduction disease would be all causes of sudden death [6, 7]. On the other hand, DM2 patients with abnormal conductions or cardiac devices seem to be less than those in DM1 patients [8].

4.2 Pathophysiology

Since the discovery of the genetic defect of myotonic dystrophy type 1 as an expansion of CTG repeats in the 3′ untranslated region of myotonin-protein kinase *(DMPK*) gene on chromosome 19q13.3 [9–12], many hypotheses have been proposed for the pathomechanism of its cardiac involvement.

Initially the functional alteration of DMPK protein, a Ser/Thr protein kinase, was suspected. For example, Annane et al. [13] measured myocardial metabolic rate for glucose (MMRGlu) and demonstrated that reductions in MMRGlu and phosphorylation were inversely linked to the length of the repeat size. They suggested that an impaired modulation of DMPK might be involved in myocardial hexokinase

activation. Later, a study using *Dmpk* knockout mice revealed abnormal sodium channel gating in cardiac myocytes [14, 15]. However, it remained unanswered why the repeat expansion located at noncoding region affects protein function.

Since the identification of second gene responsible for DM, great advance has been made in the understanding of DM pathomechanism. DM2 has been shown to result from a tetra- (CCTG) nucleotide expansion within intron 1 of the CCHC-type zinc finger cellular nucleic acid-binding protein (*CNBP*) gene on chromosome 3q21. Mutations both in DM1 and DM2 involve untranslated genomic region, suggesting the pre-RNA with expanded repeats could play an important role. As described in Chap. 2, RNA-mediated mechanism has been established.

Similar to skeletal muscle or other affected organs in DM (see Chap. 3), a variety of missplicing have been reported in functionally important genes. Cytoskeletal components including troponin (*TNNT2*), dystrophin (*DMD*), dystrobrevin (*DTNA*), and titin (*TTN*) have been shown to be aberrantly spliced in DM heart [16–19]. Interestingly a potassium channel gene (*KCNAB1*) has also been reported to be aberrantly spliced in DM heart [17]. Wahbi et al. [20] suggested a splicing error of SCN5A could play a potential role of DM1 with Brugada ECG (Fig. 4.1). Their role for the generation of DM cardiac phenotype remained unelucidated. What is the most important molecule which contributes to the cardiac involvements in DM?

An internationally collaborated study between Japan, Germany, America, and France revealed an unsuccessful switch of alternative splicing in the *SCN5A* gene in DM heart samples [21]. It was reported that MBNL proteins modulate the switch from *SCN5A* exon 6A in fetal heart to exon 6B in adult. In DM1, therefore, interruption of MBNL by mutant RNA containing expanded repeats contributes to express a fetal *SCN5A* form. The "immature" proteins still work on the myocaridium as functional channel. But the "immature" sodium channels with loss of function by impaired activation seem inappropriate to adult heart function, ultimately resulting in cardiac conduction delay and heart arrhythmias (Fig. 4.2) [21]. Loss of function type SCN5A mutations are well-known to cause not only Brugada

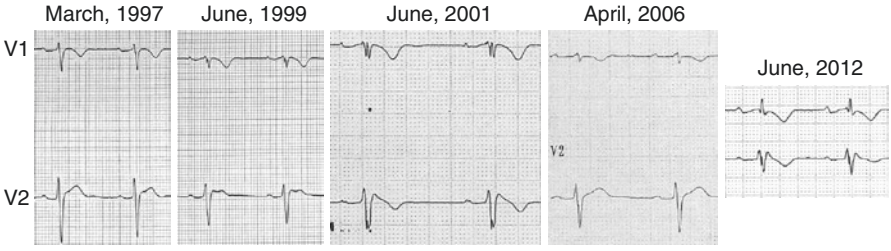

Fig. 4.1 Brugada ECG in DM1. Brugada ECG in DM1. ECGs were variable through his lifetime. In this 13-year-old patient, "saddleback" type 2 ECG appeared in June 1999 and showed a high tale-off ST-segment elevation with J-wave amplitude >2 mm. In June 2001, ST-segment type changed to coved-type ECG known as typical ECG observed in Brugada syndrome and displayed J-wave amplitude and ST-segment elevation ≥2 mm followed by a negative T-wave [37]. In April 2006, ST segment normalized, but coved ST-segment elevation with incomplete RBBB again appeared in June 2012. Electrocardiograms in this figure are original

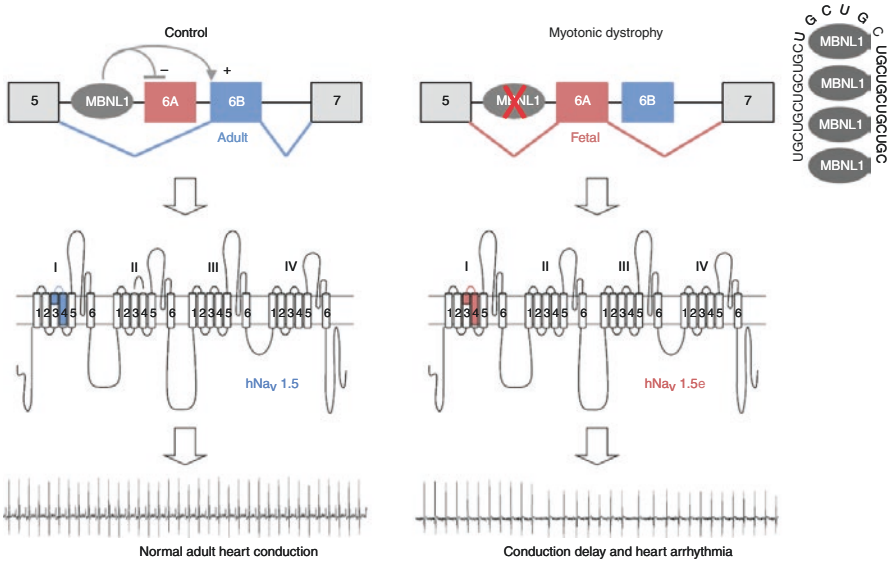

Fig. 4.2 Molecular mechanism of conduction diseases in DM. Model of splicing alteration of the cardiac sodium channel, *SCN5A*, in DM. MBNL proteins modulate the switch from *SCN5A* exon 6A in fetal heart to exon 6B in adult. In DM1, therefore, interruption of MBNL by mutant RNA containing expanded repeats contributes to express a fetal splicing form of *SCN5A*. The "immature" proteins still work on the myocardium as functional channel. But the "immature" sodium channels with loss of function by impaired activation seem inappropriate to adult heart function

syndrome [22–24] but also conduction disease or atrial fibrillation, representing cardiac sodium channelopathy [22]. Thus DM1 may demonstrate a phenocopy of cardiac sodium channelopathy with loss of function (Fig. 4.3) [23–31].

Though there have been some reports that reviewed relations between CTG repeats and electrocardiographic findings, it remains controversial whether the length of CTG repeats could be associated with the severity of cardiac involvements even now. According to the recent report, Chong-Nguyen et al. [32] clarified cardiac involvements corresponding to CTG expansion size for DM1 patients. They had discovered various conduction defects on electrocardiography and left ventricular (LV) systolic dysfunction on echocardiography and PR and QRS intervals corresponding to larger mutations by analysis of variance of ranks. The frequencies of sudden death, supraventricular arrhythmias, or pacemaker implantation were significantly higher in DM1 patients with larger mutations than others.

4.3 Electrocardiograms

Figures 4.1 and 4.4 show typical ECG courses in DM1 patients, e.g., atrial fibrillation, atrial flutter [33], or conduction diseases. All degrees of cardiac conduction defects are commonly observed in DM1 patients. The intraventricular block

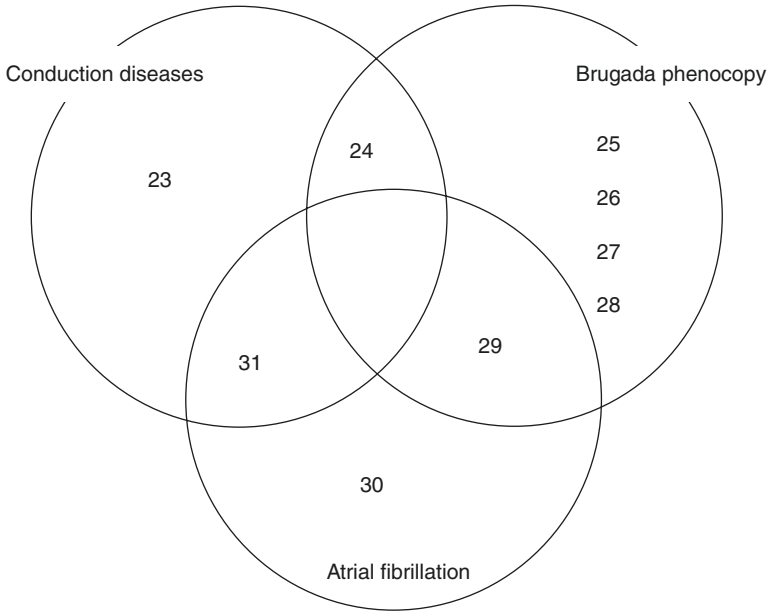

Fig. 4.3 Clinical phenotypes associated with loss of function of the cardiac sodium channel. Brugada syndrome, progressive conduction diseases, or familial atrial fibrillation has been reported to be associated with loss of function in the cardiac sodium channel. Each number indicates the corresponding reference. Cardiac involvements in DM1 may be a phenocopy of "cardiac sodium channelopathy"

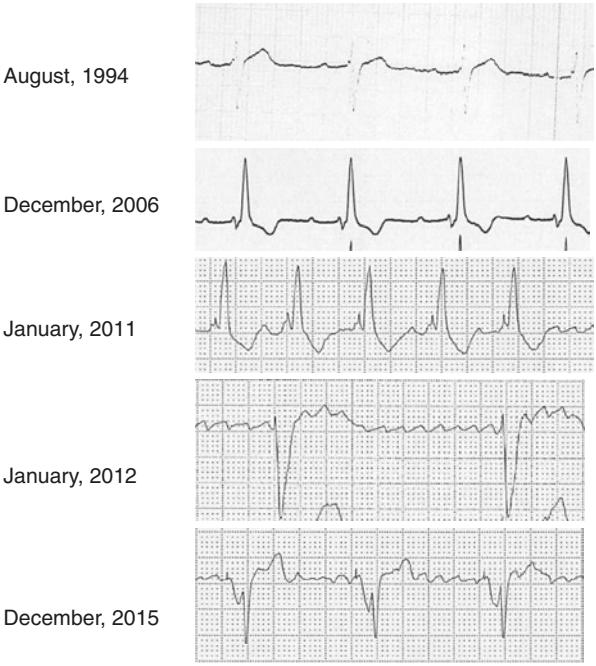

Fig. 4.4 Typical ECG course in DM1. ECG showed normal conduction in August 1994. Prolonged PQ interval and complete right bundle branch block appeared in December 2006, and atrial fibrillation to be characterized as F-wave at baseline occurred in January 2011. In January 2012, severe bradycardia (heart rate 33 bpm) occurred with and ventricular escape beats following complete AV block. Permanent pacemaker with a ventricular lead was implanted in December 2015. Electrocardiograms in this figure are original

including fascicular blocks or bundle branch blocks frequently occur along with AV blocks. Sick sinus syndrome is a comparatively rare than the AV block in DM1 patients, but it is important as one cause of the sudden death similarly. All bradyarrhythmias thus have to be carefully followed in DM1 patients because sudden death frequently happens in DM1 patients. Indeed, it remains a big issue to examine the relation between ECG abnormalities and sudden death. Groh et al. [34] reported that atrial fibrillation, PR interval over 240 ms, QRS duration over 120 ms, and second- or third-degree AV block could be a predictor of sudden death.

Figure 4.1 shows variable ST segments in a DM1 case. The recent articles [35, 36] had shown DM1 cases with the unique ST-segment elevation in leads II, III, and aV_F, "Brugada ECG." Brugada syndrome is an inherited arrhythmogenic syndrome without structural heart disease and causes unexpected nocturnal sudden death [37]. About 20% of this syndrome have been reported to be caused by the genetic background associated with cardiac ion channels, e.g., the cardiac sodium channel in the *SCN5A* (BrS1), *SCN1B* (BrS5), or *SCN10A* (BrS18), in the cardiac calcium channel in the *CACNA1C* (BrS3), *CACNB2b* (BrS4), *CACNA2D1* (BrS10), etc. [38]. Sodium channel blockers manifest "latent" mutation carriers in families with Brugada syndrome [39], while class Ic drugs, strong sodium channel blocker, have been also reported to elicit type 1 coved Brugada ECG in 18% of DM1 patients [36]. Though it remains unclear whether Brugada ECG could be associated with fatal cardiac events in DM1 patients, DM1 may be considered as a neuromuscular disease with "Brugada phenocopy" [40] or "phenocopy of progressive cardiac conduction diseases, PCCD, Lenegre's [41] or Lev's [42] disease" under considering the mechanism of sodium channelopathy as mentioned previously [21]. PCCD indeed has been also reported to be associated with mutations in the *SCN5A*, *SCN1B*, or *SCN10A*, as similar to causative genes in Brugada syndrome [43].

4.4 Arrhythmias

Variable types of brady- and tachyarrhythmias are associated with DM1. The AV block is a representative disorder in DM1. Wahbi et al. [44] reported major conduction defects such as third-degree AV block, type II second-degree AV block, or sick sinus node dysfunction requiring permanent pacing as the incidence of 19.3% in 12 years and that the major conduction disorders were associated with conduction defects on the ECG, a personal history of syncope, or atrial fibrillation. They also described that first-degree AV block and right bundle branch block were not independent predictors of sudden death, but that these conduction defects and left bundle branch block were associated with the development of major conduction defects.

Bradyarrhythmias are considered to be mainly due to the disease-specific pathological changes. The pathological changes such as fibrosis, fatty and lymphocyte filtration, and atrophy of cardiac conduction system have been reported in myotonic dystrophy with autopsy for half a century. Nguyen et al. [45] investigated the relationship of the ECG abnormalities and the histopathological changes of cardiac conduction system in 12 autopsy cases. In their report, the fibrosis of left bundle

branch was observed in nine, branching portion of the His bundle in eight, AV node and internodal approaches in seven, and sinus node in six cases. The penetrating of the His bundle was more often at the site of fatty infiltration than fibrosis. The first-degree AV block was present in five cases with excessive fibrosis of AV node. Whereas the first-degree AV block with intraventricular conduction delay was present in six cases with fatty filtration or excessive fibrosis of the His bundle, left bundle branch block was not present in six cases with fibrotic left bundle branch.

AV node conduction is easily influenced by autonomic tone as compared with the conductions of His bundle and Purkinje fibers. Therefore, widened QRS duration depends on the pathological abnormality, while PR prolongation follows both physiological conditions and pathological changes. Sick sinus syndrome is a minor disease among DM1 patients, while a case report indicated the pathological changes including atrophy, vacuolar degeneration, or interstitial fibrosis underlying in the right myocardium [46].

Atrial fibrillation (Fig. 4.4) is frequently observed with the percentage about 10–20% and important for the evaluation of sudden death because atrial fibrillation is an independent factor to be associated with sudden death as same as cardiac conduction disorders. Atrial flutter (Fig. 4.5) is also common [47], and catheter ablation of isthmus between tricuspid valve and inferior vena cava should be considered.

As shown in Fig. 4.6, ventricular tachyarrhythmias must be cared especially among DM1 patients with conduction disease, atrial fibrillation, or higher ages. Sustained ventricular tachyarrhythmias occurred in 2.3% of DM1, and the independent predictor

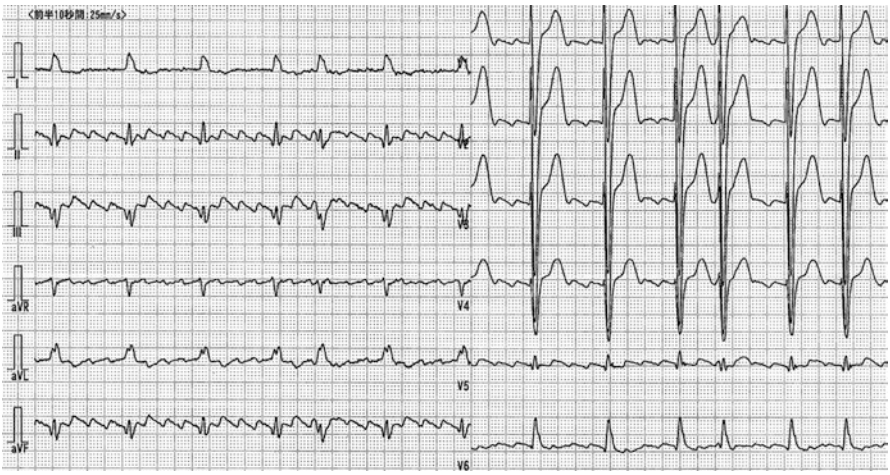

Fig. 4.5 Atrial flutter in a DM1 patient. Atrial flutter is also common among DM1 patients along with atrial fibrillation. The flutter waves are seen in leads II, III, and aV_F as a specific ECG finding in atrial flutter. In the reentrant circuit of atrial flutter, cardiac activation passes with a delayed conduction between inferior vena cava and tricuspid valve following the faster conduction in right atrium. The initial deflection of flutter waves gradually falls downward and then after that goes upward abruptly. Flutter shapes looks like "saw tooth" appearance. Flutter rate is generally between 250/min and 300/min, and cardiac heart rate depends on the ratio of AV conduction [33]. Electrocardiograms in this figure are original

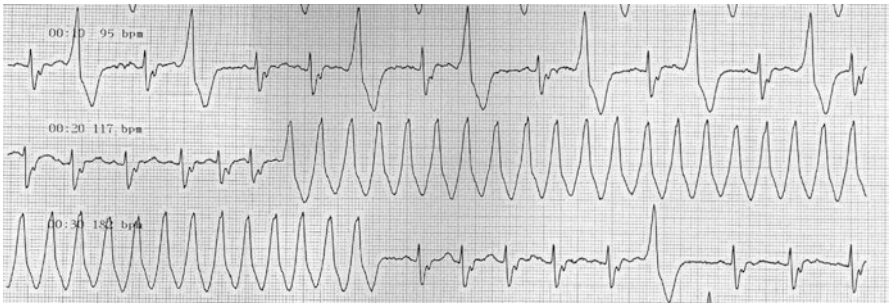

Fig. 4.6 Ventricular tachycardia in DM1. Ventricular tachycardia in a 34-year-old man with DM1. The ECG at baseline showed abnormal conduction with the widened QRS. Electrocardiograms in this figure are original

was only a personal history of non-sustained ventricular tachyarrhythmias [44]. Bundle branch reentrant tachycardia with wide QRS complex is characteristic in DM1 patients [48]. This tachycardia can occur in the origin of both bundle branches and one fascicle. Catheter ablation has been reported to be effective for the treatment of this arrhythmia [49].

4.5 Cardiac Function

Cardiac function may remain minor issue compared with conduction diseases and tachyarrhythmias in DM1. LV dysfunction with ejection fraction <50% was noted in 7.2% of DM1 patients [6], requiring more careful follow-up. Increasing age, male sex, conduction abnormalities on ECG, presence of atrial and ventricular arrhythmias, and implanted devices are more often associated with LV systolic dysfunction or heart failure, while LV dysfunction was independent from CTG repeat length and neuromuscular severity score [50]. LV systolic dysfunction or heart failure was significantly associated with all-cause death or cardiac death [51].

4.6 Management

4.6.1 Daily Care

Regular ECG recordings are recommended in DM1 patients. Physicians should take account of PQ interval, QRS duration, QT interval, RR interval, and electrical axis. Twenty-four-hour Holter ECG or external loop recorder is even more useful to detect the incidental arrhythmias such as severe bradyarrhythmia even if patients are asymptomatic.

Recently a scientific statement has been published from the American Heart Association regarding the management of cardiac involvement in neuromuscular diseases [52]. For DM patients with normal cardiac function who lack the features including palpitations, dizziness, syncope, non-sinus rhythm, PR interval over 240 ms, QRS duration over 120 ms, or second- or third-degree AV block, it is stated reasonable to assess by examination, ECG, and ambulatory ECG monitoring annually and by echocardiogram every 2–4 years. For young DM1 patients, it has been mentioned that exercise stress testing and signal-averaged ECGs may be possible options.

4.6.2 Medical Treatment

Angiotensin-converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) are recommended in neuromuscular disease patients with reduced LVEF to prevent from the LV remodeling [52], while we have no clinical trials to demonstrate the efficacy of ACEI and ARB corresponding DM1 patients. β-Blockers are also recommended in neuromuscular disease patients with reduced LVEF as same as ACEI or ARB, while we should take care of considering bradyarrhythmias or conduction blocks before start of β-blocker therapy. Diuretics, anticoagulants, or antiarrhythmic drugs should be considered under clinical conditions in each case. It is noted that class Ic antiarrhythmic drugs may not be preferable to DM1 patients because these drugs could induce Brugada ECG frequently than expectedly [36].

4.6.3 Catheter Ablation

Catheter ablation may give a great benefit for DM1 patients with tachyarrhythmias because β-blocker or antiarrhythmic drugs are often hard to be used for DM1 patients with a potential risk of bradyarrhythmias. Previous reports have shown the effects of catheter ablation in DM1 patients with ventricular tachycardia [49, 53, 54] or atrial flutter [55, 56].

4.6.4 Device Treatment

4.6.4.1 Pacemaker

According to the aforementioned recommendation, DM patients with palpitations, dizziness, syncope, non-sinus rhythm, PR interval over 240 ms, QRS duration over 120 ms, or second- or third-degree AV block should be evaluated at least annually

and considered for invasive electrophysiology study [57] for assessing an indication of pacemaker or ICD [52].

The multinational study demonstrated that HV interval over 70 ms should be under the consideration of prophylactic pacing devices [58]. In this report, Laurent et al. reported that the prognosis of prophylactic pacemaker implanted group with HV interval over 70 ms was similar to that of non-implanted group. Of 19 patients with pacemakers for paroxysmal third-degree AV block, 13 had non-sustained ventricular tachycardias. One patient who was treated with amiodarone for the paroxysmal atrial fibrillation suddenly died [59]. The prophylactic pacing may prevent DM1 patients from fatal arrhythmic events.

4.6.4.2 Implantable Cardioverter Defibrillator (ICD)

For the prevention of sudden death, ICD should be considered for DM1 patients with ventricular tachycardias at risk. The recent AHA guideline [52] also shows that ICD therapy may be appropriate upon thoughtful considerations of patients' conditions. The upgrade from pacemakers to ICD should be considered in DM1 patients with ventricular tachyarrhythmias, LV dysfunction, or progressive conduction abnormality [60]. Over 30% of DM1 patients with ICD therapy for the primary prevention had the incidence of ventricular tachyarrhythmias, and we need to care the presence of progressive conduction to be associated with the occurrence of ventricular tachyarrhythmias [61]. On the other hand, physicians have to carefully consider whether ICD would be beneficial in each DM1 patient because ICD-related complications such as inappropriate shocks, lead failure, or infection are more frequent than device complications in DM1 patients with pacemakers [62]. Bhakta et al. reported that ICD failed to improve the prognosis of DM1 patients with progressive conduction disease and we have to note that DM1 patients sometimes die suddenly even after ICD or PM implantations [60].

4.6.4.3 Cardiac Resynchronization Therapy (CRT)

Regarding some case reports, we need to consider the CRT therapy in DM1 patients with the conduction diseases associated with systolic dysfunction [63, 64]. CRT may be useful for the bridge therapy to heart transplantation in DM1 patients at end-stage heart failure.

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Disclosures. None.

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Chapter 5
Clinical Features of the Central Nervous System

Haruo Fujino, Shugo Suwazono, and Yuhei Takado

Abstract Progressive muscular weakness is a typical symptom of myotonic dystrophy, but more recently, central nervous system (CNS) involvement has become a critical issue in the disorder. Recent studies have suggested the importance of cerebral involvement in myotonic dystrophy, which influences patients' quality of life and functioning. CNS dysfunction in myotonic dystrophy has been investigated using various approaches, including cognitive (neuropsychological), neurophysiological, and neuroimaging studies. Studies have suggested that cognitive impairment in the disorder is variable, but several domains of cognition are frequently affected. Neurophysiological studies have examined the pathomechanisms of the disorder using various electrophysiological methods and modalities, such as somatosensory, visual, and auditory. Neuroimaging studies using different techniques have demonstrated that both white and gray matter of the brain are involved in the pathomechanisms of the disorder. Further accumulation of knowledge about the CNS involvement in myotonic dystrophy is required. Future possible directions of research are also discussed from each aspect in this chapter.

Keywords Myotonic dystrophy · Central nervous system · Brain · Cognitive impairment · Evoked potentials · Event-related potentials · Channelopathies · Hyperexcitability · Magnetic resonance imaging · Positron emission tomography

H. Fujino (✉)
Department of Special Needs Education, Oita University, Oita, Japan
e-mail: fjinoh@oita-u.ac.jp

S. Suwazono
Department of Neurology and Center for Clinical Neuroscience, National Hospital Organization Okinawa Hospital, Okinawa, Japan

Y. Takado
Department of Functional Brain Imaging Research, National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba, Japan

M. P. Takahashi, T. Matsumura (eds.), *Myotonic Dystrophy*,
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5.1 Introduction

The central nervous system (CNS) is an essential determinant of various human behaviors, including daily activities, participation in social situations, and interpersonal relationships. Recent studies have used various approaches to investigate several aspects of these issues. However, most previous studies of myotonic dystrophy have focused on muscular or functional impairments or organ dysfunction while overlooking CNS involvement. Despite a lack of evidence regarding the role of CNS dysfunction in myotonic dystrophy, this factor has become a major research focus in the past decade [1, 2]. Here, we review the literature regarding three essential aspects of CNS dysfunction in myotonic dystrophy: cognitive (neuropsychological), neurophysiological, and neuroimaging.

5.2 Cognitive/Neuropsychological Aspects of Myotonic Dystrophy

5.2.1 Cognition

An individual's ability to function in daily life is closely associated with their level of cognitive function. Cognition encompasses a wide variety of processes, including the perception of stimuli, distribution of attentional resources, verbal learning, and executive functions, all of which are necessary in everyday situations. Therefore, cognitive function plays an important role in the abilities of individuals to function in daily and social scenarios.

Although everyday life requires a very wide range of cognitive functions, the cognition domains included in this chapter are mainly limited to those functions measured by standardized cognitive tests (e.g., Trail Making Test [TMT]). In the general population, individuals may receive better or worse scores on different cognitive tests while largely remaining within normal ranges. By contrast, patients with myotonic dystrophy frequently receive performance scores below the normal limits, which suggests the presence of cognitive impairment.

5.2.2 Cognitive Impairment in Myotonic Dystrophy

Previous reports have described cognitive impairment in patients with myotonic dystrophy, with the above-described effects on social functioning, capacity to work, and quality of life. However, most studies have focused on myotonic dystrophy type 1 (DM1), rather than myotonic dystrophy type 2 (DM2). Therefore, this section will mainly summarize the literature concerning DM1. As a few such studies recently

demonstrated an association of cognitive impairment with quality of life [3–5], daily practice may need to incorporate assessments of cognitive function to improve the quality of life in patients with myotonic dystrophy. This is particularly important because medications are currently not available to treat CNS factors associated with myotonic dystrophy.

5.2.2.1 Affected Cognitive Domains

Patients with myotonic dystrophy exhibit a wide range of performance outcomes during neuropsychological tests [6]. Several studies have investigated impairments in different cognitive domains on patients with DM1, as summarized in Table 5.1 [5–15]. The focuses of most of these studies reflect the wide variety of cognitive domains affected by DM1, among which executive function is the most frequently reported domain. Executive function is the set of abilities required to plan actions and regulate behaviors and includes the organization of information and plans, the initiation of and focus on certain tasks, switching of focus, and inhibition of responses [16]. Patients with compromised executive function may experience difficulties in certain situations, such as planning activities or switching focus while talking. Several studies have reported impairments in abstract reasoning, processing speed, visual memory, and visuo-constructive ability. Abstract reasoning includes the abilities to identify background concepts and form concepts from materials, while processing speed includes the ability to process information rapidly, as well as executive control and response selection. Visuo-constructive ability includes the ability to see a stimulus as a set of parts and use these parts to form a design. By contrast, impairments in other functions have been reported with relatively less frequency. In addition to the classical cognitive domains, studies have also investigated impairments in social cognition (e.g., theory of mind and facial emotion recognition) [17–21], and the outcomes have suggested reduced function in this domain among patients with DM1.

Currently, no consensus has been reached regarding the cognition domains affected by DM1, although several domains appear to be more frequently impaired in patients. This lack of consensus among studies may be due to the characteristics of included patients (e.g., age range, clinical form, and severity).

To date, the natural history of cognitive impairments associated with DM1 has not been well investigated. However, a few longitudinal studies have examined the trajectory of cognitive functions in patients with myotonic dystrophy. Modoni et al. reported slight declines in language and fluency from the baseline after a 4-year interval ($N = 34$) [22]. Sansone et al. reported that both attention (visual) and the processing speed declined at 2–10 years after an initial assessment ($N = 20$) [13]. Winblad et al. reported declines in the cognitive domains of processing speed, attention, executive function, verbal memory, visual attention, and visuo-constructive ability (TMT A and B, Rey Auditory Verbal Learning Test, Spatial Span, and Block Design) in patients with classical DM1 at 5 years after a baseline assessment

Table 5.1 Cognitive domains affected by myotonic dystrophy type 1 (DM1)

	Major cognitive domains								
Study	Attention (verbal)	Attention (visual)	Abstract reasoning	Executive function	Fluency	Processing speed	Verbal memory	Visual memory	Visuo-constructive ability
Meola et al. [10]	NA			x			NA	NA	NA
Modoni et al. [11]		x	x	x	x	x	x	x	x
Gaul et al. [6]	NA		NA	x	x		NA		NA
Winblad et al. [15, 21]	x	NA	x	x		x		x	x
Sansone et al. [13]		x		x	NA				
Baldanzi et al. [7]	x	x	x	x	x	x	x	x	NA
Sistiaga et al. [14]	x		x	x		NA	x		x
Rakocevic-Stojanovic et al. [5]		x	x	x		x	x	x	x
Peric et al. [12]	NA		x	x		x		x	x
Gallais et al. [9]	x	x	x	x	x	x	x	x	x
Fujino et al. [8]	x	x	x	x	x	x	NA	NA	x

Check marks indicate studies that indicated cognitive impairment in the domain
NA not assessed

(N = 37) [23]. Recently, a 9-year longitudinal study including a relatively large number of patients (N = 115) observed declines in verbal memory, attention (visual), and processing speed throughout the study period [9]. This study also suggested that the patients' executive function and language and visual memory abilities were affected during early adulthood, whereas verbal memory, visual attention, and processing speed decreased later in life. Taken together, these studies suggest that cognitive impairments are progressive and that cognitive functions may exhibit different patterns of decline.

As noted above, fewer studies have reported the cognitive characteristics of patients with DM2 relative to those with DM1. Although one study failed to detect significant differences [6], another reported such differences between the conditions [12]. Several studies have observed executive dysfunction in patients with DM2 [10, 12, 13]. This discrepancy may be attributable to a difference in the sample sizes of the studies. Peric et al. [12] reported that patients with DM1 generally performed worse than did those with DM2.

The first and second Outcome Measures in Myotonic Dystrophy type 1 (OMMYD) meetings led to the proposal of relevant cognitive tests for the assessment of cognitive functions in patients with DM1. These tests include the TMT A and B, Color-Word Stroop Test, phonological fluency test (FAS), and the Block Design subtest of Wechsler Adult Intelligence Scale-Revised. Although the Frontal Systems Behavior Scale and Cambridge Brain Sciences computerized tests have been suggested as additional possible measures [24, 25], consensus batteries remain under consideration. Further research is needed to establish appropriate batteries for the assessment of cognitive impairment in patients with myotonic dystrophy.

5.2.2.2 Relevant Factors Affecting Cognition in Patients with Myotonic Dystrophy

Although considerable evidence confirms the existence of cognitive impairment in patients with myotonic dystrophy, various factors may affect cognitive functions in this population. One of the most influential factors is aging. Particularly, abilities such as the processing speed and perceptual reasoning decline with age, whereas others, including verbal comprehension and knowledge, are less affected. Studies of patients with myotonic dystrophy must therefore account for this highly relevant but non-specific factor.

Other major relevant factors affecting patients with myotonic dystrophy include fatigue, daytime sleepiness, apathy, and mood (depression) [26–29], all of which were discussed by the OMMYD-2 special interest group for common CNS symptoms [24]. Several medical conditions, such as cardiorespiratory disease, cataracts, and hearing problems, may also affect patients' task performances in tests of cognitive functions. These problems may affect cognitive test performance because the disease involves organs in multiple systems. Other factors that may correlate with the severity of cognitive impairment include the

number of cytosine-thymine-guanine (CTG) repeats and the clinical form of the disease [15, 30].

This wide range of factors makes it difficult to interpret impairments or declines in performance on cognitive tests among patients with myotonic dystrophy. A progressive decline in cognitive performance might be attributable to progressive muscular weakness and physical impairment. However, brain imaging studies have suggested that cognitive impairments related to this disorder can be attributed to brain structures and functions, as discussed later in this chapter.

Although no effective treatment has been established to address the cognitive impairment associated with myotonic dystrophy, interventions such as cognitive remediation therapy and cognitive enhancement therapy may improve cognitive functions. Cognitive behavior therapy may also be an effective psychosocial treatment for patients with myotonic dystrophy. A study investigating the effectiveness of cognitive behavior therapy is currently underway [31].

5.2.3 A Short Summary of this Section

In summary, cognitive function is an important predictor of social functioning and the quality of life in patients with myotonic dystrophy. Several cognition domains, including executive function, abstract reasoning, processing speed, visual memory, and visuo-constructive ability, are frequently impaired in individuals with this disorder. Of these, declines in some domains are observed in early adulthood, whereas other declines occur later. Although these declines appear to correlate with disease progression, more research is needed. Furthermore, treatments and modes of support for cognitive impairment and associated problems are needed, as these will improve the quality of life of patients with myotonic dystrophy.

5.3 Neurophysiological Aspects of Myotonic Dystrophy

In the peripheral nervous system (PNS), including from the peripheral nerve to the muscle, DM1 patients frequently present important and obvious neurophysiological abnormalities of great diagnostic value that are easy to confirm. However, what about the CNS? In this section, electrophysiological reports concerning the CNS will be overviewed and discussed, in conjunction with a dialogue about what kind of research is needed in the future, for the purpose of understanding the pathomechanisms of symptoms caused by CNS abnormalities in patients with DM1. Because sleep-related disorders are very important and serious problems and it would take up many pages to fully discuss them, they are not included in this section.

5.3.1 Previous Reports Examined with Various Modalities of Electrophysiology of the CNS

5.3.1.1 Electroencephalography

The slowing of background activity is described in many reports on the CNS in patients with myotonic dystrophy. In one of the earliest studies involving 18 cases (mean age, 45.2 years), 61% of the patients diagnosed as having myotonic dystrophy presented abnormal electroencephalograms (EEGs) [32]. This percentage was much higher as compared with those of other forms of muscular dystrophy, including Duchenne type (15%) or limb-girdle type (14%). In another report examining 84 cases (59 males; mean age, 36 years; standard deviation (SD), 12 years), 45% were judged as having no abnormality, 31% demonstrated a slowing of the dominant rhythm, 12% presented diffuse theta activity, 6% presented low-voltage EEGs, and 2% presented with focal epileptic abnormalities [33]. The mean amplitude of alpha rhythm was 44 μV and the SD was 14.4 μV. The mean frequency of the alpha rhythm was 8.5 Hz, which the authors pointed out might be shifted to a slower range, even considering the age of the patients. There was a tendency that the longer the duration of disease or the more advanced the stage was, then the slower the dominant rhythm was. A coherence analysis of alpha activity showed considerably high synchronization in both the lateral and anterior-posterior directions, which suggested a smaller number of neurons per unit area contribute to the generation of the alpha rhythms than normal [33].

5.3.1.2 Evoked Potentials, Following Somatosensory Stimulation

Among 15 cases (9 males; mean age, 35.8 years), 2 cases showed abnormality of somatosensory evoked responses following median nerve stimulation; 1 case showed a delay in P15, while the other showed a delay in N19 [34]. Because the patients with abnormal somatosensory evoked potentials (SEPs) showed normal peripheral nerve conduction, the authors discussed a possible relation between the SEP abnormality and pathological abnormality in the thalamus. Plural brain pathology reports have described inclusion bodies in the thalamus (as well as in the substantia nigra) of patients with DM1 [35–37]. In a different report of SEP evaluation following median nerve stimulation, an abnormal delay of peak latency was detected in 33% of participants including central and peripheral abnormalities among 21 cases (8 males; mean age, 34.4 years; SD, 11.8 years), and the authors discussed the possibility that abnormal muscle afferents contribute to SEP abnormalities in patients with DM1 [38]. SEP study following posterior tibial nerve stimulation showed a higher abnormal ratio (66% among 18 cases; mean age, 33.7 years) as compared with those observed following median nerve stimulation, but a correlation between age and abnormality was not clear [39]. Abnormal hyperactivity in recovery cycles was reported in N20-P25 and P25-N33 components of median SEP, indicating disinhibited cortical excitability [40].

5.3.1.3 Evoked Potentials, Following Visual or Auditory Stimulation

Abnormalities of pattern-shift visual evoked potentials (VEPs) have been reported in 10 out of 17 cases, which demonstrated 20/30 or better visual acuity [41]. In a report of results from 20 cases (mean age, 42 years; SD, 13.4 years), the P100 component of VEP became delayed in peak latency, and its amplitude decreased (versus N1); however, these abnormalities were not correlated with either the severity or duration of the disease [42]. This report also examined electroretinogram (ERG) findings, and 13 out of 20 cases showed no abnormalities in either VEP or ERG (e.g., small amplitude of b1 wave or a wave), while 3 cases showed abnormality only in ERG and 2 cases showed abnormality only in VEP. The authors discussed the importance of the retina as a possible site of lesion. Brainstem auditory evoked potentials (BAEP) study from 15 cases (9 males; mean age, 35.8 years; SD, 11.4 years) using rarefaction click sound as stimuli revealed a delay of interpeak latencies (I–III or III–V or I–V) in 8 cases and no abnormality in 7 cases [34]. The authors supposed abnormality at the upper pons and midbrain. In this report, five cases that presented with abnormal hearing showed a delay in peak latency of the wave I. It is noteworthy that, in patients with DM1, there might be subclinical cochlear damage [43] and an unexpectedly high prevalence of hearing disturbances in both high frequencies and the frequencies used for speech communication [44]. Arakawa et al. examined middle latency responses in eight cases (mean age, 56.9 years; SD, 6.7 years) and reported significant correlations between peak latency of the Nb component and CTG repeat number, but the amplitude of the Na and Pa components showed no correlation with CTG repeat number or intelligence quotient, although there were significant increments of Na and Pa amplitudes as compared with those from age-matched controls [45]. In this report, the reason for the delay in peak latency of the Nb component was attributed to a temporal lobe white matter lesion, where magnetic resonance imaging abnormalities are frequently observed [46]. As for the amplitude increment of the Na and Pa components, they discussed previous reports about Na-Pa amplitude increments following resection of the anterior temporal lobe in patients with intractable epilepsy [47] or as seen with the aging effect in normal subjects [48]. Examining multimodal evoked responses, visual and auditory evoked responses showed more frequent abnormalities than somatosensory modality [49].

5.3.1.4 Event-Related Potentials (ERP)

An auditory ERP study, using an “oddball” task (button press to 20% target tone) in eight cases (six mild cases, two moderate cases, aged 32–52 years), reported that the peak latency of the P300 component was delayed more than 3 SD as compared with that obtained from age-matched controls and that the amplitude of the P300 component was normal [50]. This report examined reproducibility in two sessions on 1 day, confirming that the abnormality was not derived from the sleepiness of the patients. Another group reported decreased amplitude and normal latency of the

P300 component [51, 52]. Although DM1 patients are frequently complicated with respiratory hypofunction, it was reported that only respiratory hypofunction cannot explain the P300 alteration [53]. Tanaka et al. observed prolonged N1 latency and attenuated N2/P3 amplitudes and reported significant hypoactivities at the orbitofrontal and medial temporal lobes, cingulate, and insula, using a "low-resolution brain electromagnetic tomography" method [54]. The waveforms in the figures by Kazis et al. [55] are comparable between the controls and the patients until P2 including N1; however, N2 and later components look different between the controls and the patients (specifically, they are remarkably delayed and with lower amplitudes). This dissociation between earlier responses and later responses cannot be explained by the abnormal skull thickness in DM1 patients [46, 56]; rather, it indicates some differences in information processing at N2 or later stages, and therefore, some cognitive declines are supposedly underlying in such ERP findings. Regarding the question of what kinds of higher function(s) are evidenced as abnormal via electrophysiological methodology, no satisfactory answers exist yet. Further elaborate research projects are definitely needed to elucidate the exact nature of cognitive disturbances in patients with DM1, including those that combine or include a detailed cognitive/behavioral assessment and ERP measurements.

5.3.2 Questions to Be Addressed

5.3.2.1 Hyperexcitability?

Considering the observations generated by needle electromyography, one might expect similar hyperexcitability in the CNS also. Hyperexcitability of the CNS is usually discussed when a high amplitude of evoked potentials is obtained. Only limited reports clearly demonstrate electrophysiological hyperexcitability of the CNS in patients with DM1, Mochizuki et al. [40] in the somatosensory modality and Arakawa et al. [45] in the auditory modality. It is not clear yet if these observations are ubiquitous in the CNS in terms of location and sensory modality, although there are only limited case reports of epilepsy in patients with DM1 (e.g., [57]), and no reports with intracranial EEG recording are available.

5.3.2.2 Channelopathy?

There are two interesting physiological techniques to investigate channelopathies (in vivo) of the PNS in patients with DM1. In a biopsied muscle from a patient with DM1, abnormally frequent late reopenings of sodium channels were reported [58]. In vivo muscle membrane property measurement via muscle velocity recovery cycle analysis helps to evaluate supernormality caused by a decrement of sodium current [59]. If "CNS channelopathy" is assumed, and some confirmation/evaluation method of it becomes available (similarly to those for the "PNS channelopathy"),

then more rational therapy (although symptomatic) can theoretically be proposed and might be implemented more widely (c.f., [60]).

5.3.3 A Short Summary of This Section

As described above, that available knowledge is too limited and insufficient to draw a comprehensive and precise schema about the ongoing processes in the CNSs of patients with DM1. Are those findings that were described in the above review correlated with (too fast) aging of the brain, severity of molecular/metabolite abnormality, or specified localization of advanced imaging abnormality? These and other issues remain to be considered or addressed. Considering the problems related with the adherence to the medical care at various stages of their life, well-designed management methods based on a better understanding of CNS problems may play a crucial role in ensuring better care of patients with DM1.

5.4 Neuroimaging Aspects of Myotonic Dystrophy

While muscle weakness is one of the main symptoms of DM1, cognitive impairment is also an important issue that significantly influences a patient's quality of life. CNS alterations are generally investigated by neuroimaging techniques, such as magnetic resonance imaging (MRI) and positron emission topography (PET) imaging. MRI techniques include structural imaging, diffusion tensor imaging (DTI), functional MRI (fMRI), and magnetic resonance spectroscopy (MRS). A limited number of PET studies on patients with DM1 use 18F-fluoro-2-deoxy-d-glucose (FDG) or H2(15)O. On the other hand, there are an increasing number of neuroimaging studies on patients with DM1 that use different MRI techniques, leading to a deeper understanding of the mechanisms underlying CNS alterations.

In this neuroimaging section, we summarize neuroimaging studies for each imaging modality to deepen the understanding of the mechanism underlying cognitive impairment in patients with DM1.

5.4.1 Structural MRI

Among neuroimaging studies of patients with DM1 using structural MRI, anterior temporal white matter (WM) lesions in T2-weighted images (T2WI) is a common finding [61]. Because similar lesions in the temporal lobe of T2WI are observed in other diseases such as CADASIL [56], those WM lesions are not specific to patients with DM1. Nevertheless, a high prevalence of WM hyperintensities (WMHs) in patients with DM1, increasing with age and disease duration, is demonstrated [62]. Lesion probability maps quantitatively show that WMHs are mainly located in the anterior

temporal, frontal, parieto-occipital, and periventricular WM regions [62]. Postmortem studies of patients with DM1 indicate that a disordered arrangement of myelin sheaths/axons, fibrillar gliosis, and increased interfascicular and perivascular space are likely to be the main substrates of WMHs [62]. These authors suggest that myelin alterations and changes in intraxonal water content lead to the increased T2 relaxation times. This may explain the scarcity of information on the pathology of lesions with WMHs because water is not preserved during histopathological analysis.

Dilated Virchow–Robin spaces are also investigated in patients with DM1 [63]. Di Costanzo et al. studied 41 patients and 41 healthy controls using MRI [63]. The results revealed that convexity Virchow–Robin spaces are more frequent (68% vs. 34%; $p < 0.01$) and severe (median scores, 4 vs. 0; $p < 0.01$) in patients with DM1 than in controls. The reason why Virchow–Robin spaces are dilated in patients with DM1 has yet to be clarified.

Global brain atrophy is considered to be associated with cognitive impairment in patients with DM1. Two independent studies showed that gray matter (GM) atrophy occurred in several brain regions, including temporal and frontal lobes, hippocampi, and thalami using the voxel-based morphometry (VBM) technique [64, 65]. Furthermore, one study showed a significant negative relationship between left temporal atrophy and verbal memory [66].

Cortical and subcortical GM and WM atrophy in patients with DM1 is associated with cognitive impairment, depression, and daytime sleepiness [65]. When the VBM results of the DM1 patient group were compared with those of healthy subjects, the results revealed that the DM1 group showed ventricular enlargement and supratentorial GM and WM atrophy. WM was reduced in patients with DM1 in the splenium of the corpus callosum and in the left-hemispheric WM adjacent to the pre- and postcentral gyrus. The study showed that GM and WM atrophy was significant in patients with DM1, indicating that pathological regions are localized both in GM and WM.

Using VBM and DTI, Caso et al. studied 51 patients with DM1 (17 juvenile DM1 and 34 classic adult DM1) and 34 healthy controls. Intriguingly, while classic adult DM1 had severe patterns of GM atrophy and WM tract damage, in juvenile patients with DM1, WM abnormalities exceeded GM involvement. Thus, both GM and WM are affected in patients with DM1, and the level of impairment in each region could be influenced by the onset age of the DM1 symptoms [62]. This led the authors to suggest a degenerative origin of GM abnormalities along with developmental changes of microstructural WM alterations in patients with DM1 [62]. Since studies involving both juvenile and classic adult DM1 patients are scarce, this hypothesis remains to be validated in future studies. Also, longitudinal studies will be important to confirm a degenerative origin of the GM.

5.4.2 DTI

DTI is utilized for detecting fine structural changes that cannot be detected by conventional MRI such as T2WI. Minnerop et al. claim that DM1 is a predominant WM disease using DTI and VBM [64]. Using tractography, Wozniak et al. demonstrated

that DM1 has diffuse WM abnormalities [67]. The other study also detected the involvement of normal appearance WM using tract-based spatial statistics [66].

Taken together, most DTI studies claim that DM1 is a predominant WM disease. However, some studies show other regions can be affected besides WM. Cabada et al. studied a total of 42 patients with DM1 and 42 healthy controls by MRI examinations and clinical evaluations. The results demonstrated that patients with DM1 had diffuse WM DTI abnormalities, subcortical structure volume loss, and increased medial diffusivity, suggesting the affected regions are not limited to WM [68].

There is evidence that GM is also affected in patients with DM1 based on structural imaging studies, and the claim that DM1 is a predominant WM disease could be simply due to the technical issue of DTI analysis. Indeed, a similar situation is found in Alzheimer's disease research, for which most DTI studies focused more on WM than GM until recently [69]. While defining a region of interest (ROI) in GM is not an easy task due to the partial volume effect of cerebrospinal fluid, it is not very difficult to define a ROI in WM based on brain anatomy.

The precise mechanism of changes in parameters of DTI such as fractional anisotropy and mean diffusivity is not fully understood in patients with DM1. A correlation study between imaging and postmortem examination is warranted in the future.

5.4.3 MRS

MRS can measure the contents of brain metabolites in vivo, noninvasively. Using metabolites' information, one can speculate on pathological changes in patients with DM1. N-acetylaspartate (NAA) is known to be produced in neuron mitochondria and can be used as a marker of neuronal function. Choline (Cho) is believed to reflect membrane turnover, while myo-inositol (MI) is present mainly in astrocytes. Using Cho and MI as markers, cell proliferation as well as gliosis can be predicted. With the recent progress of the MRS technique, neurotransmitters, such as glutamate (Glu) and gamma-aminobutyric acid (GABA), can now also be measured. While the involvement of Glu has been demonstrated previously [70], GABA has not yet been investigated in patients with DM1.

There are several MRS studies in the brain of patients with DM1 [70–74], and there has also been a debate in the MRS research field regarding the idea of DM1 being a WM or GM disease. Takado et al. addressed this question directly in their study along with their goal of identifying the affected brain regions in patients with DM1. In their study, they used 3T MRS and MRS imaging (MRSI) [70]. MRSI revealed a reduced NAA to creatine (Cr) ratio (NAA/Cr) in multiple brain regions (average 24%), suggesting diffuse brain abnormalities among patients with DM1. Single-voxel MRS in patients with DM1 showed the following: (1) reduced NAA in both the frontal cortex (23%) and frontal WM (31%) and unaltered MI, suggesting neuronal abnormalities without significant gliosis, and (2) elevated glutamine in the frontal cortex (36%) and reduced Glu in the frontal WM (20%) in patients with DM1, suggesting abnormalities in the glutamatergic system in the brain of patients

with DM1. The study demonstrated that both GM and WM are affected globally in patients with DM1.

Although several MRS studies showed evidence of diffuse abnormalities in the brain of patients with DM1 [70–74], the pathomechanism of the brains of patients with DM1 is still unknown. Some researchers claim multiple factors could be involved in the pathomechanism including RNAopathy, tauopathy, and spliceopathy [75]. Among those, the influence of tau on cognitive impairment can be assessed in vivo by tau PET imaging [76], which has not been performed yet in patients with DM1.

5.4.4 Resting-State fMRI (rsfMRI)

rsfMRI has been used for investigating the brain function of patients with DM1. Although some abnormalities were detected, the brain findings are not specific to DM1 [77, 78]. Thus, the limitation of this methodology is the lack of specific information by rsfMRI alone. However, combined with more specific technologies such as PET, described in the next paragraph, rsfMRI can be useful.

5.4.5 PET

PET can provide another type of pathophysiology information. So far, only a few ligands including FDG and H2(15)O have been used in patients with DM1. Peric et al. demonstrated that brain PET in patients with DM1 and DM2 showed the most prominent glucose hypometabolism in prefrontal, temporal, and pericentral regions in both DM1 and DM2 patients [79]. In patients with DM1, they found an association between right frontotemporal hypometabolism and executive dysfunction ($p < 0.05$).

Another study used H2(15)O PET in patients with DM1 for investigating cerebral blood flow [80]. The results revealed that DM1 had widespread hypoperfusion that extended to the dorsolateral frontal cortex and subcortical regions.

Although FDG and H2(15)O PET are useful for investigating general brain physiology, using more specific radioligands such as tau ligand would be beneficial [76]. Since this technique is available for testing in vivo patients, it would be useful for investigating the association between tau and patients with DM1 cognitive impairment.

5.4.6 A Short Summary of This Section

In this section, we described imaging studies in patients with DM1 to have a better understanding of cerebral symptoms in patients with DM1. Different imaging techniques showed that both GM and WM are involved in the pathomechanisms of the

brains of patients with DM1, and the respective distribution of GM and WM may be influenced by the age at DM1 onset. Because open questions remain pertaining to cognitive impairment of patients with DM1, in vivo neuroimaging techniques can provide further insights to identify efficient therapeutic strategies in the future.

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Chapter 6
Brain Pathology in Myotonic Dystrophy

Yasushi Iwasaki

Abstract Although patients with myotonic dystrophy (MyD) present with various neurological and psychiatric symptoms, the pathogenesis has not been elucidated. Therefore, we performed a detailed clinicopathological examination of 34 patients with MyD (24 men and 10 women) and statistical analyses. The average age at death was 57.6 years (range, 41–74 years). Mild proportional atrophy generally occurred in the cerebrum, although some cases exhibited normal external appearances. The mean brain weight was 1152.6 g (range, 900–1490 g). Neurofibrillary tangles (NFTs) were observed in the entorhinal to transentorhinal cortices in all cases and were generally extensively distributed. Although the observed NFTs were considered abnormal when patient ages were considered, the number and distribution varied between the cases. Regarding the NFT staging, as described by Braak et al., 8 cases were Stage I, 16 cases were Stage II, 9 cases were Stage III, 1 case was Stage IV, and there were no Stage V or Stage VI cases. In contrast to NFTs, senile plaques were rarely observed. Characteristic eosinophilic cytoplasmic inclusions in the thalamic nuclei (thalamic inclusions) were detected in most cases, although the numbers also varied between patients. Similar eosinophilic inclusions were also detected in the substantia nigra of some patients. Furthermore, the number of Marinesco bodies generally increased. Additionally, myelin pallor was observed in the deep cerebral white matter, and dilatation of the perivascular spaces and hyalinized vessels was remarkable in the cerebral white matter in some cases. These findings suggest that various neuropathological changes contribute to the neuropsychiatric symptoms of MyD. Our statistical analyses indicated that the number of CTG repeats and Braak NFT stages were not significantly correlated, whereas both age at death and disease duration were significantly correlated with Braak NFT stages. The number of CTG repeats was not significantly correlated with either age at disease onset or disease duration, whereas disease duration and brain weights were significantly correlated. The present investigation demonstrated that neuropathological changes are

Y. Iwasaki
Department of Neuropathology, Institute for Medical Science of Aging,
Aichi Medical University, Nagakute, Aichi, Japan
e-mail: iwasaki@sc4.so-net.ne.jp

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heterogeneous in patients with MyD, suggesting that the pathological mechanisms of MyD are complex and multifactorial.

Keywords Neurofibrillary tangle · Thalamic inclusion · Marinesco body · Hyperostosis frontalis interna · Aging pathology

6.1 Introduction

Myotonic dystrophy (MyD) is an autosomal dominant genetic disorder; it is the most common type of adult muscular dystrophy, affecting approximately 1 in 8000 people [1]. MyD affects multiple organ systems and is characterized by myotonia, progressive muscle weakness, and multisystem complications including cataracts, cardiac conduction defects, hypogonadism, and endocrinopathies such as frontal baldness and insulin resistance (Fig. 6.1a). MyD also affects the central nervous system and presents with various neurological and psychiatric symptoms including cognitive impairment (dementia), lack of awareness regarding the disease, hypersomnia, and personality changes [1, 2]. However, the presentation and severity of symptoms varies. Furthermore, the pathological mechanisms of MyD have not been fully elucidated [2]. MyD is categorized into two genetic types. The majority of MyD cases are type 1 (DM1), and result from mutations in the gene for dystrophia myotonica protein kinase (DMPK), which is located on the long arm of chromosome 19. More rarely, MyD type 2 results from an unstable tetranucleotide repeat expansion, CCTG, in intron 1 of the gene for cellular nucleic acid-binding protein

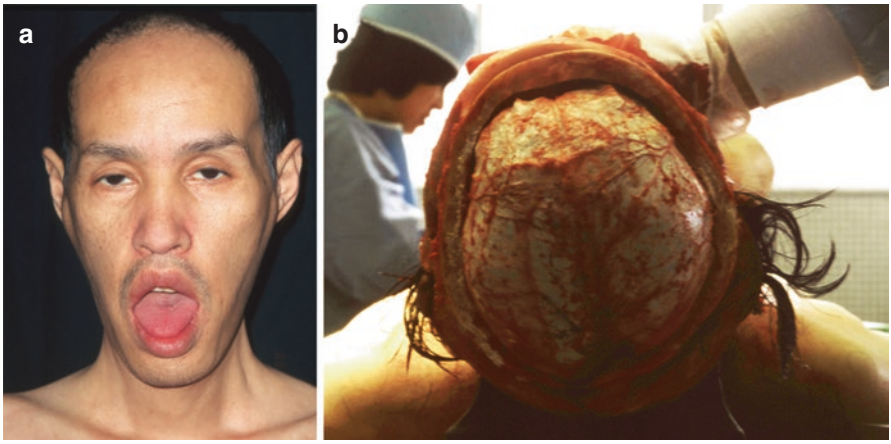

Fig. 6.1 Characteristic hatched face and hyperostosis frontalis interna. (**a**) Hatched face appearance with frontal baldness, ptosis, and muscular atrophy of the sternocleidomastoid in a patient with myotonic dystrophy (MyD). Permission to present this photograph was obtained from the patient. (**b**) Hyperostosis frontalis interna recognized at autopsy of a patient with MyD from the present series. Symmetrical thickening of the frontal bone of the skull was apparent. (**a**), Case 17; (**b**), Case 16

(CNBP), which is located on the long arm of chromosome 3 [1]. DM1 is associated with an expansion of the CTG (cytosine-thymine-guanine) trinucleotide repeats in the gene for DMPK.

There is no neuropathological diagnostic hallmark for MyD, in contrast to features such as tufted astrocytes in progressive supranuclear palsy or astrocytic plaques in corticobasal degeneration. Therefore, definitive diagnosis of MyD is not currently possible by neuropathological investigation alone. However, several reports have described various neuropathological features of MyD, including neurofibrillary tangles (NFTs), intracytoplasmic inclusion bodies in the thalamus, vascular changes, and Marinesco bodies [1, 2]. Therefore, to investigate the underlying neuropathogenesis in patients with MyD, we assessed clinical and neuropathological findings in autopsied patients, including performing statistical analyses.

6.2 Clinicopathological Investigations

6.2.1 Participants

We examined 34 formalin-fixed brain specimens from clinicopathologically confirmed Japanese patients with MyD, which were obtained from the Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University. The primary facility of our research institute is the department of neurology, which resulted in no cases of congenital MyD. All cases were assumed to be DM1, although DMPK gene analysis was not performed for all patients.

This study was approved by the Ethics Committee of Aichi Medical University.

6.2.2 Clinical Features

The features of each case were retrospectively reviewed using clinical records, particularly including the following factors: year of death, age at disease onset, age at death, disease duration, gender, and the number of CTG repeats (examined using peripheral blood prior to death). We also examined whether tube feeding (including performed gastrostomy) or tracheotomy was performed, as well as whether a respirator (mechanical ventilator) was used during the disease. Regarding respirator use, use only during the terminal phase was not included in these analyses. Brain weights were obtained from autopsy records.

When the clinical record did not report on a particular variable, we classified it as “N.D. (not described)” in the relevant data column. Data are expressed as means ± standard deviations or as medians and ranges where appropriate.

6.2.3 Neuropathological Examinations

Neuropathological examinations were performed on 8-μm brain sections, using hematoxylin-eosin (HE), Klüver-Barrera (KB), and Gallyas-Braak (GB) silver stains. Immunohistochemical analyses of selected sections were also conducted for anti-amyloid β (Dako, Glostrup, Denmark; 1:100) and anti-hyperphosphorylated tau (AT-8; Innogenetics, Ghent, Belgium; 1:1000), as well as anti-3 repeat (3R) and anti-4 repeat (4R) tau (RD3 and RD4, respectively; Upstate, EMD Millipore, Billerica, MS, USA; 1:5000 and 1:100, respectively). RD3 and RD4 immunostaining occurred after pretreatment in a microwave oven and formic acid for 10 min. Primary antibody binding was detected using the labeled streptavidin-biotin method (Dako LSAB kit; Dako). Peroxidase-conjugated streptavidin was visualized using 3,3′-diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as the chromogen. The immunostained sections were counterstained lightly with Mayer's hematoxylin.

Neuropathological findings were investigated, particularly in relation to the following factors: macroscopic findings including brain atrophy, microscopic findings including cerebral cortical involvement, NFTs and related pathology, intracytoplasmic inclusion bodies in the thalamus (thalamic inclusions), vascular lesions, and additional factors including Marinesco bodies. GB silver staining was primarily used to investigate NFTs and senile plaques. The distributions of NFTs were assessed by the criteria proposed by Braak et al. (Braak NFT staging) [3]. We semi-quantitatively classified the appearance of thalamic inclusions and Marinesco bodies, as sparse, moderate, and many.

6.2.4 Statistical Analyses

Spearman's rank correlation coefficient was used to assess the relationships between Braak NFT stages and each of the following factors: number of CTG repeats, age at death, and disease duration. The relationships between the number of CTG repeats and both age at disease onset and disease duration were also assessed. Additionally, the relationships between brain weight and both disease duration and age at death were assessed. Statistical analyses were performed using Excel 2010 (Microsoft, Redmond, WA, USA) and Statcel 2 add-in software (OMS, Tokyo, Japan). Statistical significance was set at $p < 0.05$.

6.3 Clinicopathological Findings

6.3.1 Clinical Findings and Brain Weight

Clinical findings for each patient, arranged by year of death from 1987 to 2016, are shown in Table 6.1. This series included 24 men and 10 women. The mean age at death was 57.6 ± 7.3 years ($n = 34$; median, 58 years; range, 41–74 years), with an

Table 6.1 Clinical and neuropathological findings of the present myotonic dystrophy series

Clinical findings										Neuropathological findings											
Case no.	Year of death	Age at disease onset	Age at death	Disease duration (years)	Gender	Tube feeding	Tracheotomy	Mechanical ventilation	Number of CTG repeats	Brain weight	Brain atrophy	Braak NFT stage	Senile plaque	Cerebral amyloid angiopathy	Thalamic inclusion	Lewy body	Argyrophilic grain	Marinesco body	Vascular lesions	White matter degeneration	Additional findings
1	1987	51	58	7	M	–	–	–	N.E.	1460	–	I	–	–	Sparse	–	–	Sparse	–	–	
2	1988	33	41	8	M	Nasal tube	+	+	N.E.	1280	Moderate	I	–	–	Sparse	–	–	Sparse	–	–	
3	1988	29	51	22	M	–	–	–	N.E.	1490	–	II	–	–	Moderate	–	–	Moderate	–	–	Calcification of globus pallidus, APNB
4	1992	N.D.	45	N.D.	M	Nasal tube	+	+	N.E.	1210	Mild	I	–	–	Sparse	–	–	Sparse	–	–	
5	1993	39	58	19	F	Nasal tube	–	–	N.E.	1095	Mild	II	–	–	Sparse	–	–	Sparse	–	–	
6	1995	21	61	40	M	–	–	–	N.E.	1220	Mild	II	–	–	–	–	–	Sparse	–	Mild	
7	1996	52	65	13	M	–	+	+	N.E.	1110	Mild	II	–	–	Moderate	–	–	Moderate	–	–	APNB
8	1996	40	62	22	M	–	–	–	N.E.	1200	Mild	III	–	–	Moderate	–	–	Moderate	–	Mild	Callosal thinning, BC
9	1996	12	53	41	M	–	+	–	N.E.	1160	Mild	II	–	–	Moderate	–	–	Sparse	MCA embolism	Moderate	Unilateral PTD
10	1997	31	54	23	M	–	–	–	N.E.	1060	Moderate	II	–	–	Moderate	–	–	Sparse	–	–	
11	1997	15	50	35	M	–	–	–	1000	990	Moderate	III	–	–	Sparse	–	–	Moderate	Moderate	Moderate	BC, unilateral PTD

(continued)

Table 6.1 (continued)

Clinical findings										Neuropathological findings											
Case no.	Year of death	Age at disease onset	Age at death	Disease duration (years)	Gender	Tube feeding	Tracheotomy	Mechanical ventilation	Number of CTG repeats	Brain weight	Brain atrophy	Braak NFT stage	Senile plaque	Cerebral amyloid angiopathy	Thalamic inclusion	Lewy body	Argyrophilic grain	Marinesco body	Vascular lesions	White matter degeneration	Additional findings
12	1997	N.D.	67	N.D.	M	N.D.	N.D.	N.D.	N.E.	1155	Mild	II	–	–	Moderate	–	–	Sparse	Mild	Mild	
13	1998	36	51	15	M	–	–	–	N.E.	1270	Mild	II	–	–	Moderate	–	–	Sparse	–	Mild	
14	1998	40	66	26	M	–	–	–	1066	1190	Mild	II	–	–	Moderate	–	–	Many	–	–	
15	1998	30	65	35	F	–	–	–	N.E.	1030	Moderate	IV	–	–	Sparse	–	–	Sparse	Moderate	Moderate	
16	1998	10	51	41	F	–	–	–	1500	1100	Mild	III	–	–	Moderate	–	–	Sparse	–	–	APNB
17	1999	41	52	11	M	Gastros-tomy	+	–	N.E.	1370	–	I	–	–	Moderate	–	–	Moderate	Mild	Mild	
18	1999	45	64	19	M	Nasal tube	–	–	933	1200	Mild	II	–	–	Moderate	–	–	Sparse	–	–	
19	1999	34	61	27	F	–	–	–	1100	900	Moderate	III	–	–	Moderate	–	–	Moderate	–	–	Mild CPM
20	2001	41	59	18	M	–	–	–	N.E.	1180	Moderate	II	–	–	Moderate	–	–	Moderate	–	–	
21	2002	46	64	18	M	–	+	–	1400	1100	Mild	II	–	–	–	–	–	Sparse	–	–	Calcification of globus pallidus
22	2002	32	60	28	F	–	–	–	1200	1150	Mild	III	–	–	Many	–	–	Many	–	–	
23	2003	30	52	22	M	Gastros-tomy	+	+	2900	1070	Moderate	I	–	–	Moderate	–	–	Sparse	Mild	Mild	BC, unilateral PTD
24	2003	N.D.	62	N.D.	M	N.D.	N.D.	N.D.	2600	1020	Moderate	III	–	–	Moderate	–	–	Moderate	Mild	Mild	
25	2004	36	54	18	M	–	–	–	N.E.	1240	–	II	–	–	Sparse	–	–	Sparse	–	–	
26	2004	34	56	24	M	–	–	–	3500	1200	Mild	III	–	–	Many	–	–	Moderate	–	–	Calcification of globus pallidus
27	2006	55	74	19	F	Nasal tube	–	–	N.E.	1030	Mild	III	–	–	Many	–	–	Moderate	MCA embolism	–	
28	2007	28	53	25	M	–	–	–	N.E.	1165	Mild	I	–	–	Sparse	–	–	Moderate	–	–	

29	2007	32	58	26	F	Gastrostomy	+	+	500	1175	Mild	II	–	–	Sparse	–	–	Moderate	–	–	
30	2007	N.D.	45	N.D.	M	N.D.	N.D.	N.D.	N.E.	1080	Mild	I	–	–	Moderate	–	–	Many	–	–	
31	2010	45	59	24	F	Gastrostomy	–	+	N.E.	1080	Mild	II	–	–	Many	–	–	Many	–	–	
32	2013	39	69	30	F	Nasal tube	+	+	N.E.	1010	Mild	III	–	–	Moderate	–	–	Many	–	–	
33	2015	38	57	19	F	Nasal tube	–	–	N.E.	1130	Mild	II	–	–	Moderate	–	–	Sparse	–	–	
34	2016	40	63	23	M	Intestinal fistula	+	–	N.E.	1070	Mild	I	Mild	Mild	Moderate	–	–	Sparse	Moderate	–	

F female, *M male*, *N. D.* not described, *N.E.* not examined, *NFT* neurofibrillary tangle, *PTD* pyramidal tract degeneration, *CPM* central pontine myelinolysis, *APNB* aberrant peripheral nerve bundle, *BC* brain contusion

average disease duration of 23.3 ± 8.8 years (n = 30; median, 22.5 years; range, 7–41 years). The mean number of CTG repeats in the gene for DMPK was 1609.0 ± 951.8 repeats (n = 11; median, 1200; range, 500–3500). The mean brain weight was 1152.6 ± 124.7 g (n = 34; median, 1152.5 g; range, 900–1490 g).

In the present series of patients with MyD, at least 12 cases included tube feeding due to dysphagia; 5 of these cases also had gastrostomy or intestinal fistulas. Furthermore, at least 10 cases included tracheotomy due to respiratory failure or enucleation difficulty of the sputum, and at least seven cases included mechanical ventilation.

6.3.2 Macroscopic Findings

Neuropathological findings for each patient with MyD are also shown in Table 6.1. Hyperostosis frontalis interna indicated bilateral and symmetrical skull hypertrophy in several cases at autopsy (Fig. 6.1b), although the measured value of definite skull thickness was not recorded.

No macroscopic abnormalities of the gyrus or malformations of the cerebrum, cerebellum, or brainstem were observed, and the proportions were normal. The appearance of the cerebrum generally demonstrated diffuse mild to moderate proportional and symmetrical atrophy, with mild widening of the cerebral sulci (Fig. 6.2a), although some cases had no apparent cerebral atrophy. When present, cerebral atrophy was minimally observed in the frontal lobes. The brainstem and the cerebellum were spared from observable atrophy. Atrophy was also not apparent in the spinal cord in most cases, although there was minimal proportional spinal cord atrophy in some cases. Additionally, atrophy was not indicated in the anterior and posterior spinal nerve roots.

Symmetrical lateral ventricular enlargement was observed in coronal sections of the cerebrum, particularly in the anterior and inferior horns (Fig. 6.2b). Compared to the cerebral appearance, dilatation of the lateral ventricle was remarkable; however, dilatation of the third ventricle was not remarkable compared to that of the lateral ventricle. Furthermore, no apparent atrophy was observed in the basal ganglia or thalamus, and hippocampal atrophy was either absent or mild. Axial sections of the brainstem generally revealed no depigmentation of the substantia nigra or locus coeruleus, although there were minimal effects in some cases. Dilatation of the fourth ventricle was not observed. In sagittal sections of the cerebellum, atrophy was not indicated in the cortex or white matter, and abnormalities were not observed in the dentate nucleus.

6.3.3 Microscopic Appearance

In general, the microscopic appearance of the cerebral neocortex did not indicate gliosis or neuron loss, regardless of disease duration (Fig. 6.3a). Furthermore, the laminar architecture of the cerebral neocortex and the number of Betz cells in the

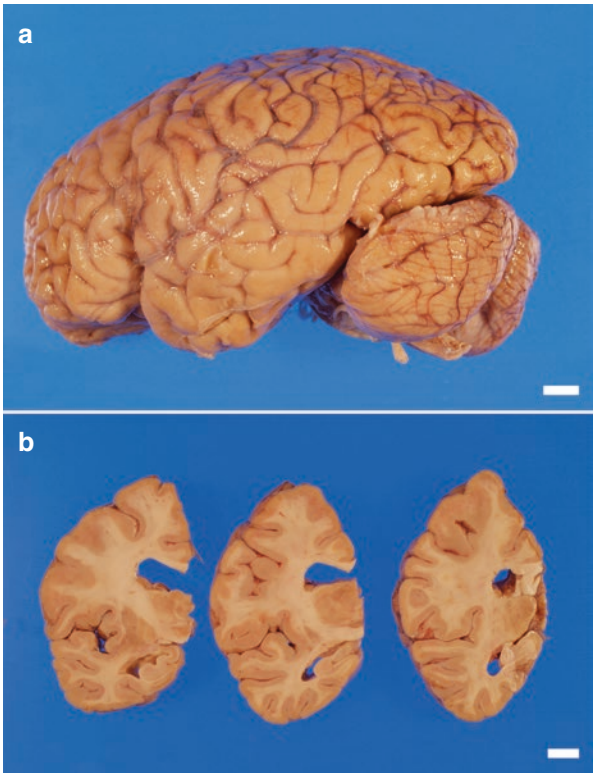

Fig. 6.2 Macroscopic appearance of the formalin-fixed brain of a patient with myotonic dystrophy. (**a**) Diffuse cerebral atrophy, particularly in the frontal lobe. The cerebellum is spared from atrophy. (**b**) Coronal sections of the left cerebral hemisphere indicate lateral ventricular dilatation, particularly in the anterior horn. The basal ganglia, the thalamus, and the hippocampus were relatively spared from atrophy. Case 32. Scale bars: (**a**, **b**), 10 mm

precentral gyrus were preserved (Fig. 6.3b). Additionally, the hippocampus was relatively protected from atrophy (Fig. 6.3c); no apparent neuronal loss occurred in the hippocampus, subiculum, or dentate gyrus of any patient. However, there was mild to moderate neuronal loss, as well as gliosis in the entorhinal and transentorhinal cortices of some patients with numerous NFTs. Additionally, gliosis and neuron loss were not observed in the basal ganglia (Fig. 6.3d) or thalamus.

The number of brainstem neurons was preserved in our patients with MyD. The substantia nigra and locus coeruleus had mild gliosis in some cases, although neuron loss was minimal (Fig. 6.3e). Additionally, the oculomotor nucleus, red nucleus, pontine nucleus, facial nucleus, hypoglossal nucleus, inferior olivary nucleus, and dorsal nucleus of the vagal nerve were protected from neuron loss (Fig. 6.3f). Furthermore, Lewy bodies were not observed on HE staining.

The cerebellum was also generally protected from gliosis and neuron loss. Additionally, the molecular, Purkinje, and granule cell layers of the cerebellar

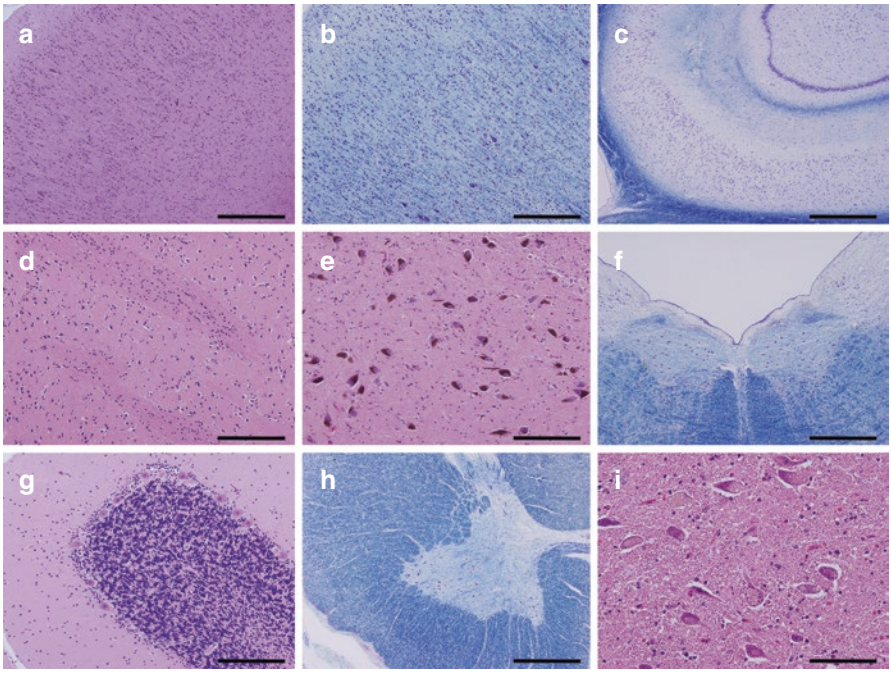

Fig. 6.3 Neuropathological findings in representative brain regions of patients with myotonic dystrophy. (**a**) Neuron loss or gliosis was not indicated in the cerebral neocortex. The laminar architecture was preserved (superior temporal gyrus). (**b**) The number of Betz cells in the precentral gyrus was consistently preserved. (**c**) The hippocampus was relatively spared from neuron loss or gliosis. (**d**) Gliosis or neuron loss was not indicated in the putamen. Pencil fibers were also well-preserved. (**e**) Mild gliosis was observed in the substantia nigra, although neuron loss was not apparent. Lewy bodies were not observed. (**f**) The tegmentum of the medulla oblongata did not exhibit neuron loss, which was also extended to the hypoglossal nucleus and the dorsal nucleus of the vagal nerve. (**g**) In the cerebellar cortex, the molecular, Purkinje, and granule cell layers were well-preserved. (**h**) The spinal anterior horn was generally spared from atrophy. Pyramidal tract degeneration was not observed. (**i**) The number of large motor neurons in the spinal anterior horn was essentially preserved. Additionally, the quantity of lipofuscin in the neurons appears to have increased. (**a**, **d**, **e**, **g**, **i**), hematoxylin-eosin staining; (**b**, **c**, **f**, **h**), Klüver-Barrera staining. (**a**), Case 26; (**b**–**i**), Case 32. Scale bars: (**a**, **b**), 500 μm; (**c**, **f**, **h**), 1 mm; (**d**, **e**, **g**), 200 μm; (**i**), 100 μm

cortex were generally preserved (Fig. 6.3g). Specifically, the cerebellar white matter and dentate nucleus did not exhibit any abnormalities.

In the spinal cord, the pyramidal tract was preserved (Fig. 6.3h). However, some cases exhibited secondary degeneration due to cerebral infarction. The number of large motor neurons in the anterior horn was preserved (Fig. 6.3i). The quantity of lipofuscin generally increased in the motor neurons, and central chromatolysis was also frequently observed. Furthermore, Bunina bodies were not observed, and the intermediolateral column, Clark's nucleus, and posterior horn were protected from gliosis and neuron loss. Finally, Onuf's nucleus was also preserved.

6.3.4 *Neurofibrillary Tangles and Related Pathology*

NFTs were observed in all cases included in the present MyD series (Figs. 6.4a and 6.5a), although the degree of appearance varied. In contrast, senile plaques were rare (Fig. 6.4b) and argyrophilic grains were not observed. In some cases, ghost tangles were remarkable in the superficial layer of the entorhinal and transentorhinal cortices. NFT staging, as described by Braak et al., was as follows: Stage I, 8 cases; Stage II, 16 cases; Stage III, 9 cases; Stage IV, 1 case, and no Stage V or Stage VI cases. No cases met the pathological diagnosis criteria for Alzheimer's disease (AD) [4].

NFTs were not prevalent, although they were widely distributed in the central nervous system. NFTs were observed in the hippocampus, subiculum, entorhinal and transentorhinal cortices (Fig. 6.4c, d), amygdala, and basal nucleus of Meynert; they were also observed in the putamen, caudate nucleus (Fig. 6.5b), globus pallidus, thalamus, subthalamic nucleus, nucleus accumbens, hypothalamus, substantia nigra (Fig. 6.5c), locus coeruleus, tegmentum of the medulla oblongata, cerebellar dentate nucleus, spinal anterior horn (Fig. 6.5d), sympathetic ganglia, and olfactory bulb. Neuropil threads and coiled bodies were also extensively observed in the areas with NFTs and in more extensive regions without NFTs. The number of neuropil threads was generally more frequent compared to NFTs. A few neuropil threads and coiled bodies were also extensively observed in the white matter of the cerebrum, cerebellum, brainstem, and spinal cord. Tau-positive/Gallyas-positive tuft-shaped astrocyte-like glial inclusions were occasionally identified in these regions.

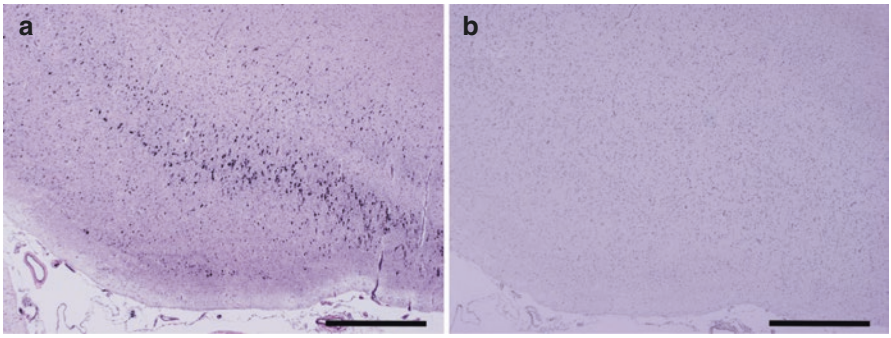

Fig. 6.4 Neuropathological findings regarding neurofibrillary tangles (NFTs) and senile plaques. Numerous NFTs were observed in the transentorhinal cortex (**a**), although senile plaques were not observed (**b**). In the transentorhinal cortex, numerous argyrophilic and tau-positive structures included NFTs, neuropil threads, and glial inclusions, which were observed using Gallyas-Braak silver staining (**c**) and AT-8 tau immunostaining (**d**). Tau-positive structures were stained strongly with RD3 immunostaining (**e**) and with RD4 immunostaining to a lesser extent (**f**). (**a**, **c**), Gallyas-Braak silver staining; (**b**), anti-amyloid β immunostaining; (**d**), anti-hyperphosphorylated tau (AT-8) immunostaining; (**e**), anti-3 repeat-tau (RD3) immunostaining; (**f**), anti-4 repeat-tau (RD4) immunostaining. Case 32. Scale bars: (**a**, **b**), 1 mm; (**c**–**f**), 100 μm

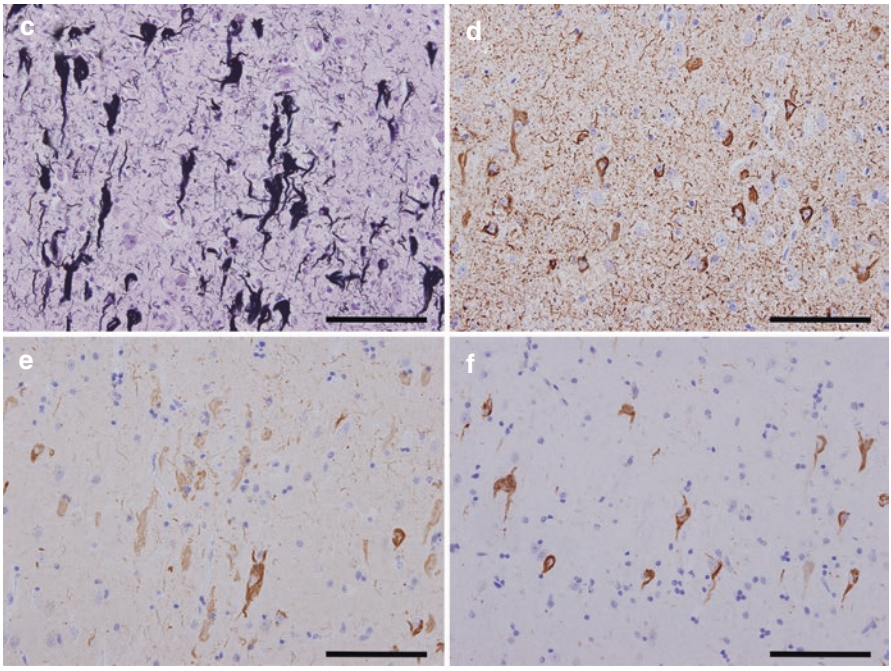

Fig. 6.4 (continued)

NFTs were immunopositive for both RD3 and RD4, although RD3 reactivity was predominant (Fig. 6.4e, f).

6.3.5 Intracytoplasmic Inclusion Bodies in the Thalamus

In the present series, intracytoplasmic inclusion bodies in the thalamus, termed "thalamic inclusions," were detected in 32 of 34 cases (Fig. 6.5a), although the numbers varied considerably between cases. The inclusions were oval with smooth and sharply defined contours located at the periphery of the nerve cell body. Each body had a maximum diameter of approximately 4–8 μm. The thalamic inclusions were particularly prominent in the thalamic anterior nucleus and dorsal medial nucleus. Furthermore, these inclusions were also observed on KB staining (Fig. 6.5b), although they were negative for GB silver staining or anti-phosphorylated tau (AT-8) immunostaining.

Additionally, irregular intracytoplasmic inclusion bodies, which were 1–8 μm in maximum diameter, were also observed at the periphery or within neuromelanin granule accumulations in the pigmented cells of the substantia nigra (nigral inclusions) (Fig. 6.5c).

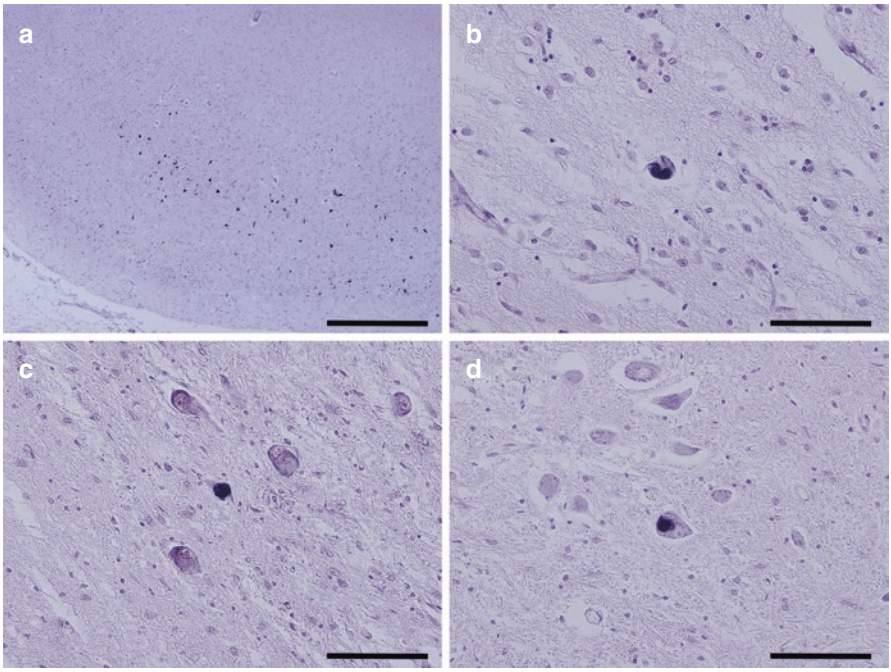

Fig. 6.5 Neurofibrillary tangle (NFT) distribution in a representative case of myotonic dystrophy. (**a**) A small number of NFTs were observed in the transentorhinal cortex, which corresponds to Braak NFT stage I. However, NFTs are extensively and disproportionately observed in the central nervous system, although the total number was small. A few NFTs were observed in the caudate nucleus (**b**), substantia nigra (**c**), and spinal anterior horn (**d**). (**a**–**d**): Gallyas-Braak silver staining. Case 23. Scale bars: (**a**), 1 mm; (**b**–**d**), 100 μm

6.3.6 Vascular Lesions and White Matter Degeneration

In the present series of patients with MyD, myelin pallor in the cerebral white matter was frequently observed, to varying degrees. The regions of myelin pallor were observed with broad or scattering distributions in the cerebral white matter (Fig. 6.7a, b); myelin pallor was more conspicuous in the deeper white matter, and U-fibers were generally preserved. Some cases demonstrated thinning of the corpus callosum, which likely resulted from cerebral white matter degeneration. Although there were differences in degree between cases, dilatation of the perivascular space (état criblé) was frequently observed (Fig. 6.7c). These état criblés were commonly located in the basal ganglia and included capillary hyalinization, fibrillary gliosis, and loss of adjacent myelin. Small infarctions and arteriolosclerosis of the small vessels were also observed in several cases, to varying degrees (Fig. 6.7d).

Two cases exhibited extensive cerebral embolism of the middle cerebral artery perfusion area, due to atrial fibrillation.

6.3.7 *Additional Findings*

Marinesco bodies, which are nuclear inclusions in the pigmented neurons of the substantia nigra, were frequently observed in the present series of patients with MyD (Fig. 6.6d). Hirano bodies, which are rod-shaped eosinophilic intracytoplasmic inclusions in the neuronal processes of CA1 to the subiculum, were also relatively abundant.

Three cases had what appeared to be historical brain contusions, likely due to falls or other accidental injuries. Interestingly, NFTs and neuropil threads were often found in areas adjacent to the contusion.

Calcifications of the vessel walls were identified in some cases, particularly in the basal ganglia. Additionally, spheroids were recognized in the gracile nucleus in several cases. Furthermore, aberrant peripheral nerve bundles were also observed in the spinal cord of several patients.

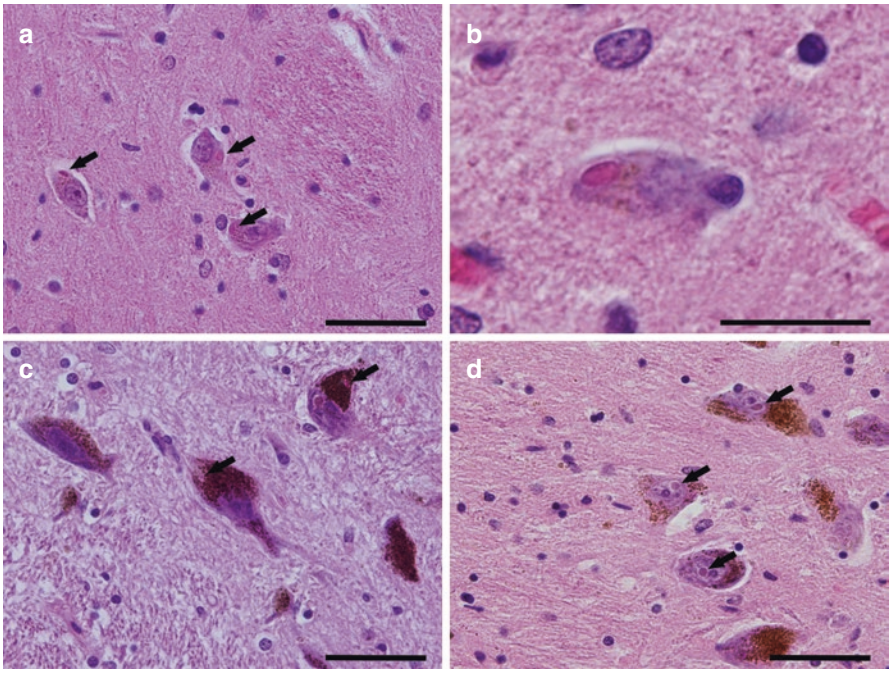

Fig. 6.6 Thalamic inclusions, nigral inclusions, and Marinesco bodies. (**a**) Three thalamic inclusions (arrows) are shown in the medial thalamic nucleus. The inclusions are oval with smooth and sharply defined contours and are located at the periphery of the nerve cell body. (**b**) A typical thalamic inclusion at high magnification (×100). (**c**) Two nigral inclusions (arrows) are shown in the pigmented cells of the substantia nigra. Irregular intracytoplasmic inclusion bodies surrounded by a halo occur within accumulations of neuromelanin granules. (**d**) Three Marinesco bodies (arrows) are shown in the pigmented neurons of the substantia nigra. Hematoxylin-eosin staining. (**a**), Case 31; (**b**, **d**), Case 32; (**c**), Case 24. Scale bars: (**a**, **c**, **d**), 50 μm; B, 20 μm

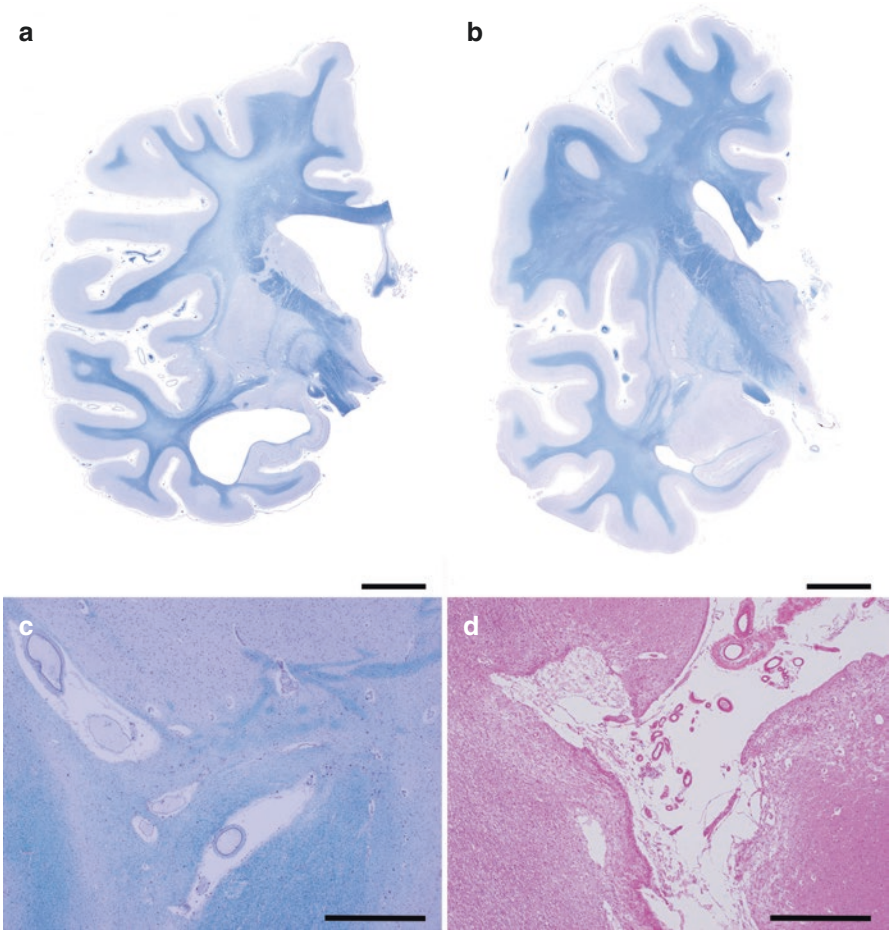

Fig. 6.7 Vascular lesions and white matter degeneration. (**a**) Moderate myelin pallor was extensively observed in the cerebral white matter. U-fibers were generally preserved. The anterior and inferior horns of the lateral ventricle were enlarged. (**b**) Several dispersed regions with mild myelin pallor were observed in the cerebral white matter. Lateral ventricular dilatation was not apparent. (**c**) État criblé and arteriolosclerosis of the small vessels with myelin pallor of adjacent regions. (**d**) A relatively large historical infarction in the cerebral cortex (middle frontal cortex). Arteriolosclerosis of the leptomeningeal vessels was also apparent. (**a**–**c**), Klüver-Barrera staining; (**d**), hematoxylin-eosin staining. (**a**), Case 15; (**b**, **c**), Case 24; (**c**), Case 11. Scale bars: (**a**, **b**), 10 mm; (**c**, **d**), 1 mm

6.3.8 Statistical Analyses

The results of our statistical analyses are presented in Table 6.2, including *p* values and correlation coefficients (signified by rs). The number of cases varies between variables since some factors did not apply to all patients.

The number of CTG repeats and Braak NFT stages was not significantly correlated ($n = 11$; $p = 0.52$, rs = 0.20), whereas age at death and Braak NFT stages were

Table 6.2 Results of statistical analyses

Two groups which were statistically compared	The number of cases which could be analyzed	*p* value	Correlation coefficient (signified by rs)
1. The number of CTG repeats and Braak NFT stages	11	N.S. ($p = 0.52$)	0.20
2. Age at death and Braak NFT stages	34	$p = 0.01$	0.43
3. Disease duration and Braak NFT stages	30	$p < 0.01$	0.53
4. The number of CTG repeats and age at disease onset	10	N.S. ($p = 0.65$)	−0.15
5. The number of CTG repeats and disease duration	10	N.S. ($p = 0.80$)	−0.09
6. Disease duration and brain weights	30	$p = 0.01$	−0.46
7. Age at death and brain weights	34	N.S. ($p = 0.06$)	−0.33

Spearman's rank correlation coefficient was used
N.S. statistically not significant

significantly correlated ($n = 34$; $p = 0.01$, rs = 0.43). Disease duration was also significantly associated with Braak NFT stages ($n = 30$; $p < 0.01$, rs = 0.53).

The numbers of CTG repeats were not significantly correlated with either age at disease onset or disease duration ($n = 10$; $p = 0.65$, rs = −0.15; $n = 10$; $p = 0.80$, rs = −0.09, respectively). Disease duration and brain weights were significantly correlated ($n = 30$; $p = 0.01$, rs = −0.46), whereas age at death and brain weights were not significantly correlated ($n = 34$, $p = 0.06$, rs = −0.33).

6.4 Bibliographical Considerations

Hyperostosis frontalis interna was identified in several of our MyD cases. In general, hyperostosis frontalis interna indicators are non-disease-specific changes and are occasionally observed in healthy participants [5]. Although the aetiology is unknown, hyperostosis frontalis interna is most commonly observed in older women; occurrence in patients with MyD is probably related to multiple organ system involvement in MyD.

Regarding brain atrophy in the present MyD series, although brain weights were generally reduced in relation to expected healthy brain weights, macroscopic cerebral atrophy was generally mild to moderate and not readily apparent in some cases. We found that it was not possible to specify significant pathological changes that explain the cerebral atrophy. This proportionally small brain is thought to reflect a generally reduced cerebrum, which is termed "kozukuri (小作り)" in Japanese. Similar findings have been reported in several triplet repeat diseases, such as denta-

torubral-pallidoluysian atrophy (DRPLA) and Machado-Joseph disease (MJD), and may be related to CTG repeat prolongation during MyD. Furthermore, the brainstem and cerebellum were consistently spared from atrophy in all the present MyD cases; therefore, the developmentally more recent brain regions may be vulnerable to atrophy, whereas older regions may be resistant to degradation. Alternatively, the cerebrum may be smaller in patients with MyD, beginning at early stages of development.

NFTs are characteristic neuronal intracytoplasmic inclusions that are observed in AD and other neurodegenerative disorders. The distribution of NFTs in the brain varies among tauopathies. We found that NFTs were consistently observed in all our MyD cases, whereas senile plaques were rarely observed. These findings are abnormal in relation to patient's ages; however, pathological diagnosis criteria for AD were not met [4]. Although the appearance of NFTs was generally within Braak NFT stages I to III, NFTs were distributed more extensively within specific brain areas such as the basal ganglia, brainstem, and spinal cord. These pathological findings are similar to those of senile dementia of neurofibrillary tangles (SD-NFTs) or "primary age-related tauopathy," which is a recently suggested term to describe the pathology that is commonly observed in the brains of aged individuals [6]. Furthermore, neuropil threads and coiled bodies were more extensively observed not only in the areas with NFT expression but also in the more extensive regions without NFTs.

The aging-related pathology observed in the present study may partially result from prolonged disease duration, because Japanese patients with neurodegenerative diseases, including MyD, have longer survival durations than Caucasian patients in general; Japanese patients receive intensive symptomatic treatment such as tracheotomy, tube feeding, gastrostomy, and mechanical ventilation. Our statistical analyses revealed a significant correlation between both age at death and disease duration and Braak NFT stages. Disease duration and brain weights were also significantly correlated, whereas age at death and brain weights were not significantly correlated.

Characteristic eosinophilic intracytoplasmic inclusion bodies in the thalamus, termed "thalamic inclusions," have been emphasized as features of MyD for >30 years [7, 8]. These thalamic inclusions were observed in most of the present MyD cases (32 of 34 cases, 94.1%). The appearance of thalamic inclusions in MyD is thought to be a significant pathological finding, but thalamic inclusions are also observed in healthy brains and are not specific to MyD [7, 8]. However, thalamic inclusions are minimal in healthy brains and increase in the brains of patients with MyD [7, 8].

Marinesco bodies were frequently observed in the present MyD series, but the number varied between cases. Marinesco bodies are nuclear inclusions found in the pigmented neurons of the substantia nigra. The frequency of these inclusions increases with advancing age, although no pathological associations have been established; therefore, the increased number of Marinesco bodies in the present MyD series is likely to be an aging-related pathology. Similar relationships are also

likely explanations for our observations of Hirano bodies in CA1 to subiculum areas, spheroids in the gracile nucleus, and aberrant peripheral nerve bundles in the spinal cord. In several of our MyD cases, dilatation of the perivascular space was remarkable, particularly in the basal ganglia. These vascular alterations are indicative of perivascular ischemic changes and may be related to the pathology of aging.

Although MyD generally includes muscle weakness, as well as facial, tongue, respiratory muscle, and extremity atrophy [1], motor neurons including Betz cells, as well as brainstem and spinal motor neurons, were well-preserved in the present MyD series. These findings suggest that muscle weakness and atrophy are fundamental muscular dystrophy characteristics rather than neurogenic changes.

Recent studies suggest that the severity of organ involvement and age of MyD onset is strongly correlated with the number of CTG repeats [1]. Longer repeats are usually associated with earlier onset and more severe symptoms [1]. In the present study, we did not find a statistically significant correlation between the number of CTG repeats and Braak NFT stages. Furthermore, no statistically significant correlation occurred between the number of CTG repeats and either age at disease onset or disease duration.

Although there is no neuropathological diagnostic hallmark for MyD, neuropathological changes such as brain atrophy, neurofibrillary tangles, thalamic inclusions, Marinesco bodies, and vascular changes were observed to varying degrees in the present MyD series. Many of these pathologic changes are associated with general aging pathology, suggesting that MyD may be a form of progeria. However, senile plaques, argyrophilic grains, and Lewy bodies, which are also associated with age-related pathology, were not prominent in the present study; therefore, the observed changes may not reflect simple aging pathology.

6.5 Conclusion

The present investigation demonstrated that neuropathological changes are heterogeneous in patients with MyD, suggesting that the pathological mechanisms of MyD are complex and multifactorial.

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The author has no conflicts of interest to declare.

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Chapter 7
Molecular Defects in the DM Central Nervous System

Takashi Kimura

Abstract The central nervous manifestation of myotonic dystrophy (DM) is one of the most devastating features of this disorder, along with muscle- and heart-related symptoms. Neuropathologically, neurofibrillary degeneration accompanying hyperphosphorylated microtubule-associated tau and C(C)UG nucleotide repeat accumulation in the nucleus are observed in DM brains. CTG repeat is the longest in the cerebral cortex of DM type 1 but the shortest in the cerebellum. Non-replication processes, including transcription and repair after DNA damage, may play significant roles in repeat expansion in the central nervous system because neurons are non-mitotic. Mismatch repair protein as a trans-factor and abnormal methylation at the CTCF binding site upstream of the CTG repeat as a cis-element have been postulated as the causes of somatic instability. Because C(C)TG repeat expansion occurs in noncoding regions, the transcripts exert a toxic effect, mainly through an RNA gain-of-function mechanism. Expanded C(C)UG repeat transcripts are accumulated in the nuclei of the brain of DM patients (foci). These RNA foci sequester RNA-binding proteins MBNL1 and MBNL2, which normally regulate the splicing transition from fetal to adult isoforms, and this sequestration in the nuclei of neuronal cells leads to the retention of fetal transcripts. Although Mbnl1 knockout mice displayed DM skeletal muscle phenotypes, they only exhibit mild splicing changes in the brain. On the contrary, Mbnl2 knockout mice showed DM-CNS symptoms, indicating a major role of MBNL2 in the DM brain. However, loss of functions of both Mbnl1 and Mbnl2, demonstrated by conditional double-knockout mice, leads to dramatic splicing switch to fetal patterns, including Mapt that encodes Tau proteins. These molecular defects may contribute to synaptic dysfunction and Tau-related toxicity in the DM brain. Based on these evidences, therapeutic approaches are being tested at different levels, including clinical trials.

Keywords Myotonic dystrophy · Central nervous system · MBNL · CELF · MAPT · Alternative splicing · RNA-mediated disease

T. Kimura
Division of Neurology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan
e-mail: kimura@hyo-med.ac.jp

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7.1 Clinical Manifestation

Myotonic dystrophy types 1 and 2 (DM1 and DM2, respectively) are progressive and multisystemic disorders characterized by myotonia, muscle weakness, early-onset cataract, frontal baldness, cardiac conduction abnormalities, endocrine disturbances, and central nervous system (CNS) involvement. DM1 is caused by the unstable expansion of a CTG repeat within the 3′ untranslated region (UTR) of DM protein kinase (DMPK) gene. DM2 is caused by the expansion of a CCTG repeat within the first intron of the cellular nucleic acid-binding protein (CNBP) gene [1]. Adult-onset DM1 and DM2 present as cognitive and behavioral abnormalities, including visual spatial and attention deficits, dysexecutive syndrome, apathy, avoidant behavior, and excessive daytime sleepiness (reviewed in [2]) (see Chap. 5 "Clinical Features of the Central Nervous System"). In addition, compared with DM2, DM1 demonstrates congenital- and childhood-onset forms with reduced IQ, speech and language delay, deficit in cognitive levels and adaptive skills, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder, problems with communication, and social anxiety [2–7].

7.2 Pathology (See Chap. 6 "Pathological Changes in DM Brains")

Neuropathological studies have demonstrated neurofibrillary degeneration (NFD) in the amygdala, hippocampus, entorhinal cortex, and temporal cortex of both DM1 and DM2 brains. This spatial distribution of NFD is similar to that of Alzheimer's disease (AD); however, no amyloid/senile plaques have been observed in DM [8–12]. Moreover, "état criblé" (indicative of increased perivascular Virchow–Robin spaces) has been reported in deep white matter and suggested to be compatible with subcortical dementia in DM patients [9].

7.3 Magnetic Resonance Imaging

Conventional magnetic resonance imaging analyses have demonstrated cortical atrophy, white matter lesions, and increased Virchow–Robin spaces, with more significant involvement of anterior temporal lesions [13–17].

Recent quantitative volumetric studies have shown a decrease in the gray matter volume of widespread regions, including all four lobes (frontal, temporal, parietal, and occipital) as well as the basal ganglia and cerebellum. Diffusion tensor imaging studies have revealed widespread reduction of fractional anisotropy in all major association, projection, and commissural fibers. There have been variable degrees of correlation for imaging findings with CTG repeat number, age, and duration of disease [18–29] (reviewed in [30]).

7.4 CTG Repeat Instability in the Brain

DM1 patients exhibit a high degree of somatic instability of CTG repeats in the DMPK gene, throughout their development and lifetime (reviewed in [31]). This somatic instability is most severe in the skeletal muscle, heart, and brain [32]. In addition, there is region-specific somatic instability in the brain. The repeat size is particularly small in the cerebellar cortex [33]. In fact, other repeat expansion diseases, such as Huntington's disease (HD), spinocerebellar ataxias 1 and 3, and dentatorubral–pallidoluysian atrophy, also exhibit the shortest repeat expansion in the cerebellum [33–39]. Except for the cerebellum, the repeat length did not significantly differ among any regions in the CNS, and no correlation exists between the extent of neuropathological changes and the length of CTG repeats in each region of the cerebrum [9].

How does this region-specific difference in somatic instability occur in DM1? Somatic instability presumably occurs through DNA replication, repair, recombination, and transcription. In DM1 skeletal muscles, repeat expansion is likely to arise during myoblast proliferation and stop after terminal differentiation [40, 41] (reviewed in [31]). Given that CNS neurons are non-mitotic and display higher instability, the role of non-replication processes, such as transcription and repair after DNA damage, must be important.

A group of factors leading to repeat instability is mismatch repair proteins. The factors include MSH2, MSH3, MSH6, and PMS2, which originally protect incorrect deletions and insertions but play a mutagenic role in CTG/CAG repeat expansion. MSH2-, MSH3-, and PMS2-null mice crossed with DM1 (with CTG repeat in the human DMPK or mouse Dmpk gene), or HD model mice display repeat stability or contractions. By contrast, MSH6-null mice crossed with DM1 mice (with CTG repeat in the mouse Dmpk gene) exhibit somatic expansions [31, 42–46]. However, the expression levels of these mismatch repair proteins are not attributable to the relatively short CTG/CAG repeat expansion in DM1 and HD cerebellums because the expression levels of MSH2, MSH3, and MSH6 in cerebellums are higher than those in the cerebrum [47, 48].

Another factor associated with repeat instability is the cis-element close to the CTG repeat and its methylation status. DNA methylation plays a crucial role in epigenetic modification of the genome, and many human diseases are associated with an abnormal methylation status (reviewed in [49]). The binding sites for CCCTC binding factor (CTCF) are identified upstream and downstream of the CTG repeat and form a methylation-sensitive insulator at the DM1 locus. CpG methylation at the DM1 locus inhibits the binding of CTCF to these sites [50]. Abnormal CpG methylation at the CTCF sites upstream of the CTG repeat has been reported in leukocytes from congenital DM1 (CDM) patients and in the heart, liver, and cerebral cortex, but not in the cerebellum, kidney, and skeletal muscle of adult DM1 patients [51, 52]. Therefore, shortest repeat expansion in DM1 cerebellums might correlate with the lack of methylation. However, the causal relationship between hypomethylation and shorter repeat length remains undetermined. On the other hand, the somatic instability of CCTG repeats in DM2 brains has not been reported so far.

7.5 Foci

Regarding the mechanisms of trinucleotide expansion in the untranslated region, Taneja et al. hypothesized that these expanded trinucleotide repeat sequences alter the subcellular distribution of transcripts, leading to aberrant function (RNA gain of function). To prove this hypothesis, they used oligonucleotide probes to explore the site of DMPK transcripts and detected transcripts containing extended CUG repeats in the nuclei of fibroblasts from DM1 muscle biopsies [53]. Similarly, mutant transcripts were also accumulated in DM2 [1], implicating that the mislocalization of mutant transcripts may play a role in disease mechanism. To examine this hypothesis, Mankodi et al. generated the transgenic *HSA*^{LR} model expressing an untranslated CUG repeat driven by the human skeletal muscle actin (HSA) gene. The mice developed DM phenotypes and also showed RNA foci in the nuclei, supporting the role of RNA gain of function in DM pathogenesis [54]. These accumulated transcripts were also observed in other tissues such as the smooth muscle [55] and heart of DM1 patients [56]. In the CNS, Jiang et al. firstly reported that RNA foci were detectable in all cortical layers and confined to neurons in the cerebral cortex, hippocampus, dentate gyrus, thalamus, substantia nigra, and brain stem tegmentum. The main exception was the cerebellar cortex, where small foci were detected in some Purkinje cells but not in the neurons of the molecular or granular cell layers [57]. They also reported that a 3.1-fold greater number of RNA foci were found in frontal cortical neurons compared with in the skeletal muscle from the same patient.

7.6 Aberrant Splicing

Aberrant splicing of several exons has been discovered in DM1 tissues, which includes exons 2, 3, and 10 exclusion for MAPT in DM1 brains, exon 5 inclusion for cTNT in DM1 cardiac muscle, exon 11 exclusion for IR in skeletal muscle, and exon 7a inclusion for ClC1 in *HSA*^{LR} mice and DM1 skeletal muscle [58–62]. These splicing changes in DM1 tissues are shifted toward the splicing isoform that is normally expressed in fetal tissues. Therefore, it is postulated that CUG repeat transcripts inhibit the splicing shift from fetal to adult isoforms.

7.7 RNA-Binding Proteins Colocalized with Foci

The next question was “What were the pathological effects generated by RNA foci? Do they have anything to do with aberrant splicing in DM?” RNA foci influence many transcription factors and RNA-binding proteins. For example, CUG-BP- and ETR-3-like factor (CELF) 1 promotes fetal splicing patterns, and its steady-state protein levels are increased in the DM1 muscle and heart [63], but

this factor does not colocalize with foci. Miller et al. identified MBNL proteins as candidates that may be sequestered in CUG RNA foci. They specifically bind to double-stranded CUG RNA, and the binding is proportional to the length of CUG repeat RNA hairpins. MBNL proteins are homologue to drosophila muscle blind, which play an essential role in the terminal differentiation of muscle and photoreceptor cells [64, 65]. Different from other transcription factors and RNA-binding proteins (HnRNPs H and F, p68/DDX5, Staufen1) [57, 66–69], MBNL proteins are the only ones that colocalize with C(C)UG repeat transcripts in the nuclear foci of all cultured DM1 cells [64, 70], DM1 and DM2 skeletal muscle [71], and DM1 neurons in the brain [57] .

7.8 MBNL-Dependent Splicing Changes in the DM Muscle and Brain

To determine whether loss of function of MBNL accounts for the development of DM phenotypes, Kanadia et al. generated Mbnl1 knockout mice, which showed myotonia, cataracts, and RNA splicing defects that are characteristic of DM [72]. Rescue experiments using AAV-mediated MBNL1 overexpression also reverse myotonia and splicing abnormalities in *HSA*^{LR} mice, indicating that MBNL1 sequestration is the key pathogenic factor in this transgenic model [73]. Additionally, Du et al. compared the skeletal muscles of two DM1 model mice *HSA*^{LR} transgenic and MBNL1 knockout mice using splicing-sensitive microarray and observed strong correlation in the splicing changes [74]. Unlike numerous targets found from muscles, microarray analysis of Mbnl1 knockout brains identified only 14 candidates for aberrant splicing, and 3 of these events were misregulated in DM1 postmortem brains. In addition, several splicing events—including Grin exon 4 and Mapt exons 3 and 9 (homologous to human exon 10), which have been reported to be mis-spliced in human DM1—were normally regulated in Mbnl1 knockout brains, suggesting that factors other than MBNL1 may also contribute to aberrant splicing in the DM1 brain [75]. In addition to MBNL1, MBNL2 is colocalized with RNA foci in the DM1 brain [57]. Although ubiquitously expressed, MBNL2 is highly expressed in CNS, suggesting a spatial and temporal expression difference compared with MBNL1 (mainly in adult skeletal muscle and heart) and MBNL3 (fetal stage and placenta) [70]. To prove if the loss of MBNL2 function contributes to the DM-CNS pathogenesis, Charizanis et al. generated Mbnl2 knockout mice that exhibited the anomalous splicing of at least hundreds of exons, including Grin exon 4 and Mapt exons 3 and 9. The top-ranking mis-splicing events on RNA-seq and microarray lists were validated in the knockouts and also in DM1 brains. In addition, Mbnl2 knockout mice demonstrated CNS manifestations of DM such as an abnormal REM sleep propensity and deficits in spatial memory [76]. Similar to the upregulation of MBNL2 observed in the skeletal muscle of Mbnl1 knockout mice, the compensatory upregulation of MBNL1 was also found in Mbnl2 knockout

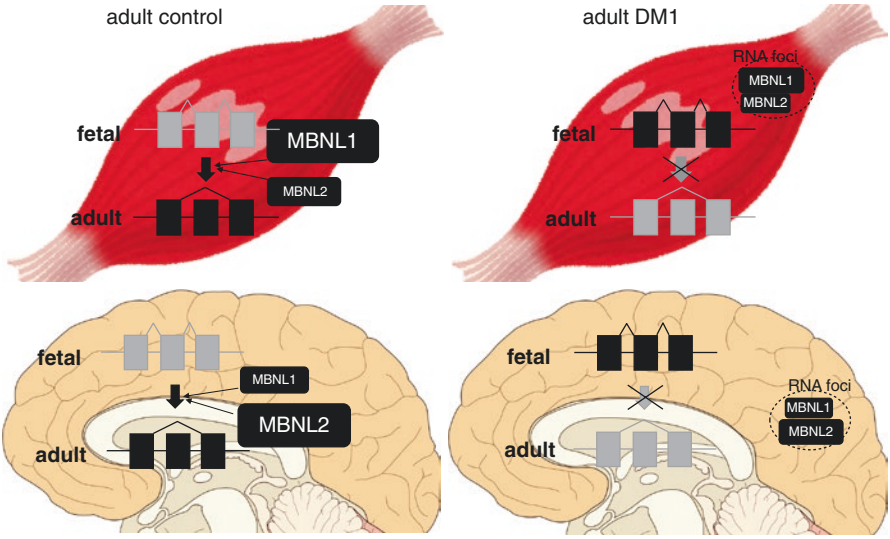

Fig. 7.1 Contribution of MBNL1 and MBNL2 to the developmentally regulated splicing transition and their different effects in the skeletal muscle and brain. Because MBNL1 is predominantly expressed in the skeletal muscle, Mbnl1 knockout mice mainly developed mis-splicing and manifestations (myotonia and muscle weakness) in the muscle. By contrast, because Mbnl2 is predominantly expressed in the brain, MBNL2 knockout mice exhibited brain-specific splicing changes and CNS phenotypes. Additionally, both proteins compensated for each other in both tissues, and thus the compound loss of both proteins caused more severe mis-splicing and phenotypes in the brain and skeletal muscle

brain. As expected, combined loss of function of Mbnl1 and Mbnl2 in neuron-specific double-knockout mice enhanced splicing changes that were similar to splicing patterns observed in DM1 brains [77]. Critical DM1 brain targets that were not identified in Mbnl2 knockout were also recapitulated (e.g., MAPT exon 10) in double knockouts. These results reminiscent the severe phenotypes caused by combined loss of MBNL1 and 2 in the skeletal muscle and indicate their indispensable roles in the CNS [78] (Figs. 7.1 and 7.2).

How do MBNL1 and MBNL2 regulate alternative splicing? Both proteins recognize the YGCY (Y = C/U) motif identified in pre-mRNAs adjacent to many alternatively spliced exons that are regulated by MBNL1 and MBNL2. Generally, YGCY is clustered in upstream intronic regions for MBNL-dependent exon skipping and in downstream intronic regions for exon inclusion ([74, 76, 79, 80] reviewed in [81]). MBNL binding sites were also mapped at 3′-UTR identified by high-throughput sequencing–cross-linking immunoprecipitation (HIT-CLIP) experiments using postmortem DM brains, suggesting that this factor may also influence alternative polyadenylation and RNA localization [78] similar to that seen in the DM1 skeletal muscle [82]. No difference in binding capability between MBNL1 and MBNL2 has been reported, and therefore the contribution of each protein to aberrant splicing is probably expression level dependent.

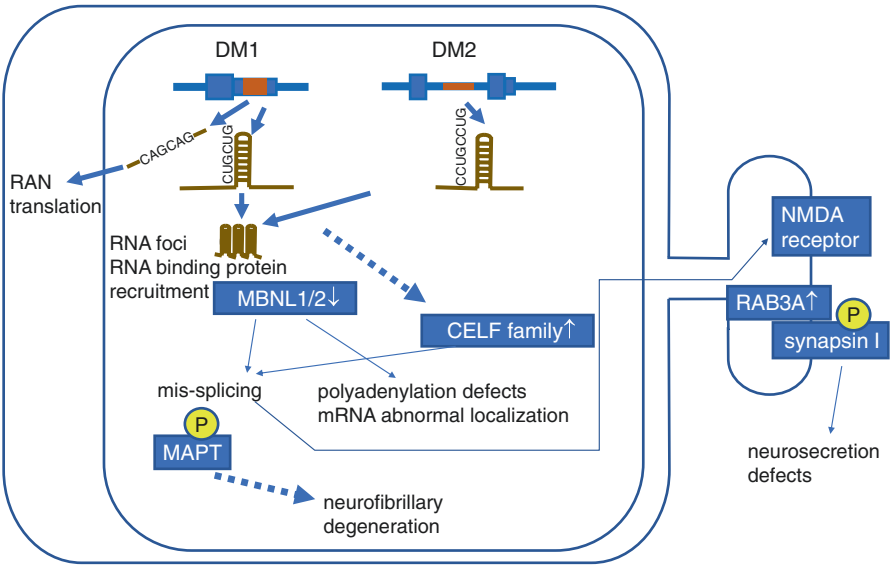

Fig. 7.2 Pathogenic mechanisms in DM CNS. C(C)TG repeat expansions in the 3′-UTR of DMPK gene and the intron of CNBP gene are transcribed to C(C)UG mRNA. These transcripts form a hairpin structure and accumulate in the nucleus. The RNA-binding proteins, such as MBNL, are recruited by these hairpins and are therefore unable to regulate splicing, polyadenylation, and mRNA localization. As reported in DM1 skeletal muscle, repeat-induced upregulation of CELF protein might lead to mis-splicing in the brain (7.10 CELF and Mis-splicing of MAPT). A candidate of pathogenic mis-splicing is NMDA receptor, which may contribute to aberrant synaptic transmission. These synaptic defects are also caused by RAB3A upregulation and the hyperphosphorylation of synapsin I, which are reported in DMSXL mice (7.12 Other Mis-splicing). Another candidate is MAPT, whose mis-splicing and consequent hyperphosphorylation may be involved in neurofibrillary degeneration. In DM1, bidirectional CAG-expanded transcripts are translated to polyglutamine expansion proteins without a non-ATG start codon (7.13 RAN translation)

The splicing defect in *HSA*^{LR} mice selectively targets a group of exons that share a common temporal pattern of developmental regulation. During the postnatal interval, MBNL1 translocates from the cytoplasm to the nucleus. Herein, MBNL1 is depleted from the nucleoplasm as a result of sequestration in the nuclear foci in DM1 muscle [83]. Similarly, MBNL2 is distributed preferentially to the nuclei in the neurons of the hippocampus, dentate gyrus, and cerebellar Purkinje cells, while it is expressed in both the nucleus and cytoplasm in other regions of the adult mouse brain. As is the case with MBNL1 in skeletal muscle, it is proposed that MBNL2 acts as a splicing factor during postnatal development of the brain [76], although the localization of MBNL2 during postnatal development has not been examined. Interestingly, new evidence using a mouse model expressing 960 CUG RNA repeats in the postnatal brain has been reported. This study showed that learning disability, impaired synaptic potentiation, and reduced expression of cytoplasmic MBNL1 on the dendrites occur before the reduction of MBNL2 expression and MBNL-related splicing defects [84]. Notably, the contribution of each splicing factor to DM pathogenesis should carefully

be interpreted because CTG repeat instability, RNA metabolism, synaptic function, and brain structure change with disease progression.

7.9 Molecular Defects in the Brains of CTG Transgenic Mice

The Gourdon group generated transgenic mice carrying >300 CTG repeats downstream of their native human promoter [85]. The transgenic mice demonstrated marked intergenerational repeat expansion and developed a mouse line carrying >1000 CTG repeats (DMSXL mice) that showed more behavioral and cognitive defects and deficits in short-term synaptic plasticity [86, 87]. Although DM1 brain splicing alteration was not clearly seen in this line, DMXSL mice exhibited RAB3A upregulation and synapsin I hyperphosphorylation, which could also be observed in the DM1 brain [88].

7.10 CELF and the Mis-splicing of MAPT

As reported in skeletal muscle, CELF may be involved in the mis-splicing of some exons in the DM1 brain. While CELF2 and 4 modify the splicing in exons 2 and 10 of MAPT [89–91], CELF5 and 6 change the splicing pattern in exon 6 of MAPT [89]. The expression levels of CELF1 and 2 are highly variable among the brains of DM1 patients. In addition, only the brains with increased expression of CELF2 show mis-splicing of MAPT exon 10 [91]. Moreover, increased expression of CELF1 and 2 has been reported in the frontal brain of DM1 and DMSXL mice [88]. CELF4 expression in DM1 brains is relatively similar to that in controls [91]. The phosphorylation status of CELF proteins in DM brains is not yet determined (reviewed by Marie-Lure Caillet-Boudin [92]). Taken together, the upregulation of CELF2 in DM brains may be involved in some aberrant splicing, such as that of exon 10 of MAPT (Fig. 7.2). To make the contribution of CELF family clear, an in vivo evidence for CELF-related DM brain phenotype needs to be established.

7.11 Mis-splicing of MAPT and Tauopathy

Neurofibrillary tangles (NFTs) are characterized by intracellular aggregates of hyperphosphorylated and insoluble tau, mostly in neuron cell bodies and less in neuropil threads. These constituents are observed in normal aging and are significantly conspicuous in many tauopathies, including AD. NFTs are also detected in the hippocampus, the entorhinal cortex, and most temporal areas of DM1 patients [11]. In adult brains, six MAPT splicing isoforms are expressed with the inclusion

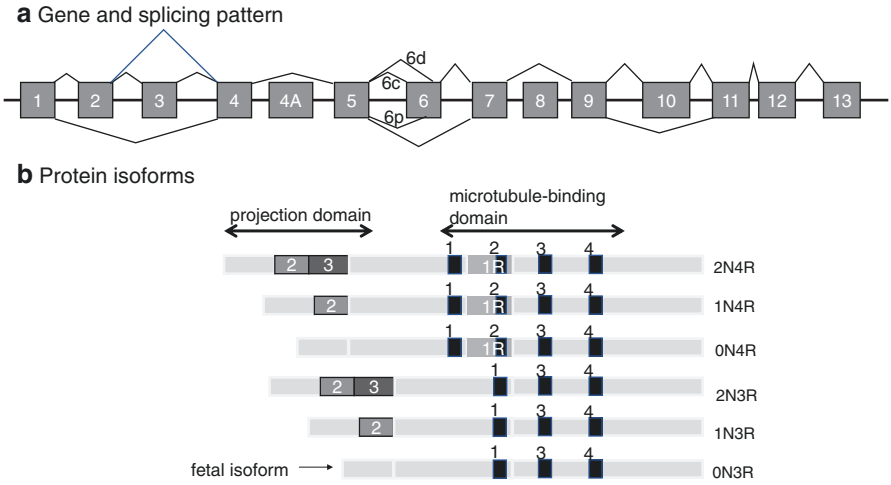

Fig. 7.3 Schematic diagrams of human microtubule-associated tau gene, splicing pattern, and protein isoforms. (**a**) The structure of MAPT gene and schematic representation of MAPT mRNAs. Exons 4A and 8 are mostly excluded in the CNS. For exon 6, three distinct splice sites can be used (6c, 6p, and 6d), although these isoforms are rarely expressed in the brain. Exons 2, 3, and 10 are alternatively spliced. (**b**) The six MAPT isoforms expressed in the brain. The noted amino acid sequences are encoded by exons 2, 3, and 10. The isoforms are termed depending on the presence or absence of N- and C-terminal sequences as 0-2N and 3-4R (drawn from [92, 109])

or exclusion of exons 2, 3, and 10 (Fig. 7.3). These isoforms are named according to the presence of N-terminal exons 2 and 3: 0N (without both exons 2 and 3), 1N (with exon 2), and 2N (with both exons 2 and 3), and the C-terminal 4-repeat region: 3R (without exon 10) and 4R (with exon 10). Interestingly, isoforms vary in different brain disease: both 3R and 4R Tau isoforms are aggregated in AD, whereas 3R is predominantly accumulated in diseases such as frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), and 4R is mainly deposited in diseases, such as progressive supranuclear palsy (PSP). N-terminal exons 2 and 3 belong to the projection domain through which MAPT interacts with the plasma membrane [93]. On the other hand, C-terminal four-repeat region encoded by exons 9–12 belongs to the microtubule-binding domain [94]. MAPT is supposed to promote microtubule assembly and axonal development. In vitro experiments showed that adult 4R MAPT plays this role more efficiently than fetal 3R [95].

The aberrant splicing of exons 2, 3, 6, and 10 has been reported in DM1 patients. A decrease in the inclusion of exons 2, 3, and 10, a decrease in exon 6c, and an increase in 6d have been demonstrated in DM1 brains [11, 57, 89–91]. Consequently, the fetal 0N3R isoform is predominantly expressed in DM1 brain NFTs [11]. The exclusion of exons 2, 3, and 10 might protect from Tau aggregation and contribute to a variation in microtubule bundle organization and axonal transport in DM1 brains (reviewed in [92]).

Mbnl2 knockout mice exhibited the preferential exclusion of Mapt exons 2 and 3 [76], and 3R isoform was predominant in Mbnl1/Mbnl2 conditional double-knockout mice [78]. Thus, Mbnl1/Mbnl2 conditional double-knockout mice

recapitulate Mapt splicing more in the brain, although this aberrant splicing does not accompany the hyperphosphorylation and aggregation of tau that are observed in DM1 brains. This discrepancy might be explained by the difference in life span between model mice and human patients. In fact, it is still debated whether Tau aggregates are toxic or protective in tauopathy (reviewed in [96]). For example, in a mouse model expressing nonmutant human Tau, cell death can occur before NFT formation [97].

7.12 Other Mis-splicing

Although many mis-spliced events are found in DM1 and mouse model brains, the detailed mechanisms underlying the causing relationship between splicing deficit and brain dysfunction in DM1 remain to be investigated. The MBNL2 knockout mice exhibit deficits in spatial memory and impaired hippocampal synaptic plasticity. This may be explained by the preferable inclusion of exon 5 of GRIN1, which is a subunit of NMDA receptor that is contributed to neuronal plasticity and memory. Because GRIN1 splice variants without exon 5 are reported to be expressed in dendrites [98], exon 5 inclusion may give rise to decreased dendritic localization, deleterious hippocampal synaptic transmission, and spatial memory disturbance. This synaptic dysfunction may also be affected by RAB3A upregulation and synapsin I hyperphosphorylation which is reported in DMSXL mice [88] (Fig. 7.2).

7.13 RAN Translation

Recently, another interesting pathogenic mechanism has been proposed as an RNA gain of function. The CAG/CTG repeat expansion can be translated to homopolymeric polyglutamine, polyalanine, and polyserine proteins without an ATG start codon, termed repeat-associated non-ATG translation (RAN translation) in lentiviral-transduced cells and brains. In addition, RAN translation results in the accumulation of polyglutamine expansion proteins in DM1 mouse models and human tissues. Thus, these new toxic proteins should be considered in the pathogenic models of DM1, although the downstream mechanisms remain to be investigated [99, 100].

7.14 Therapeutic Approach

Based on these accumulated evidences for DM brain, several therapeutic approaches have been proposed: [A] the silencing of toxic C(C)UG-expanded RNA, [B] the prevention of interaction between toxic RNA and RNA-binding protein, and [C] the

modification of other downstream pathways of toxic RNA [101]. An example for [A] is antisense oligonucleotides, including CAG repeats targeting the toxic CUG RNA. These molecules reduced the DMPK RNA in DM1 cells [102] and in the skeletal muscle of *HSA*^{LR} mice [103]. More specifically, with antisense oligonucleotides promoting a RNase-H-mediated degradation, treatment of either human DMPK transgenic mice or monkeys inhibited human DMPK mRNA up to 70% [104]. The antisense drug entered phase 1–2a clinical trials in patients with DM, but this trial failed to validate its efficacy in muscles (ClinicalTrials.gov, identifier: NCT02312011). An example for [C] is tideglusib, a glycogen synthase kinase-3β (GSK-3β) inhibitor. GSK-3β has been recognized as the main kinase for MAPT hyperphosphorylation [105, 106]. Tideglusib is being tested in phase 2 clinical trials for tauopathies, including AD, PSP, and DM [107, 108] (ClinicalTrials.gov, identifier: NCT02858908).

Compared with the skeletal muscle and heart, the delivery of these molecules to the brain is significantly more challenging because of the blood–brain barrier. Considering that the brain is a heterogeneous organ and that CNS symptoms such as excessive daytime sleepiness are rather specific unlike overall impairment, a more specific approach such as targeting synaptic transmission would be more effective for the treatment of CNS symptoms in DM.

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Chapter 8
Respiratory Feature in Myotonic Dystrophy

Satoshi Kuru

Abstract Respiratory involvement is common in myotonic dystrophy (DM), and respiratory failure is the leading cause of death in DM. DM is associated with a slowly progressive impairment of lung function. Respiratory function is serially assessed by measuring the vital capacity, oxyhemoglobin saturation, peak cough flow, and end-tidal CO_2 level. Sleep-disordered breathing (SDB) is also common in DM, including obstructive and/or central sleep apnea and nocturnal hypoventilation. In DM, excessive daytime sleepiness (EDS) is not necessarily attributable to SDB, and it might be caused by a dysfunction in central sleep regulation. The respiratory insufficiency is caused by the combination of muscle impairment and altered central ventilatory control. For the treatment of respiratory failure, noninvasive ventilation (NIV) is recommended, but it might be hampered by cognitive impairment, altered personality, and unawareness of the disease. Improving compliance of the NIV is essential for enhancing the effectiveness of treatment for these patients. In addition, there are various factors that exacerbate respiratory failure, including ineffective airway clearance, infection, obesity, and perioperative pulmonary complication. Multidisciplinary care should be provided for the successful management of these respiratory complications.

Keywords Respiratory failure · Sleep-disordered breathing · Excessive daytime sleepiness · Noninvasive ventilation · Multidisciplinary care

8.1 Introduction

Myotonic dystrophy (DM) has two genetically distinct forms: DM1 and DM2. Based on the age at onset, DM1 can be classified into four different clinical phenotypic forms: congenital, childhood, classic (adult), and late onset [1]. Each form shows a distinct clinical picture, and there are differences in the onset, progression,

S. Kuru
National Hospital Organization Suzuka National Hospital, Suzuka, Japan
e-mail: kuru@suzulan.hosp.go.jp

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and severity of the respiratory impairment of each form. In this chapter, we mainly focus on adult-onset DM1.

8.2 Clinical Manifestations

Respiratory involvement is the leading cause of death in DM [2], and it is the major factor in the high morbidity and mortality in DM1. The natural history of the evolution of respiratory failure in DM is not predictable due to complicated pathological mechanism and variations in its clinical course, which is different from those of Duchenne muscular dystrophy [3]. According to a previous study of longitudinal course in DM1, lung function was slowly and progressively impaired in DM1 compared with other neuromuscular disorders [4]. Monteiro et al. reported that the CTG repeat length correlated with lung function and that it might be useful for the prediction of ventilatory involvement in DM1 [5].

Respiratory impairment usually occurs during middle age in the adult-onset type of DM1, and the development of respiratory failure is insidious. A lack of a correlation between the severity of respiratory involvement and motor performance was suggested [6]. Therefore, respiratory failure could be the first presenting feature of DM1 when generalized motor dysfunction is mild.

A practitioner should pay attention to subtle indicative symptoms and signs to detect an insidious occurrence of respiratory failure. It should be noted that symptoms of chronic respiratory failure, such as fatigue, morning headache, poor appetite, weight loss, and difficulty concentrating, are often nonspecific and generalized [7]. A checklist of these symptoms provided by 207th ENMC Workshop on chronic respiratory insufficiency in myotonic dystrophies is recommended for clinical use [6]. The checklist contains the following headings: (1) orthopnea, (2) dyspnea with performing ADL, (3) poor sleep, (4) morning headaches, (5) apnea, (6) decreased cognitive performance, (7) excessive daytime sleepiness (EDS), (8) fatigue, and (9) treated chest infection since last visit.

The abnormality of nocturnal ventilation reduces the quantity of sleep and impairs the quality of sleep, leading to symptoms related to those of EDS, such as morning headache and difficulty concentrating. In clinical practice, interviewing the patient's bed partner is useful to ascertain the respiratory state of the patient during sleep. EDS and fatigue are frequently occurring symptoms. Sleepiness or fatigue is not necessarily attributable to sleep-disordered breathing (SDB). While some studies noted a link between EDS and sleep apnea in DM patients [8, 9], others did not observe this link [10–13]. EDS is even found in DM1 patients without SDB. Conversely, patients with SDB do not always present with EDS. Nocturnal ventilation treatment for SDB infrequently eliminates sleepiness or fatigue. O'Donoghue et al. demonstrated that daytime hypersomnolence did not change after the withdrawal of NIV in DM patients [14].

Daytime sleepiness could be caused by a dysfunction in central sleep regulation [15], which is probably secondary to the loss of serotonin-containing neurons in the

raphe and to the loss of catecholaminergic neurons in the medullary reticular formation [16, 17]. The hypothalamic hypocretin system might also be responsible for the dysregulation of sleep, as was demonstrated by low levels of hypocretin-1 in the cerebrospinal fluid in DM1 patients [18]. Talbot et al. reported that modafinil is effective for reducing daytime sleepiness [19].

8.3 Examinations

Respiratory function should be serially assessed by measuring vital capacity, oxyhemoglobin saturation, peak cough flow, and end-tidal CO_2 level. Regular and routine examination is recommended from an early stage of the disease because of the lack of correlation between the severity of respiratory involvement and motor performance [6].

Vital capacity (VC) is a basic parameter of lung function, but it is not sensitive enough to detect early respiratory involvement. The measurement of VC is performed in both the sitting and supine positions. Diaphragm weakness can be indicated by a decrease in the VCs between the upright and supine positions by 25% [20].

Respiratory and ventilator muscle functions can be assessed by measurements of the maximal static inspiratory pressure (MIP) and maximal static expiratory pressure (MEP). The PI_{max} and PE_{max} measurements are used as parameters of inspiratory and expiratory muscle strength, respectively, but they are not suitable in DM patient with orofacial muscle weakness. For the patients, the sniff nasal inspiratory pressure (SNIP) is preferable to the PI_{max} and PE_{max}.

The transdiaphragmatic pressure (Pdi) is the difference between the esophageal and gastric pressures. The Pdi is adequate for specifically assessing diaphragm function, but it is often difficult to use for DM patients who cannot tolerate this invasive examination.

The peak cough flow (PCF) is a useful indicator of the ability to clear airway secretions. A PCF of 160 L/min is the threshold of efficacy for expectoration [21]. In particular, if the PCF is below 160 L/min, an ineffective cough predisposes patients to chest infection. If the PCF decreases to 160–270 L/min, effective expectoration is difficult during infection, and adequate intervention is thus needed.

Arterial blood gas analysis is one of the most fundamental pulmonary function tests, and it is used to assess hypoxemia and hypercapnia. A high tendency toward hypercapnia is observed in DM patients. The Pa_{CO2} is often inappropriately elevated compared to the degree of respiratory muscle dysfunction. An increase in the p(A-a) O_2 gradient is indicative of airway obstruction or involvement of the pulmonary parenchyma.

Sleep disturbances and SDB are common and insidious symptoms of DM, but they are sometimes overlooked. Therefore, careful follow-up is required to identify patients with SDB who are asymptomatic. We should take into consideration the possibility that SDB can occur irrespective of the patient's subjective symptoms,

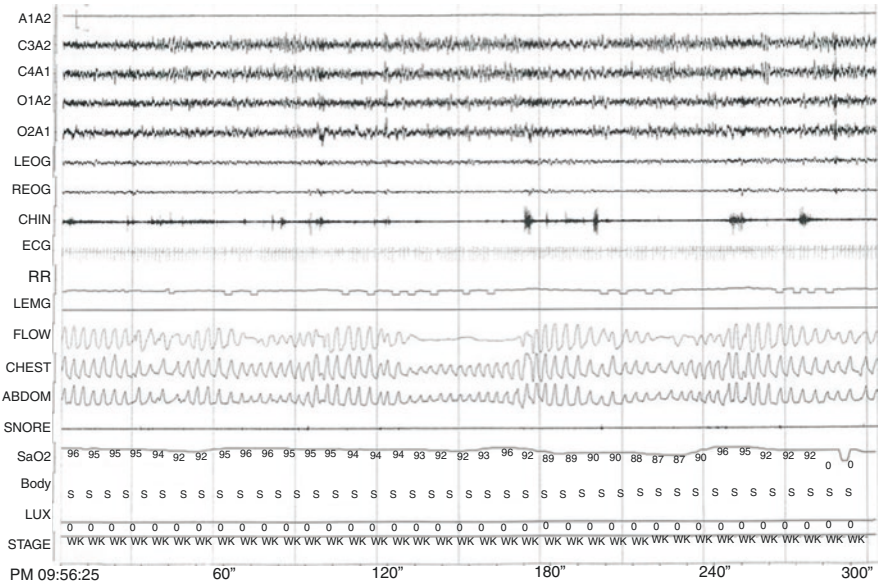

Fig. 8.1 Polysomnography

motor performance, and daytime pulmonary function. From the clinical viewpoint, studying a patient's sleep is mandatory, even in an asymptomatic patient with DM. Nocturnal desaturation in DM1 tends to be disproportionately severe relative to the degree of daytime pulmonary function. Standard type of polysomnography (EOG, EEG, EMG, ECG, and respiration) is useful for identifying the nature and severity of sleep disorders (Fig. 8.1). Ambulatory oximetry may serve as a screening tool for sleep disorders.

8.4 Case Report

(A case illustrating the beneficial effects of NIV treatment)

A 45-year-old Japanese man was referred to our hospital. He was 167 cm in height and 74 kg in weight.

At 34 years of age, he began to notice a loss of grasping power and had complaints of a morning headache and daytime sleepiness. The diagnosis of DM1 was established by gene analysis, which showed an expansion of approximately 200 (CTG)n in his leukocytes. Neurological examination revealed bilateral blepharoptosis and mild facial and distal limb weakness with myotonia. His serum CK level was elevated, and he was still ambulatory. Pulmonary function tests revealed mild restrictive ventilatory disorder with a forced vital capacity (FVC) that was 64.5% of the predicted value. His ventilatory response to CO_2 ($\Delta V_E/\Delta P_{ET}CO_2$) was 0.43 L/min/mmHg. Nocturnal polysomnography showed an increase of apnea-hypopnea

index (AHI, 23.6 events/h) (Fig. 8.2). We observed 24 obstructive apneas, 27 central apneas, 56 mixed apneas, and 117 hypopneas from the nocturnal polysomnography results. We started to treat the patient with noninvasive mechanical ventilation (NIV) using bilevel positive pressure (BiPAP) (Fig. 8.3). The initial setting that was used was an EPAP of 4 cm H_2O and an IPAP of 8 cm H_2O. After the commencement of the NIV, he noted improvement in the morning headache and EDS. Thereafter, annual assessments of nocturnal ventilation were performed for 6 years (Fig. 8.4). The deterioration of sleep apnea was shown by the comparison between the results when the patient was at 46 years old (before the NIV) and when the patient was

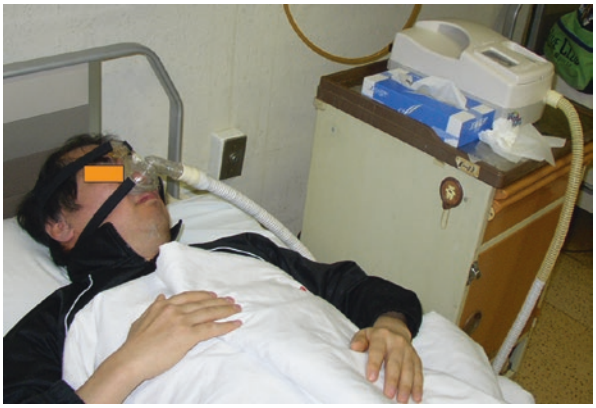

Fig. 8.2 Patient with DM who is undergoing NIV

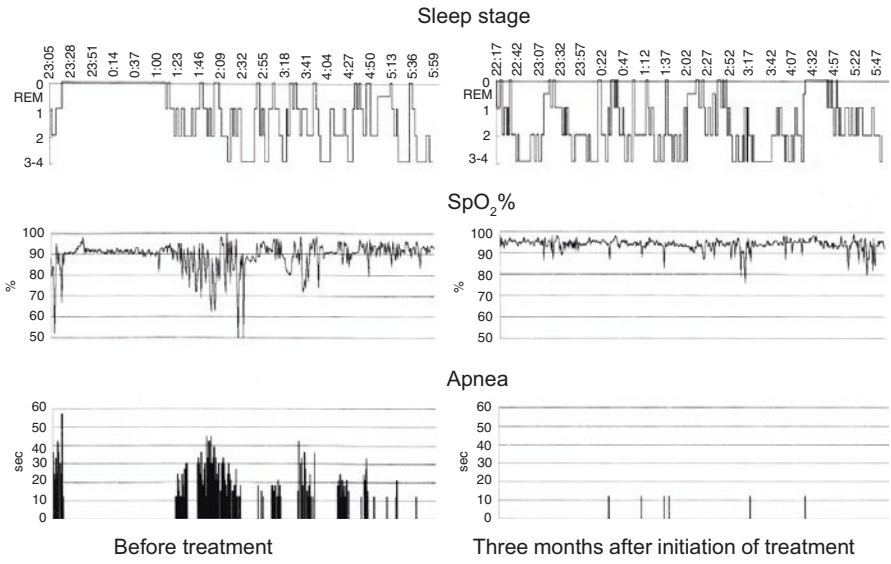

Fig. 8.3 Polysomnography

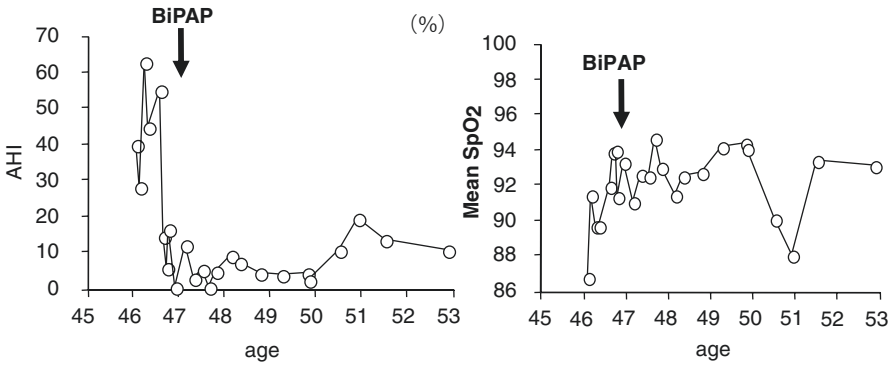

Fig. 8.4 Clinical course

Table 8.1 Sleep apnea at age 46 and 53

	46 years old (before treatment)	53 years old (withdrawal BiPAP)
CSA	27	62
Mixed type	56	81
OSA	24	46
Hypopnea	117	124
Apnea index	23.6	56.7
Mean SpO_2	89.7%	89.0%
Sleep efficacy	0.656	0.701

53 years old (temporal withdrawal) (Table 8.1). This case demonstrated the long-term beneficial effect of BiPAP for sleep apnea.

8.5 Pathophysiology

Respiratory failure may result primarily from weakness and myotonia in the respiratory and upper airway muscles, and it is probably influenced by an inherent abnormality of the central control of ventilation.

All of the inspiratory, expiratory, and upper airway muscles are involved in DM1. Weakness of the inspiratory muscles, such as the diaphragm and external intercostal, is responsible for the restriction of the rib cage, which leads to CO_2 retention and hypercapnia. On the other hand, weakness in the expiratory muscles is responsible for an ineffective cough, leading to the impairment in clearing airway secretions and a susceptibility to pulmonary infection. Weakness of the upper airway muscle enhances the risk for aspiration, asphyxia, and pneumonia.

Electrophysiological studies indicated that myotonia is observed in the respiratory muscles and may be involved in the pathological mechanisms of respiratory failure in DM [22, 23]. In addition, an association of peripheral neuropathy with

sleep-related breathing disorders in DM was reported [24]. An animal study demonstrated that the DMSXL transgenic mouse model exhibited pathological changes in the diaphragmatic muscle and a significant decrease in the number of unmyelinated phrenic afferents [25].

A neuropathological study suggested that neuronal loss in the medullary reticular formation was associated with hypoventilation in DM [17]. The central chemoreflex sensitivity can be estimated by the ventilatory response to carbon dioxide. A reduced ventilatory response to CO_2 has been shown by both the steady-state and rebreathing methods [15, 26, 27]. CO_2 sensitivity is independent of respiratory muscle and lung function [15].

An abnormality of respiratory rhythm was observed predominantly in the awake and light sleeping states, and it was not observed in the slow-wave sleeping state [28]. This finding suggests that the abnormal pattern of respiratory rhythm observed in DM patients is influenced by the "behavioral system" rather than the "metabolic system."

Weakness of both the respiratory and upper airway muscles also contributes to an increased risk for SDB in several types of muscular dystrophies [7]. SDB is more severe in DM1 than in other types of muscular dystrophies that present with a similar degree of respiratory muscle weakness [29]. In normal subjects, the tone of the skeletal muscles, except for the diaphragm and extraocular muscles, is reduced during REM sleep. The ventilation in REM sleep is predominantly dependent on the diaphragm due to atonia of the inspiratory muscles. Therefore, a reduced diaphragmatic strength augments hypoventilation during REM sleep. Furthermore, upper airway muscle weakness contributes to airway obstruction during sleep, leading to obstructive apnea.

Mild alterations in blood gases, such as a decrease in the SaO_2 level and an increase in CO_2 tension, are observed during REM sleep in normal subjects. These alterations may be exaggerated in patients with neuromuscular disorders. Hypoventilation and the abnormalities of gas exchange cause frequent arousal and reductions in sleep time and efficiency, leading to hypersomnolence and fatigue.

SDB, including obstructive and/or central sleep apnea and nocturnal hypoventilation, is common in DM patients and has a prevalence ranging from 16 to 75% [9, 29–34]. While some studies showed a predominance of OSA in DM patients [32, 35], others showed a predominance of central apnea [8, 29, 30]. Additionally, periodic limb movements and REM sleep dysregulation have been described as being symptoms in DM1 patients [36]. The severity of sleep apnea is related to the body mass index (BMI) of the patient [29, 37]. In addition, the risk for obstructive sleep apnea may be enhanced by craniofacial abnormalities, such as a narrow maxillary arch and a retrognathic mandible [38]. These abnormalities are more prominent in the congenital and early-onset forms of DM1.

SDB in chronic neuromuscular diseases has adverse effects on the cardiovascular and central nervous systems (Fig. 8.5). DM involves multiple organs, and the involvement of the disorder in each of the organs may be explained in part by a toxicity of expanded *DMPK* mRNA on the alternative splicing of related effector

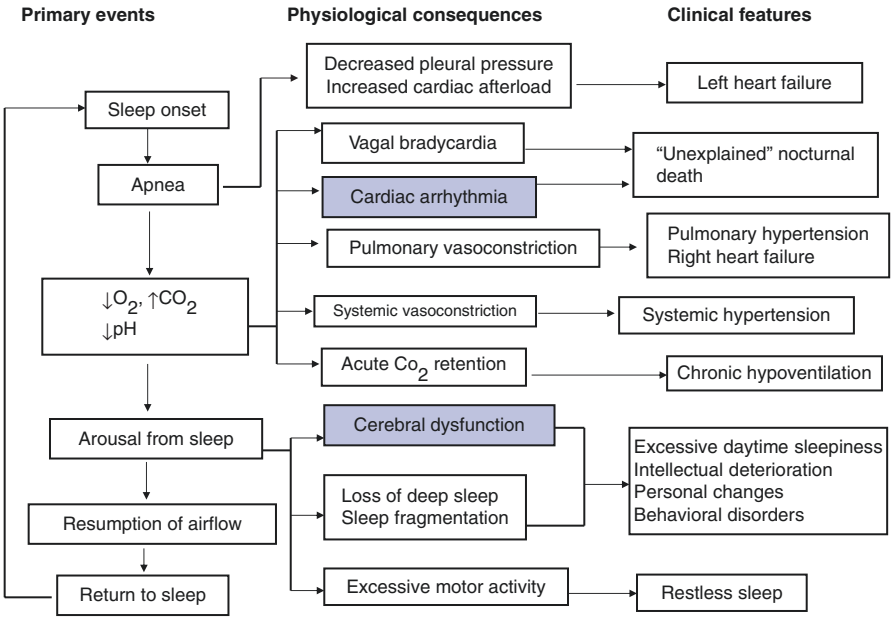

Fig. 8.5 Pathological mechanism of sleep apnea syndrome (Modified from Philipson EA [39])

genes (see Chaps. 3 and 4). Cardiac arrhythmia is frequently encountered in DM patients. A recent study showed that splicing misregulation of SCN5A may contribute to heart arrhythmias and cardiac-conduction delays in DM patients [40]. OSA generally enhances the risk for arrhythmias, particularly atrial fibrillation. Lazarus et al. suggested that sleep apnea might play the arrhythmogenic role in DM1 [41].

OSA may also contribute to cognitive dysfunctions, which are characterized by the impairment of attention, memory, and executive functions [42, 43]. In DM1, there have been a few studies evaluating the relationship between cognitive impairment and sleep apnea. Gallais et al. showed that a lower score in attention, vigilance, and executive functioning test was associated with a higher degree of AHI and longer duration of desaturation [37], while other studies suggested no significant relationship between them [44, 45]. Therefore, treatment with NIV might be able to reduce the adverse effect that SOB has on the cognitive and cardiovascular functions of DM1 patients and improve their related symptoms. However, further study is needed regarding this problem.

8.6 Management

Respiratory management should be performed according to the severity of respiratory dysfunction.

8.6.1 Airway Clearance

An effective airway clearance is crucial for preventing atelectasis and pulmonary infection. Assisted cough techniques include manually assisted coughing, insufflation using a self-inflating resuscitation bag, and mechanical insufflation/exsufflation. In addition, intrapulmonary percussive ventilation (IPV) is a method that promotes airway clearance by superimposing high-frequency mini-bursts of gas [46]. They are useful for reducing the risk for infection.

8.6.2 Noninvasive Nocturnal Ventilation

NIV was needed in 15% of the DM patients from the UK Myotonic Dystrophy Registry. Compared to the female DM patients, the male DM patients had higher frequencies of NIV [47]. Previous studies demonstrated successful treatment with NIV [48–50]. However, there have been few studies on whether NIV improves the survival of patients and when and how it starts to have beneficial effects on the QOL and prognosis of patients with DM1. Further study is needed to examine and describe natural history in a large cohort of patients who are stratified by CTG size.

8.6.2.1 Criteria for Launching

Specific criteria for launching NIV in DM1 have not been established.

The general criteria for launching NIV were proposed as follows [6, 51]:

When there is at least one or more of the daytime or nighttime symptoms suggestive of chronic respiratory insufficiency in combination with:

1. Daytime hypercapnia, $PaCO_2 > 45$ mmHg
2. FVC < 50% of predicted based on the best of three measures and MIP < 60 cmH_2O
3. Evidence of nocturnal hypoventilation, such as:
   (a) A rise in $PaCO_2 > 8$ mmHg between evening and morning ABGs or other accurate CO_2 surrogate
   (b) A rise in $TcCO_2$ or $ETCO_2 > 50$ mmHg for more than 50% of total sleep time
   (c) While not ideal—when a measure of CO_2 is not available—nocturnal oximetry demonstrating sustained desaturation (SpO_2) <88% for 5 consecutive minutes or $SpO_2 < 90\%$ for >10% of total sleep time

8.6.2.2 Ventilator and Interface

It is important to select an appropriate ventilation modality and interface for each patient. BiPAP is widely used. Continuous positive airway pressure (CPAP) can be used when BiPAP is not tolerated or, alternatively, when the contribution of the

obstruction of the upper airway predominates [6]. An initial inspiratory pressure between 12 and 22 cmH_2O and an expiratory pressure of 3–6 cmH_2O are recommended. After the introduction of NIV, it is necessary to assess the respiratory function regularly to ascertain the effectiveness of the NIV and adjust the settings of the ventilator.

At first, NIV is used intermittently, usually only during nighttime sleep. As the pulmonary dysfunction progresses, the duration of ventilator use should be gradually increased according to the efficacy and tolerance of the ventilation. Hours of use can be monitored using the respiratory chip device.

In cases of domiciliary-assisted ventilation, patients should be advised to use the equipment properly. A persistent effort by the caregivers is needed to optimize the mask fit to manage both air leakage and skin complications, for which various types of masks are now available. A nasal mask is widely used, and a nasal-oral or full-face mask may be used if necessary. The appropriate selection of the interface is one of the key elements for improving compliance of the NIV in DM patients.

8.6.2.3 Adverse Effects and Compliance

NIV is frequently associated with several adverse effects, including skin erosion and eye irritation, dryness of mucous membranes, air leaks, and aerophagia [6, 7]. The use of a chinstrap can reduce the amount of air leaking out of the mouth.

Improving compliance is essential to enhancing the effectiveness of NIV. Compliance with NIV is influenced by various factors, including subjective symptoms, excessive leaks, ventilator dysfunction, and a high BMI [52]. The efficacy of NIV may be hampered by cognitive impairment, an altered personality, and the patient's unawareness of the disease [53].

The treatment of SDB is not always satisfactory for improving EDS. Therefore, daytime sleepiness cannot be used as an outcome measure for NIV treatment in DM. Failure to improve subjective symptoms may partly account for the poor compliance to NIV in patients with DM.

8.6.3 Continuous Invasive Ventilation

Invasive ventilation (mainly with permanent tracheostomy) should be considered when the NIV is poorly tolerated or not feasible for various reasons. Invasive ventilation can provide a higher ventilator pressure and has an advantage of a secure interface and a direct airway suctioning through the tracheostomy. However, invasive ventilation is associated with many serious complications, such as hemorrhage, tracheoesophageal fistulae, upper airway obstruction by granulation tissue or stenosis, and respiratory infection [6]. Since invasive ventilation is a lifelong requirement for patients, communication and consensus among the patient, the patient's family,

and the physicians on the start of mechanical ventilation are essential from the standpoint of end-of -life care.

8.6.4 Factors That Exacerbate Respiratory Failure

There are various factors that exacerbate respiratory failure, including obesity, infection, and perioperative pulmonary complications.

8.6.4.1 Weight Control

Patients should be advised to achieve their ideal body weight. A previous large observational study showed that an overweight was found in 59% of DM1 patients and was an independent risk factor for a reduced lung volume, which presumably increased the amount of work required for breathing [54]. In the general population, obesity is one of the risk factors for OSA. Gallais et al. observed a strong relationship between BMI and AHI in patients with DM [37]. Therefore, weight control could delay the onset of respiratory failure to ameliorate obstructive sleep apnea. Adequate exercise and diet are recommended for weight control.

8.6.4.2 Swallowing Disturbance

Swallowing disturbances and dysphagia are common symptoms of DM1 and are a major cause of aspiration pneumonia (see Chap. 11).

8.6.4.3 Infection

Pulmonary infection and pneumonia frequently occur in DM1 patients, and recovering from them seems difficult due to the presence of poor cough and muscle weakness. DM patients are susceptible to an acute exacerbation of chronic respiratory insufficiency during the course of pulmonary infection.

8.6.4.4 Perioperative Pulmonary Complications

Patients with DM are exposed to a significant risk of perioperative pulmonary complications (PPC), especially those with severe muscular impairment after upper abdominal surgery [55], so it is mandatory to carefully monitor the respiration, circulation, consciousness, and glucose metabolism in these patients and pay attention to the response and side effects of anesthetic agents, as well as the depth of anesthesia, in order to prevent PPC.

For the success of respiratory management, we should recognize aspects of DM that are distinguishable from those of other neuromuscular diseases to overcome several difficulties. These aspects of DM are as follows:

1. A paucity of subjective symptoms
2. A broad spectrum and variation in the clinical course in relation to CTG expansion
3. A complicated pathophysiology of respiratory insufficiency
4. Intellectual and emotional problems
5. A poor adherence to treatment

These factors are related to each other.

Multidisciplinary care based on a long-term therapeutic strategy should be provided for managing the respiratory insufficiency in patients with DM. Medical staff of the respiratory support team are required to understand the aforementioned characteristics of DM, share information on each patient, and adequately educate the patient and the family and caregiver of the patient. From the standpoint of communication, a simple explanation according to the cognitive and behavioral needs of DM patients is desirable.

The author believes that adequate management of coping with the problems related to respiratory insufficiency can improve the survival and QOL of patients with DM.

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Chapter 9
Glucose Intolerance in Myotonic Dystrophy

Hiromi Iwahashi

Abstract Abnormality of carbohydrate metabolism is often observed in myotonic dystrophy. Hyperinsulinemia and impaired glucose metabolism in the skeletal muscle have also been reported in myotonic dystrophy 1 (DM1); thus, insulin resistance could be the primary pathophysiology leading to glucose intolerance in DM1. One of the molecular mechanisms of insulin resistance is an aberrant expression of the low-signaling IR-A in the muscle resulting from abnormal splicing caused by altered RNA binding due to the CUG expansion in the DMPK gene. It is also suggested that DMPK itself affects insulin action, and reduced DMPK causes insulin resistance. Fasting hyperinsulinemia and hypersecretion of insulin on glucose loading are observed in DM1 patients from early period before the onset of diabetes, and these may be risk of developing diabetes. Myotonic dystrophy patients have some factors for deterioration of glucose intolerance other than genetic-based insulin resistance: reduced lean body mass, relatively increased body fat, and low physical activity with progression of myopathy. Concerning treatment, it is reasonable to use insulin-sensitizing drugs such as pioglitazone and metformin. However, most of patients, especially with long duration of diabetes, need to use other medicines including insulin for keeping adequate glycemic control. In clinical practice, the goals of glycemic control should be determined individually in light of the patient's important comorbidities. Since myotonic dystrophy is one of the important comorbidities, further studies will be needed to answer how we determine the goals of glycemic control and how we should treat in safe the individuals with progressed myopathy.

Keywords Myotonic dystrophy · Glucose intolerance · Diabetes mellitus · Insulin resistance · Insulin secretion

H. Iwahashi
Department of Diabetes Care Medicine, Graduate School of Medicine,
Osaka University, Osaka, Japan
e-mail: iwahashi@endmet.med.osaka-u.ac.jp

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9.1 Prevalence of Glucose Intolerance in Myotonic Dystrophy

An abnormality of carbohydrate metabolism in myotonic dystrophy was first reported in 1950 by Caughey JE et al. [1]. Then, there has been many case reports or small studies on the association between them. Huff et al. reported an elevated fasting plasma insulin and an increased insulin response to a glucose load in myotonic dystrophy patients [2]. In addition, hyperinsulinemia and impaired glucose metabolism in the skeletal muscle had also been reported in myotonic dystrophy 1 (DM1); thus, insulin resistance could be the primary pathophysiology leading to glucose intolerance in DM1. However, the exact frequency of clinical diabetes in myotonic dystrophy has not clearly been proven so far.

Harper described in the literature [3] that the prevalence of clinical diabetes in myotonic dystrophy was 6.5% (Table 9.1). Since the data derive from the study in relative younger population, the risk of developing clinical diabetes during the lifetime should be higher than that. It is suggested in the literature that myotonic dystrophy patients have a fourfold increase in risk of clinical diabetes compared with their control series. As a while, the frequency of asymptomatic glucose intolerance in myotonic dystrophy has also been reported from Japan [4]. In that report, DM1 patients with mean age of 43.0, who had not been diagnosed as diabetes mellitus before nor treated with antidiabetic agents, were performed by 75 g oral glucose tolerance test (OGTT), and impaired glucose tolerance (IGT) and diabetic pattern (DM) were found at 14.7% and 9.5%, respectively (Table 9.2). The prevalence of diabetes mellitus was reported to be significantly higher in DM1 patients even after correcting age, BMI, and sex with the control subjects [4]. Thus, there seems to be a relative higher prevalence of asymptomatic glucose intolerance in myotonic dystrophy.

Anyway, it is no disagreement that there is a slightly increased incidence of clinical DM and a more frequent occurrence of asymptomatic glucose intolerance in DM1 patients.

Table 9.1 Clinical diabetes in myotonic dystrophy

		Clinical diabetes	Nondiabetes
Harper [3]	Myotonic dystrophy	11 (6.5%)	158 (93.5%)

Table 9.2 OGTT pattern in myotonic dystrophy without clinical diabetes

		Diabetes	IFG and/or IGT	NGT
Matsumura et al. [4]	Myotonic dystrophy	9 (9.5%)	14 (14.7%)	72 (75.8%)

9.2 Characteristics of Glucose Metabolism in DM1

Matsumura et al. performed a cross-sectional study for glucose intolerance in myotonic dystrophy and reported the characteristics of glucose metabolism in DM1 in detail [4]. In that report, they found that DM1 predisposed insulin resistance and compensatory hyperinsulinemia exist even in patients with low fasting plasma glucose. They concluded that we should pay attention to glucose intolerance of DM1 patients earlier than that of the general population. In fact, the frequency of glucose intolerance tended to be higher in DM1 patients than that in the general population whose fasting plasma glucose levels were similar (Fig. 9.1). This indicates that one of the characteristics of glucose metabolism in DM1 is hyperglycemia after glucose loading rather than on fasting.

Takada et al. reported daily glucose profiles in DM1 minutely using continuous glucose monitoring (CGM) [5]. Thirty patients with DM1 with mean age of 49.0 participated in the study. They consisted of 7 normal glucose tolerance, 15 impaired glucose tolerance, and 8 diabetic pattern subjects. According to the report, three patterns of daily glucose variability were observed: (a) the glucose value changed within reference range all day, (b) the glucose value increased gradually from morning to evening, and (c) the glucose value increased after breakfast and continued in the high level. The glucose tolerance proved to be getting worse in this order. In addition, hypoglycemia was observed in six of seven DM1 patients with normal glucose tolerance not only in nighttime but also after meals. Hyperinsulinemic hypoglycemia is sometimes observed in subjects with insulin resistance with normal glucose tolerance [6]. One supposed mechanism is excessive secretion of

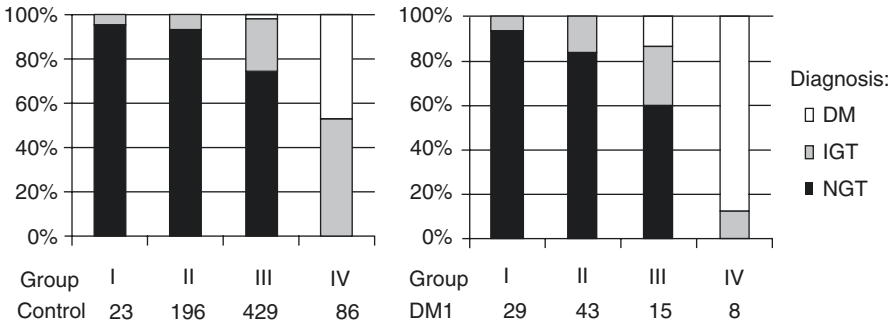

Fig. 9.1 Distribution of the diagnosis of OGTT in the literature [4]. Participants were divided into four subgroups based on their FBS (group I, FBS < 80 mg/dL; group II, 80 = <FBS < 90 mg/dL; group III, 90 = <FBS < 110 mg/dL; group IV, FBS= > 110 mg/dL). In control, DM pattern was observed in 1.9% of group III and 46.5% of group IV. In DM1, that was found in 13.3% of group III and 87.5% of group IV

insulin after glucose (or meal) loading, with resultant persistently high concentrations of insulin that ultimately suppress hepatic glucose output, leading to postprandial hypoglycemia. Hypoglycemia might usually occur in DM1 patients with normal glucose tolerance.

In summary, the characteristics of glucose metabolism in DM1 would be hyperglycemia after glucose and/or meal loading rather than on fasting and postprandial hypoglycemia with excessive response of insulin.

9.3 Insulin Resistance in DM1 Patients

There have been many reports which showed insulin resistance in DM1 patients. Matsumura et al. reported that HOMA-IR, which is an index of insulin resistance evaluated in the fasting state, was higher in DM1 patients than in control subjects significantly even in the normal fasting plasma glucose stage [4] (Fig. 9.2d). In addition, they also showed that insulin sensitivity assessed by insulin tolerance test, which represents whole-body insulin sensitivity, was low regardless of fasting plasma glucose levels in DM1 without clinical diabetes mellitus. As a while, Moxley et al. [7, 8] performed euglycemic and hyperinsulinemic clamp, which is a gold standard method in measuring insulin resistance, and showed that the insulin resistance in myotonic dystrophy is peripheral. Their studies also showed the whole-body glucose uptake is reduced even when corrected for reduced muscle mass in myotonic dystrophy, suggesting abnormal insulin signaling in the muscle of myotonic dystrophy patients.

Some hypotheses have been presented to explain molecular mechanism of insulin resistance in DM1. One of them is an altered splicing of insulin receptor pre-mRNA in skeletal muscle of DM1. Insulin receptor has the two isoforms: isoform A (IR-A) lacking exon 11 and isoform B (IR-B) including exon 11. The IR-B is a higher-signaling form and usually predominates in insulin-responsive tissues such as skeletal muscle. Savkur et al. reported that alternative splicing of the insulin receptor (IR) pre-mRNA was aberrantly regulated in DM1 muscle, resulting in predominant expression of the lower-signaling non-muscle isoform of IR-A (Fig. 9.3). This abnormal splicing may be caused by altered RNA binding due to the CUG expansion in the DMPK gene. They also reported that IR-A predominates in DM1 skeletal muscle cultures, which exhibit a decreased metabolic response to insulin relative to those from normal controls. These facts suggest that an altered splicing of insulin receptor pre-mRNA is, at least, one of the molecular mechanisms of insulin resistance in DM1 [9].

The other hypothesis is that DMPK itself is a positive modulator of insulin action. Llagostera et al. suggested using DMPK-deficient mice that reduced DMPK expression may directly influence the onset of insulin resistance. They showed that DMPK-deficient mice exhibited impaired insulin signaling in muscle tissues but not in adipose and liver tissues in which DMPK is not expressed. They also exhibited reduced glucose uptake and impaired insulin-dependent GLUT4 trafficking in the

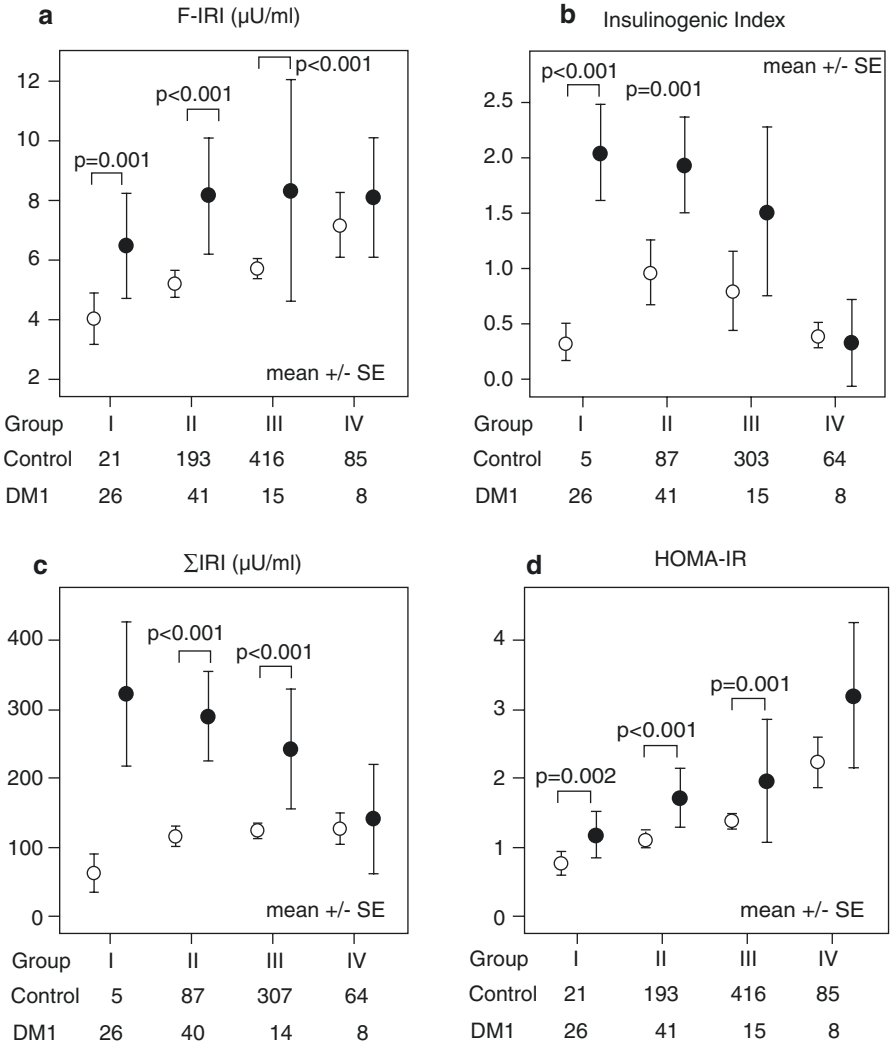

Fig. 9.2 Comparison of F-IRI, Insulinogenic Index, ΣIRI, and HOMA-IR between control and DM1 in the literature [4]. Participants were divided into four subgroups based on their FBS (group I, FBS < 80 mg/dL; group II, 80 = <FBS < 90 mg/dL; group III, 90 = <FBS < 110 mg/dL; group IV, FBS= > 110 mg/dL), and (**a**) F-IRI, (**b**) Insulinogenic Index, (**c**) ΣIRI, and (**d**) HOMA-IR were compared between control and DM1 by ANCOVA with sex, age, and BMI as covariates. When significant differences were detected, *p* value was described in the graphs

muscle. In addition, they showed DMPK is required for a correct intracellular trafficking of insulin and IGF-1 receptors. These facts suggest that DMPK itself is a positive modulator of insulin action, and reduced DMPK may directly influence the onset of insulin resistance in DM1 patients [10].

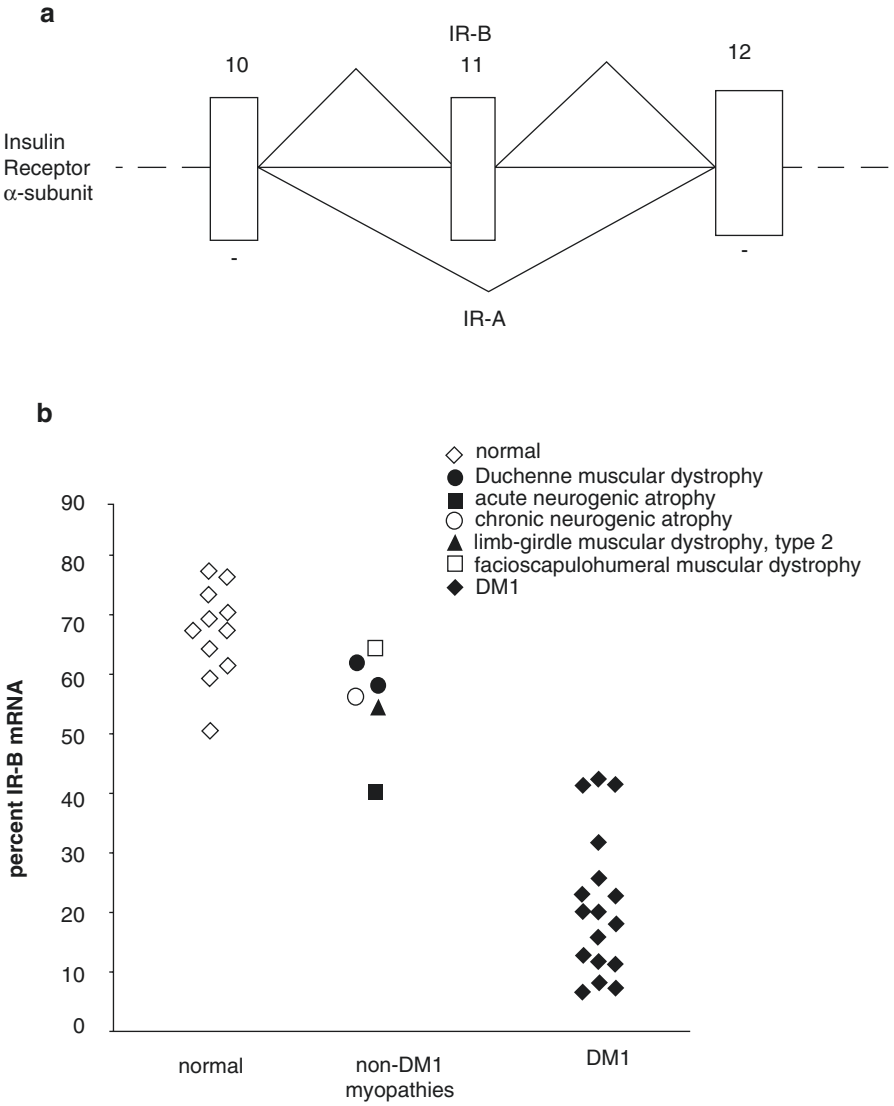

Fig. 9.3 The following graphics are quoted from the literature [9]: (**a**) Graphical representation of two isoforms of insulin receptor (IR). Human IR exon 11 is included in IR-B but not in IR-A, which is a lower-signaling isoform. (**b**) Graphical representation of RT-PCR analysis depicting percentage of IR-B mRNA in skeletal muscle tissues of normal (n = 11), non-DM1 myopathies (n = 6), and DM1 (n = 17) individuals. Statistical analyses were performed using a two-tailed unpaired Student's t-test. The p values from the comparisons between normal versus DM1 and normal versus non-DM1 myopathies are $p < 0.0001$ and $p = 0.05$, respectively

Anyway, there exactly exists insulin resistance in DM1 patients, especially in the muscle, from normal glucose tolerance stage, and this may be the primal cause for developing glucose intolerance or clinical diabetes in DM1. One of the molecular mechanisms is supposed to be an aberrant expression of the low-signaling IR-A in the muscle resulting from abnormal splicing caused by altered RNA binding due to the CUG expansion in the DMPK gene. It is also suggested that DMPK itself affects insulin action, and reduced DMPK causes insulin resistance.

9.4 Abnormal Insulin Secretion in Myotonic Dystrophy

Another metabolic alteration observed in DM1 patients is insulin hypersecretion on glucose loading. Insulinogenic Index in OGTT calculated from [immunoreactive insulin (IRI) at 30 min after glucose loading—fasting IRI]/[plasma glucose (PG) at 30 min after glucose loading—fasting PG] is an index of early insulin secretion after glucose loading. Matsumura et al. reported that in nondiabetic subjects of fasting plasma glucose under 90 mg/dL, insulinogenic indices as well as fasting IRI levels were higher in DM1 patients than in normal control (Fig. 9.2a, b). In addition, sigma IRI (sum of IRI at 0, 30, 60, and 120 min in OGTT) was also higher in DM1 patents [4] (Fig. 9.2c). Since insulin resistance was also confirmed by insulin tolerance test in those DM1 patients, they concluded that insulin resistance is a primary phenomenon, and compensatory hyperinsulinemia occurs from early stage to keep plasma glucose normal in DM1 patients. Anyway, their data indicate that insulin hypersecretion both in fasting and on glucose loading is observed in DM1 patients from early stage before the onset of diabetes.

On the other hand, Perseghin et al. reported in their small number study that abnormal insulin secretion occurred in nondiabetic myotonic dystrophy patients with normal insulin sensitivity [11]. They performed euglycemic-hyperinsulinemic clamp and OGTT in ten young nondiabetic patients with myotonic dystrophy and compared their insulin sensitivity and insulin secretion with healthy subjects. In that report, the myotonic dystrophy patients had reduced lean body mass, but peripheral insulin sensitivity was not different to that of control. As a while, increased insulin secretion during the first 30 min of OGTT was observed in them compared with normal control. In addition, abnormal plasma proinsulin levels were found in the fasting state, during the clamp and also during the OGTT in the patients. They concluded that these secretory dysfunctions of insulin were peculiar of myotonic dystrophy and might be more important than insulin resistance in determining the risk of developing diabetes. Then, how does this abnormal insulin secretion occur in nondiabetic myotonic dystrophy patients if their insulin sensitivity is normal? A possible reason is proposed in the literature [12]. Protein kinase in DMPK gene is involved in the modulation of the Ca^{2+} homeostasis in skeletal muscle. If a malfunction of the protein kinase exists and also affects the alteration of Ca^{2+} homeostasis in pancreatic beta cells, it is possible that the abnormal pattern of insulin secretion is caused by the aberration in the DMPK gene. Anyway, there might be some

patients with abnormal insulin secretion with preserved insulin sensitivity in nondiabetic myotonic dystrophy.

In summary, fasting hyperinsulinemia and hypersecretion of insulin on glucose loading are observed in nondiabetic DM1 patients from early stage before the onset of diabetes and may be risk of developing diabetes. Although the phenomenon is usually associated with insulin resistance and is considered to occur by a compensatory mechanism, there might be another independent mechanism from insulin resistance by which hypersecretion of insulin on glucose loading occurs.

9.5 Other Factors for Deterioration of Glucose Intolerance in Myotonic Dystrophy

Myotonic dystrophy patients usually have reduced lean body mass. This is accompanied with relatively increased body fat mass, enhancing the extent of insulin resistance. Furthermore, low physical activity resulting from progression of myopathy would worsen insulin resistance. These factors could cause deterioration of glucose intolerance in myotonic dystrophy (Fig. 9.4).

Neuromuscular diseases such as myotonic dystrophy are often associated with weakness, reduced mobility, and reduced work capacity; thus, they are considered to have common problems related to the effects of inactivity. In fact, Aitkens et al. reported about metabolic syndrome in neuromuscular diseases including myotonic dystrophy [13]. According to their report, patients with neuromuscular diseases were more obese and spending less time in total activity, leading to having more cardiovascular and metabolic risk factors. They concluded that patients with neuromuscular diseases are at high risk for developing metabolic syndrome and diabetes mellitus.

In addition, we often see the diabetic patients with myotonic dystrophy worsen their glucose control with progression of their myopathy. Horii et al. reported a case

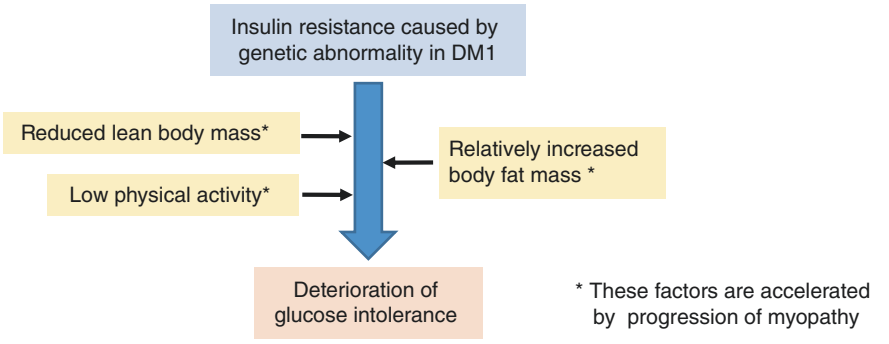

Fig. 9.4 Various factors for the deterioration of glucose intolerance in myotonic dystrophy

of myotonic dystrophy with diabetes in whom the deterioration of insulin resistance and progression of muscle weakness had been observed longitudinally for about 8 years [14]. In that case, insulin resistance was observed from the onset of diabetes. Treatment with pioglitazone and reduction of body fat improved the insulin resistance and glucose control at the onset of diabetes. However, as muscle weakness had progressed and lean body mass had been decreased, insulin resistance had been deteriorated. After 8 years, the case had to be treated with metformin and DPP-4 inhibitors in addition to pioglitazone to maintain good glucose control. Progression of muscle weakness might be one of predictors for deterioration of diabetes mellitus.

In summary, myotonic dystrophy patients have some factors for deterioration of glucose intolerance other than insulin resistance based on genetic abnormalities. These are reduced lean body mass, relatively increased body fat, and low physical activity with progression of myopathy.

9.6 Treatment of Diabetes in Myotonic Dystrophy

Since the frequency of glucose intolerance is relatively higher in DM1 patients even if their fasting plasma glucose levels are within normal range, OGTT and advice for diet and body weight control should be conducted in DM1 patients from normal FPG stage to prevent developing diabetes. After onset of diabetes mellitus, diet and exercise therapy should also be conducted, but the patients with progressed myopathy would have some difficulty in performing exercise. Concerning medicines, several groups reported that thiazolidine [15] and metformin [16] could improve hyperglycemia in myotonic dystrophy. In fact, insulin resistance evaluated by insulin tolerance test in myotonic dystrophy could be improved by pioglitazone [15]. Since insulin resistance is considered to be the primal pathophysiology in glucose intolerance with myotonic dystrophy, it is reasonable to use such insulin-sensitizing drugs. However, most patients, especially with long duration of diabetes, need to use other medicines including insulin for keeping adequate glycemic control.

Dipeptidyl peptidase-4 inhibitor (DPP4-i) is widely used for the treatment of type 2 diabetes mellitus and supposed to have an advantage of low risk of hypoglycemia and lowering postprandial hyperglycemia. Considering the characteristics of glucose metabolism in DM1 patients, DPP4-i might also be one of the suitable medicines for diabetes in myotonic dystrophy. However, the efficacy against diabetic patients with myotonic dystrophy has not been fully verified. According to the questionnaires about therapeutic situation in DM1 patients with diabetes mellitus registered to the Japanese national patient registry (Remudy), about one-third of the patients had been treated by DPP4-i. DPP4-i had been really effective for lowering hyperglycemia and weight reduction in most of the patients without major adverse effects [17]. DPP4-i could be a useful therapeutics against diabetic patients in DM1.

9.7 Clinical Questions in the Management for Glucose Intolerance Associated with Myotonic Dystrophy

In general, the objectives of diabetes management are to reduce symptoms of diabetes, to prevent development or progression of diabetic complications, and to enable affected individuals to maintain their quality of life (QOL) and life expectancy comparable to those seen in healthy individuals [18]. Glucose levels in affected individuals should be controlled as close to normal as possible. Achieving and maintaining favorable glycemic control early after initiation of treatment are likely to lead to favorable long-term outcomes [18]. Improving fasting glucose levels and HbA1c values is critically important for the prevention of microangiopathy associated with diabetes, and in addition to these, improving postprandial hyperglycemia is key to prevent macroangiopathy [19, 20]. These principles seem to be also suitable for diabetic patients with myotonic dystrophy.

In clinical practice, however, the goals of glycemic control should be determined individually in light of the patient's important comorbidities as well as duration of diabetes, life expectancy, established vascular complications, and risk for hypoglycemia [21]. Myotonic dystrophy should be one of the important comorbidities of the patients. Then, how should we manage glucose intolerance in myotonic dystrophy? To answer this question, we have to clarify the natural history of the myotonic dystrophy patients with or without diabetes mellitus. In addition, we should investigate whether insulin resistance underlying myotonic dystrophy has also atherogenic effects similarly as in obese people. Furthermore, we must find out how to treat in safe the patients with progressed myopathy, namely, lower activity of daily living or muscle weakness. Future study will be needed to answer these clinical questions for glucose intolerance underlying myotonic dystrophy.

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Chapter 10
Lipid Metabolism in Myotonic Dystrophy

Hiroto Takada

Abstract Lipid metabolism abnormality in myotonic dystrophy (DM) was reviewed. Dyslipidemia is frequently seen in patients with DM. Hypertriglyceridemia, low HDL cholesterolemia, high LDL cholesterolemia, and exceeded visceral fat accumulation are common as abnormal lipid metabolism findings in DM. Dyslipidemia is one of risk factors for metabolic syndrome or nonalcoholic fatty liver disease and is closely relevant to insulin resistance. Careful follow-up for dyslipidemia in DM, especially complicated with glucose intolerance, should be required.

Keywords Myotonic dystrophy · Dyslipidemia · Lipid metabolism · Insulin resistance · Glucose intolerance · Metabolic syndrome · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Visceral fat accumulation

10.1 Introduction

Myotonic dystrophy (DM) is a multisystemic disorder affecting the skeletal muscle with myotonia as well as the eyes, heart, and respiratory, central nervous, and endocrine system [1, 2]. The two forms of DM (types 1 and 2) with different pattern of muscle weakness are recognized. DM type 1 (DM1) is the most common muscular dystrophy in adults due to trinucleotide sequence (CTG) in the 3′-untranslated region of myotonic dystrophy protein kinase (*DMPK*) gene located at chromosome 19q 13.3 [1, 2]. DM type 2 has relatively mild and slowly progressive proximal limb muscle dominant symptoms, resulted from an unstable tetranucleotide CCTG repeat expansion in intron 1 of the nucleic acid-binding protein (*CNBP*) gene on chromosome 3q21 [1, 2]. Although recent studies on endocrine implication in DM2 are coming out, most of the endocrine system abnormalities, mainly focused on insulin resistance, refer to DM1 as yet. Insulin resistance is closely related to lipid

H. Takada
National Hospital Organization Aomori National Hospital, Aomori, Japan
e-mail: takada@aomorihosp.jp

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metabolism abnormalities, in addition to metabolic syndrome and nonalcoholic fatty liver disease. In this chapter, we therefore present what is known of dyslipidemia being relevant to metabolic syndrome and fatty liver, chiefly in DM1.

10.2 Dyslipidemia

Lipids are fats that are either absorbed from food or synthesized by the liver. Triglycerides (TGs) and cholesterol contribute most to disease, although all lipids are physiologically important. The primary function of TG is to store energy in adipocytes and muscle cells. Cholesterol is a ubiquitous constituent of cell membranes, steroids, bile acids, and signaling molecules.

Dyslipidemia is elevation of plasma cholesterol, TGs, or both, or a low high-density lipoprotein (HDL) level that contributes to the development of atherosclerosis.

Pathophysiology of dyslipidemia in DM1 has not been identified. We carried out an oral fat-loading test using 25 g of butter as 14 patients with DM1 objects [3]. Eight patients were nondiabetic, and six were diagnosed with diabetes mellitus. Serum free fatty acid (FFA) at 180 or 240 min after the loading in DM1 was significantly lower than in age- and body mass index-matched controls of nondiabetic volunteers (Fig. 10.1). There was no significant difference for serum TGs, total cholesterol, LDL cholesterol, and apolipoprotein B48 during the fat-loading between DM1 patients and controls. In comparison between nondiabetic and diabetic DM1, FFA at all point during the examination and Apo-B before the loading in nondiabetic patients was significantly lower than in diabetic patients. Based on these results, we concluded that DM1 had no specific postprandial hyperlipidemia, and the lower value of FFA in DM might reflect effects of insulin resistance due to hyperinsulinemia. Multiple factors are complicatedly entangled each other in human lipid metabolism. One of mutual keywords in DM1 is possibly insulin resistance.

Lipid metabolism abnormalities in past clinical researches are summarized in Table 10.1. Clinically, hypertriglyceridemia and low HDL cholesterolemia are often observed in patients with DM1. Hudson et al. reported the elevated fasting plasma insulin, TGs, and very low-density lipoproteins (VLDL) in spite of normal fasting plasma glucose, total cholesterol, or low- and high-density lipoproteins and suggested the role of insulin resistance affecting lipid metabolism in 1987 [4]. Moorjani et al. described similarly increased level of plasma TGs and VLDL but lower level of plasma low-density lipoprotein (LDL) cholesterol in relation to apolipoprotein E phenotypes of upregulation of LDL receptors on larger number of patients with DM1 [5]. Recently, a study for metabolic syndrome related to dyslipidemia in DM1 have been reported; hypertriglyceridemia was observed in 67% of patients and low HDL cholesterol was in 35% [8]. In our series of 91 patients with DM1, 42% of patients had hypertriglyceridemia, 15% had low HDL cholesterol, and 21% showed high LDL cholesterol [10] (Table 10.2).

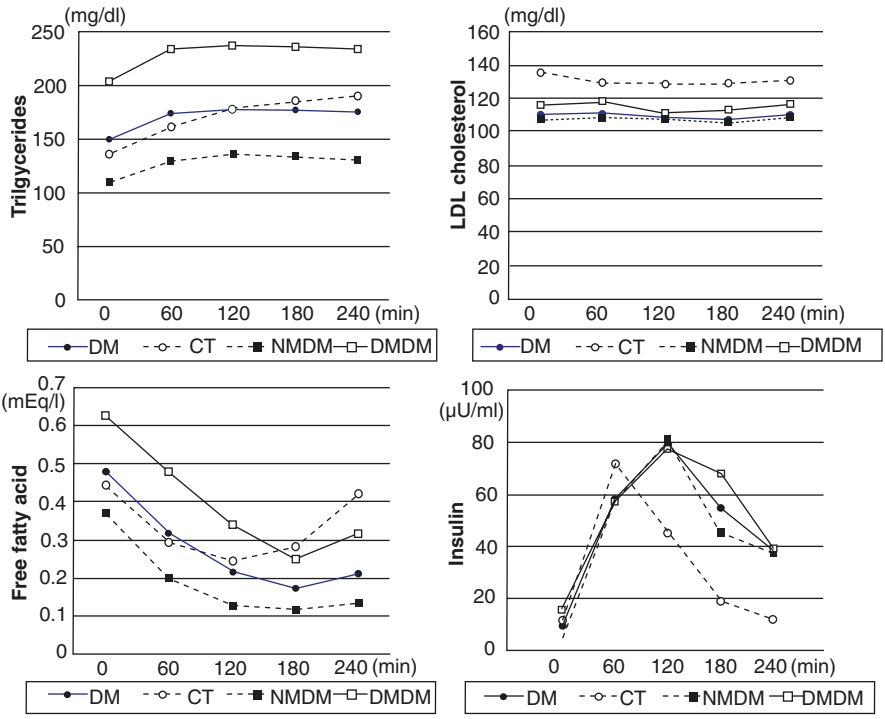

Fig. 10.1 Results of an oral fat-loading test in 14 patients with myotonic dystrophy type 1 (DM1). The abscissa of the graph is the time axis, and the ordinate represents the measurement value: upper left, triglycerides; upper right, low-density lipoprotein; lower left, free fatty acid; lower right, immunoreactive insulin. Thick solid lines indicate whole DM1 patients myotonic dystorphy (DM), and thick dotted lines are age- and body mass index-matched controls of nondiabetic volunteers control (CT). Thin dotted lines show nondiabetic patients with DM1 non-diabetic myotonic dystrophy (NMDM), and thin solid lines are diabetic DM1 patients. Serum free fatty acid at 180 or 240 min after the loading in DM1 was significantly lower than in controls. It seems to reflect the effects of insulin resistance due to long-lasting hyperinsulinemia in DM1

Table 10.1 Clinical lipid test abnormality in myotonic dystrophy type 1

Authors	Abnormal findings	Number of cases
Arthur J [4]	TGs↑, VLDL-C↑,LDL-C→	10
Moorjani S [5]	TGs↑, VLDL-C↑,LDL-C↓	70
Rakocevic Stojanovic [6]	Leptin↑, HOMA-IR↑, BMI↑	34
Daniel A [7]	Adiponectin↓, HMW oligomers↓, TGs↑, BMI↑	21
Vujnic M [8]	TGs↑(67%), HDL-C↓(35%)	66
Takada H [9]	TGs↑(43%), HDL-C↓(15%), LDL↑ (21%)	80

TGs triglycerides, *VLDL* very low-density lipoprotein, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *HOMA-IR* Homeostasis model assessment-insulin resistance, *BMI* body mass index, *HMW* high molecular weight

Table 10.2 Dyslipidemia and liver damage in 91 patients with myotonic dystrophy type 1

	Abnormality	Median value	Normal value
Vfat	48 cases (53%)	106.3	<100 cm^2
LSR	13 cases (14%)	1.26	≧1.0
AST	30 cases (33%)	30	13–33 IU/L
ALT	35 cases (39%)	Female 26, male 32	Female 6–27, male 8–42 IU/L
gamma-GTP	70 cases (77%)	88	10–47 IU/L
TGs	38 cases (42%)	129	30–149 mg/dL
LDL cho	19 cases (21%)	117	70–139 mg/dL
HDL cho	14 cases (15%)	51	40–96 mg/dL

Vfat visceral fat, *LSR* liver-spleen ratio, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* glutamyl transpeptidase, *TGs* triglycerides, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein

Studies for endocrine hormone that influence lipid metabolism in DM1 have been reported. Rakocevic Stojanovic et al. pointed out elevated serum leptin and insulin concentrations and increased body mass index [6]. They concluded the leptin overproduction correlated with insulin resistance in DM1. Leptin is a mediator of long-term regulation of energy balance, suppressing food intake and thereby inducing weight loss [11]. Although mechanism is still obscure, leptin may contribute to the development of central obesity. Daniele et al. indicated decrease in concentration of adiponectin and high molecular weight oligomers and high TGs, glucose, body mass index, and waist circumference [7]. They suspected that decreased expression of adiponectin contributed to the worsening of insulin resistance and metabolic complications in DM1. Adiponectin has been postulated to play an important role in the modulation of glucose and lipid metabolism in insulin-sensitive tissues. Low adiponectin levels have also been strongly implicated in the development of insulin resistance in mouse models of both obesity and lipoatrophy [12]. It is also reported that tumor necrosis factor (TNF) system activity is associated with insulin resistance and dyslipidemia in DM1; plasma cholesterol and TG concentrations were significantly increased, and plasma soluble TNF receptor 1 and 2 levels were strongly associated with plasma cholesterol in DM1 [13]. TNF is a multifunctional cytokine with effects on lipid metabolism, coagulation, insulin resistance, and the function of endothelial cells lining blood vessels. Moreover, vitamin D deficiency, which played a critical role in the pathophysiology of risk factors of insulin resistance and obesity [14], in DM1 was reported [15, 16]. Human fibroblast growth factor 21 (FGF21) is a regulator of lipid and glucose metabolism and a sensitive marker for mitochondrial diseases. Lovadi et al. revealed that serum FGF21 levels were significantly elevated in patients with DM1 and was influenced by insulin resistance [17].

Regarding DM2, hypertriglyceridemia and insulin insensitivity with increased prevalence of diabetes mellitus have been reported in DM2 [18]. Screen et al. described that hyperlipidemia was frequent in DM2 [19], but specific studies on metabolic risk factors in this form are still lacking.

10.3 Metabolic Syndrome

Abdominal visceral fat accumulation increased in patients with DM1 [20]. It is reported that visceral fat area measured by computer tomography (CT) scanning at the umbilical level was significantly greater in 19 patients with DM1 than in age- and body mass index-matched control subjects, and visceral fat area was positively correlated with plasma glucose area under the curve of 75 g oral glucose tolerance test, fasting plasma insulin, serum total cholesterol, and serum TGs. In our study of 91 cases, abnormally exceeding visceral fat accumulation on CT scanning was shown in 53%, and the area of visceral fat was positively correlated with the number of CTG repeat, serum TGs, LDL cholesterol levels, and serum gamma-glutamyl transferase level and negatively correlated with the liver-spleen ratio on CT scanning [10]. Visceral fat accumulation is one of the risk factors of metabolic syndrome and is closely connected with insulin resistance.

Metabolic syndrome is a group of metabolic and hemodynamic disturbances that appear together and multiply the risk of cardiovascular diseases, diabetes mellitus type 2, or stroke. Patient should have at least three of the following five criteria to be diagnosed with metabolic syndrome: abdominal obesity (which is highly correlated with insulin resistance); elevated TGs or the use of medication for hypertriglyceridemia; reduced HDL cholesterol; elevated blood pressure, diagnosis of hypertension, or the use of medication for hypertension; and elevated fasting glucose or the use of medication for hyperglycemia [21].

Several features of metabolic syndrome are very common in DM1, including insulin resistance, increased waist circumference, and dyslipidemia [7]. However, Vujnic et al. described that only a low percentage of DM1 patients met the criteria for metabolic syndrome, which results to be less frequent in DM1 than in the general population [8]. In this report, hypertriglyceridemia was seen in 67% of DM1 patients, low HDL cholesterol in 35%, hypertension in 18%, central obesity in 14%, and hyperglycemia in 9%, whereas metabolic syndrome was present in 17% of patients with DM1. We investigated a current state of metabolic syndrome in Japanese patients with DM1. We applied the Japanese criteria for metabolic syndrome which was made by the panel of Japanese eight scientific societies (the joint examination committee of Japanese diagnostic criteria for metabolic syndrome, 2005) because frequency of component factors is varied according to life habit or ethnic groups [22]. Obesity was assessed by area of visceral fat measured by CT scanning at the umbilical level instead of waist circumference. In our cases, hypertriglyceridemia was seen in 42% of DM1 patients, low HDL cholesterol in 15%, exceeding visceral fat accumulation in 53%, hyperglycemia in 26%, and hypertension in 9%, and as a result, 41% of patients had metabolic syndrome, which was not so rare [10] (Table 10.2). This divergence might be due to the variance of lifestyle or race or the difference of estimation method for obesity.

10.4 Hepatopathy

Achiron et al. indicated that liver test abnormality was frequent in patients with DM [23]. Sixty-six percent of patients showed abnormal results in at least one of six liver enzymes assays: alkaline phosphatase 50.9%, gamma-glutamyl transferase 52.8%, 5′ nucleotidase 43.4%, aspartate aminotransferase 35.8%, alanine aminotransferase 33.9%, and lactate dehydrogenase 37.7%. Liver function test results did not correlate with severity of muscle weakness, disease duration, or serum level of creatine kinase, glucose, or lipids.

Shieh et al. described that nonalcoholic fatty liver disease (NAFLD) was very common and should be considered in the management of DM1 [24]. They found abnormal liver chemistry tests in 44% of 36 patients with DM1, and 87% were attributable to NAFLD. Clinical predictors of NAFLD included increased insulin resistance by the homeostasis model assessment (HOMA), elevated fasting insulin, elevated TGs, and elevated total cholesterol.

Recently, DM cases complicated with nonalcoholic steatohepatitis (NASH) have been reported [25, 26]. NASH develops liver fat accumulation, inflammation, and fibrosis in patients with obesity, dyslipidemia, or glucose intolerance, possibly linked to insulin resistance, and can cause cirrhosis or cancer [27].

We also reported that NAFLD was not very rare and deeply relevant to dyslipidemia, visceral fat accumulation, and glucose intolerance [10] (Table 10.2).

Exceess visceral fat accumulation was shown in 53% of patients, elevated alanine aminotransferase in 39%, elevated alkaline phosphatase in 33%, elevated gamma-glutamyl transferase in 77%, and liver-spleen ratio less than 0.9 on CT scanning in 15%. There was significant correlation between visceral fat area and liver-spleen ratio, alanine aminotransferase, alkaline phosphatase, TGs, or LDL cholesterol (Fig. 10.2). Visceral fat area, serum alkaline phosphatase or LDL cholesterol showed a tendency to increase following as development of glucose intolerance (Fig. 10.3). The number of CTG repeats showed no significant difference whether in the presence of excess visceral fat accumulation or decreased liver-spleen ratio on CT examination. CT findings of our NAFLD case are shown in Fig. 10.4. NAFLD or NASH as well as atherosclerotic disease should be kept in mind in also patients with DM1, especially complicated with dyslipidemia or glucose intolerance.

10.5 Treatment

It has not been demonstrated that atherosclerotic disease such as ischemic heart disease or stroke is frequently complicated in patients with DM1. However, steps against well-documented risk factors for metabolic syndrome are important as a practical procedure today even for days when DM1 patients will take new lease of life due to prospective advanced therapeutics. Metabolic tests should be performed periodically, and treatment should be started promptly in case of DM1 coplicated with dyslipidemia.

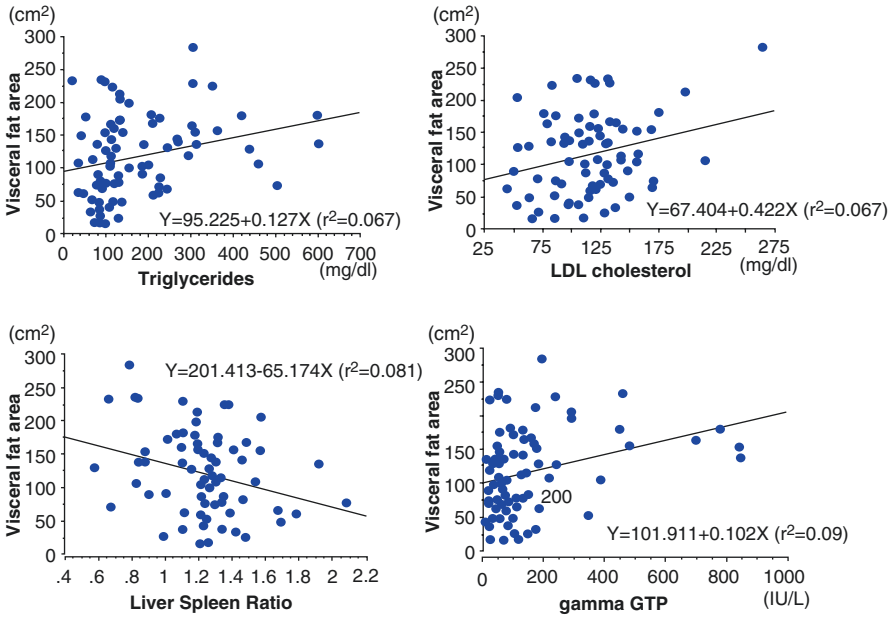

Fig. 10.2 Relation between visceral fat accumulation and triglycerides, low-density lipoprotein cholesterol, liver-spleen ratio on CT scanning, or gamma-glutamyl transpeptidase in 91 patients with myotonic dystrophy type 1 (DM1). Visceral fat area on CT scanning was significantly correlated with triglycerides (upper right), low-density lipoprotein cholesterol (upper right), liver-spleen ratio (lower left), or gamma-glutamyl transpeptidase (lower right) in DM1

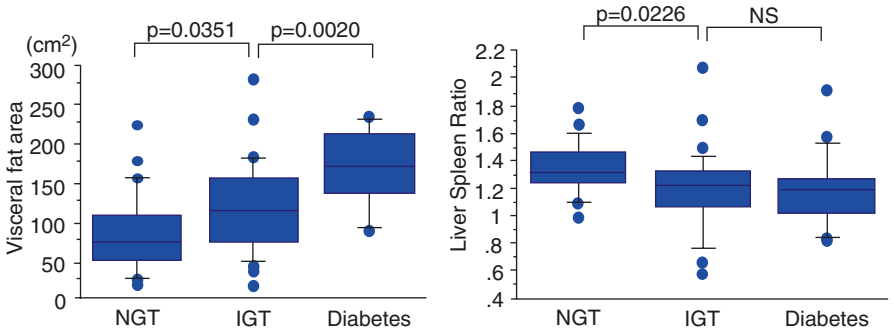

Fig. 10.3 Comparison of visceral fat area and liver-spleen ratio on CT scanning according to the degree of glucose intolerance in 91 patients with myotonic dystrophy type 1 (DM1). DM1 patients are classified into three groups by oral glucose tolerance test: normal (NGT), border line glucose intolerance (IGT), and diabetes mellitus. Visceral fat area in diabetes group was significantly greater than that in IGT group or NGT group. Liver-spleen ratio in diabetes group or IGT group was significantly lower than that in IGT group

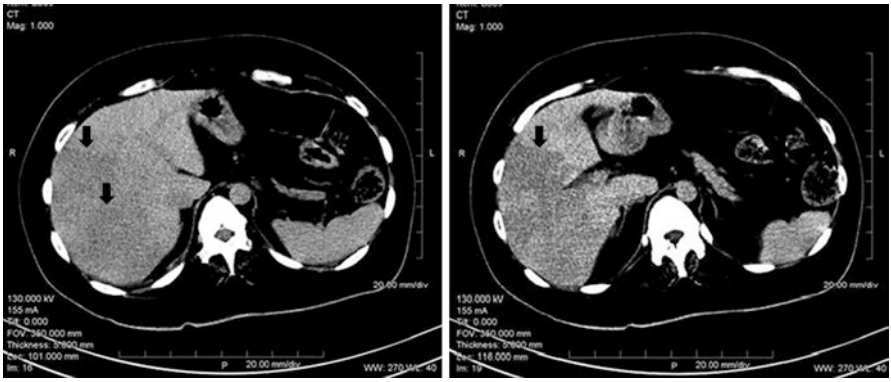

Fig. 10.4 A patient with myotonic dystrophy type 1 with nonalcoholic fatty liver disease is shown. The case is a 39-year-old female with diabetes mellitus. The number of CTG trinucleotide repeats is 2900. Visceral fat area on CT scanning is 210.1 cm^2, and liver-spleen ratio on CT is 0.841. Blood examination: aspartate aminotransferase; 102 IU/L, alanine aminotransferase; 141 IU/L, gamma-glutamyl transpeptidase; 707 IU/L, low-density lipoprotein cholesterol; 138 mg/dL, triglycerides; 125 mg/dL. Spotted low-density areas indicating exceeding fat accumulation are seen here and there

So far, a specific guideline for treatment against dyslipidemia in DM1 has not been reported. Therefore, medication for dyslipidemia in DM1 should be carried out in accordance with representative guidelines made by respective countries or areas because significant racial/ethnic differences in dyslipidemia prevalence, dyslipidemia-related mortality rates, and response to lipid-lowering agents, including lifestyle modifications, have been reported [22]. The principal strategies of medication for dyslipidemia are lifestyle modifications including dietary change or exercise, but drug treatments are required in some cases: statins, sometimes bile acid sequestrates, ezetimibe, or niacin for high LDL cholesterol and niacin, fibrates, or omega-3 fatty acids for high TGs [28]. However, adverse effects of muscle pain and rhabdomyolysis as a risk for myopathy associated with statin have been reported [29, 30]. Although Gupta reported nocebo effect for an excess rate of muscle-related adverse events in patients with hypertension [31], it is still uncertain whether the same results are produced in patients with muscular disorders. Cautious observation and handling should be taken for statin therapy.

10.6 Conclusion

Lipid metabolism abnormality is frequently seen in patients with DM. Dyslipidemia is deeply relevant to insulin resistance and is one of the risk factors for metabolic syndrome or nonalcoholic fatty liver disease. Periodical lipid test and prompt treatment for dyslipidemia are recommended in patient with DM, especially complicated with glucose intolerance.

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Chapter 11
Dysphagia in Myotonic Dystrophy

Sonoko Nozaki

Abstract In myotonic dystrophy type 1, all stages in the five swallowing model are disturbed.

Deglutition-related muscle weakness, as opposed to myotonia, was the most significant contributor to impairment.

In anticipatory stage, some patients show poor awareness of dysphagia. Aberrations of feeding behaviors and silent aspiration have been reported.

In oral preparatory and propulsive stage, force of masticatory and labia oris is degraded, but patients sometimes swallow foods without adequate chewing. Myotonic phenomena are seen in the circumferential musculature of the buccal capsule.

In pharyngeal stage, decreased pharyngeal peristalsis, delayed deglutition reflex, and epiglottis dysraphic are observed. The threshold of the cough reflex is high, and this is related to silent aspiration.

In esophageal stage, degradation of esophagus peristalsis and gastroesophageal reflux are present.

Myotonic phenomena reduce with hyperthermia treatment on the masseter muscle, and that induces easier mouth opening. A warm-up phenomenon is seen with repeated opening and closing of the mouth.

Dilatation of upper esophageal sphincter using balloon catheter is effective.

When drop in SpO_2 is revealed during meals due to respiratory disturbance, ventilation is desirable while eating.

During percutaneous endoscopic gastrostomy, we need to keep watching for aggravation of respiratory insufficiency.

Keywords Myotonic dystrophy · Dysphagia · Myotonia · Silent aspiration · Feeding behavior · Hyperthermia treatment · Respiratory failure

S. Nozaki
Department of Neurology and Rehabilitation, Kansai Rosai Hospital, Amagasaki, Hyogo, Japan
e-mail: nozaki-sonoko@kansaih.jophas.go.jp

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11.1 Introduction

DM (myotonic dystrophy) is an autosomal dominant hereditary muscle disease characterized by muscle atrophy/myotonia/multiple organ failure. There are two types of myotonic dystrophy, type 1 (DM1) and type 2 (DM2). With a prevalence of 5–6 per 100,000 people, DM1 is the most frequent adult hereditary myopathy. This pathology involves CTG triplet repeat elongation in the myotonin-protein kinase (*DMPK*) gene located on chromosome 19q13. DM2, a proximal-related muscle ankylosis myopathy, involves a CCGT repeated sequence elongation in the zinc finger protein 9 (*ZNF9*, (*CNBP*)) gene located on chromosome 3q13.

For the details of the general disease profiles of DM1 and DM2, you can refer to Chap. 1.

According to a 2015 investigation by the Japanese National Hospital Organization in 2015, the most common cause of death associated with DM was respiratory insufficiency (30%), followed by cardiac insufficiency (10%), and respiratory tract infection (10%) (Fig. 11.1). The most frequent respiratory tract infection is aspiration pneumonia, and we consider dysphagia and aspiration as strongly related to the cause of death in DM1 [1, 2].

Eating involves several related functions: food affiliation, feeding behavior, swallowing function, and respiratory coordination. The neurological mechanisms of deglutition involve sensory inputs from the buccal capsule/pharynx/larynx/esophagus and motor outputs from the deglutition reflex of the swallowing center. The deglutition center is located in the *nucleus solitarius* of the medulla oblongata and induces the deglutition reflex but is under the control of the cerebral cortex. The muscles of deglutition contributed to breathing and chewing, and the deglutition center/respiratory center/chewing center/coughing center rules over these muscles during deglutition (Fig. 11.2). Processing differs between liquid deglutition and

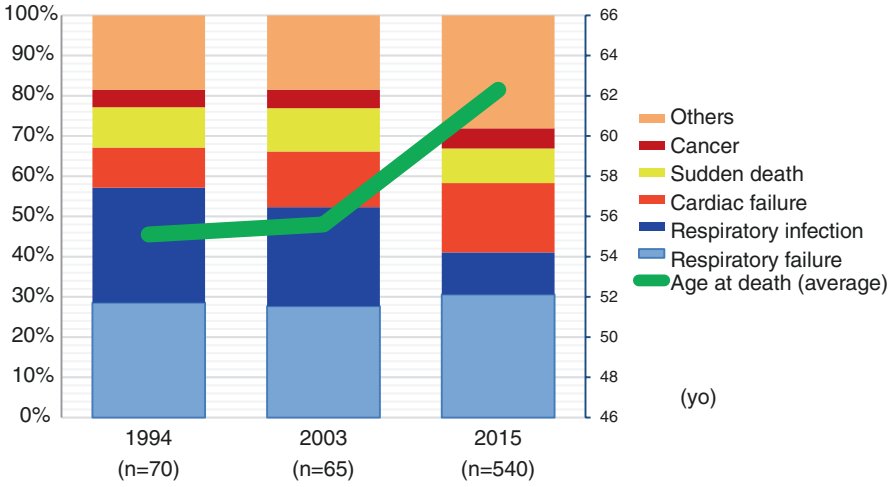


Fig. 11.1 Causes of death of DM1 according to the National Hospital Organization data

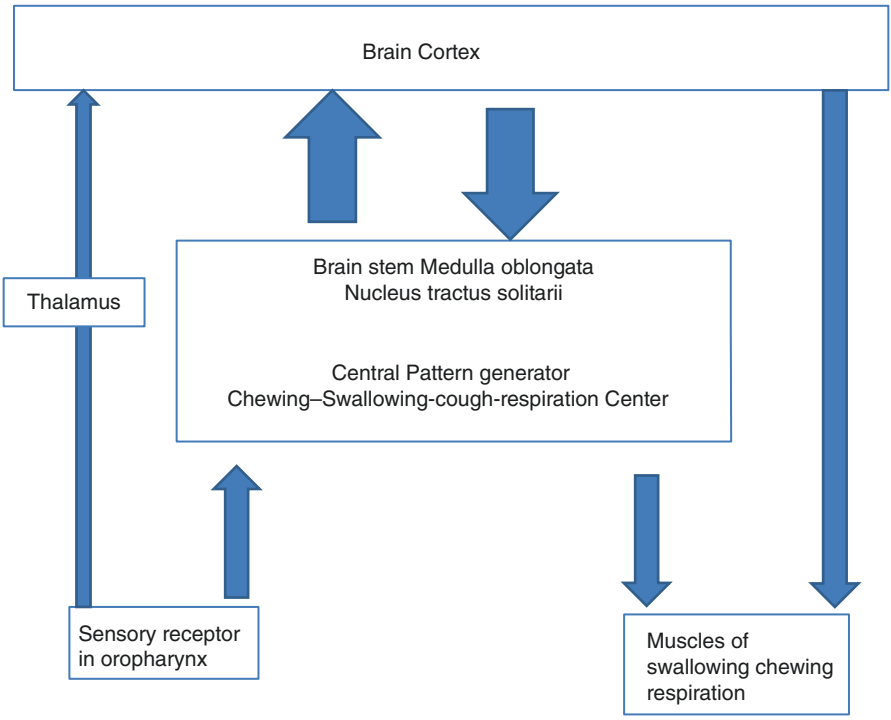


Fig. 11.2 Neurological network of swallowing

solid chewing deglutition, and models such as a four-stage model and a processing model [3] have been used to explain each process. However, we tend to drink liquids and eat solids together in daily meals, so a five-stage model (anticipatory stage, oral preparatory stage, oral propulsive stage, pharyngeal stage, and esophageal stage) has recently been used to explain disease states [4].

In DM1, all of these stages are disturbed to various degrees. In this chapter, I am going to use the five-stage model to explain the dysphagia of DM1. When evaluating dysphagia, we reach a diagnosis by completing a questionnaire and performing a screening test [5], followed by video fluorography (VF) [6] and video endoscopy (VE) [7]. In addition, cough testing with citric acid [8], cervical auscultation [9], occlusal force testing, the use of a tongue pressuremeter [10], and manometry [11] are useful.

11.2 Dysphagia in DM1

11.2.1 Characteristics of Dysphagia

In the five-stage swallowing model, disturbances are present at all stages in DM1: anticipatory stage, oral preparatory stage, oral propulsive stage, pharyngeal stage, and esophageal stage (Fig. 11.3). In DM1, the number of CTG triplet repeats is

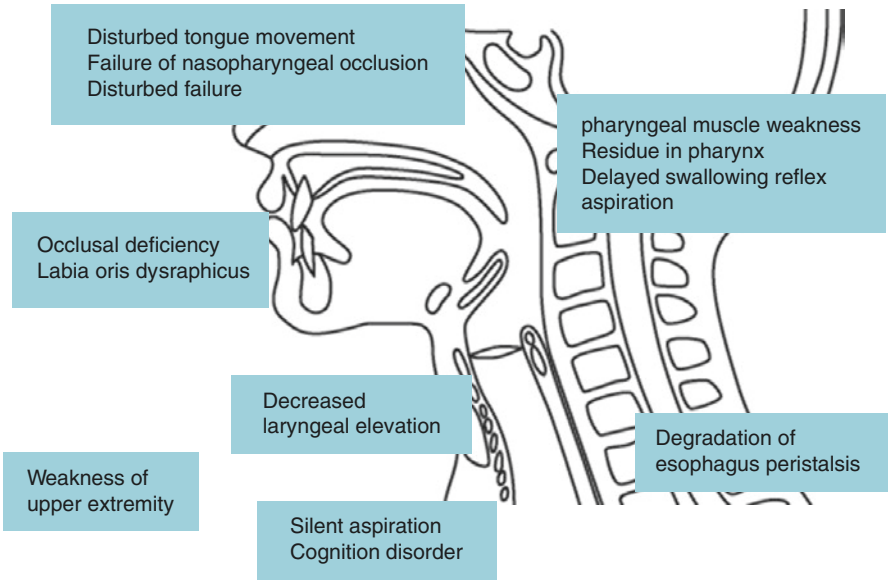


Fig. 11.3 Profile of dysphagia in DM1

associated with the degree of seriousness in video fluoroscopic findings [12]. Disease duration is also related to the severity of dysphagia [13]. Generally, several reports have indicated that deglutition-related muscle weakness, as opposed to myotonia, was the most significant contributor to impairment associated with DM1 [14, 15] .

Some reports have described sex differences in dysphagia, with greater severity in females than in males [16]. However, other papers have reported the opposite, with males affected more than females [17].

I will describe disturbances in each of the processes of deglutition, as follows.

11.2.1.1 Anticipatory Stage Affiliation Period

Some patients often show poor awareness of dysphagia [18, 19]. Aberrations of feeding behaviors such as packing food into the mouth and silent aspiration have been reported. It is thought that there is a patient ingesting with repeated aspiration. However, in DM1, discovery of dysphagia based on subjective symptoms and screening tests is difficult.

11.2.1.2 Oral Preparatory Stage and Oral Propulsive Stage [20]

Malocclusion is present in 35% of DM1, and with apertognathia, in which a front tooth and bicuspid tooth are not engaged [21] (Fig. 11.4), the occlusal force is around one-tenth that of a healthy person [22]. Due to abnormal occlusion and temporal/masseter muscle weakness and atrophy [21, 22] (Fig. 11.5), masticatory force is degraded [20, 23], but patients often have little consciousness of bite force weakness or muscular atrophy and sometimes place foods in their mouth in sequence without completing adequate chewing. That action results in suffocation [20]. With masseter muscle weakens, chewing efficiency decreases [24, 25]. The masticatory function of patients with DM1 worsened as activity of daily life (ADL) reduced [26].

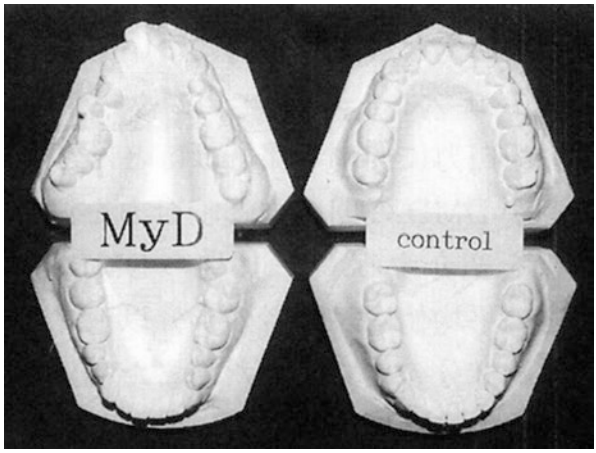


Fig. 11.4 Dental arch in DM1 ref. [21]. Diameter of the upper dental arch in DM1 was significantly decreased compared with that in control

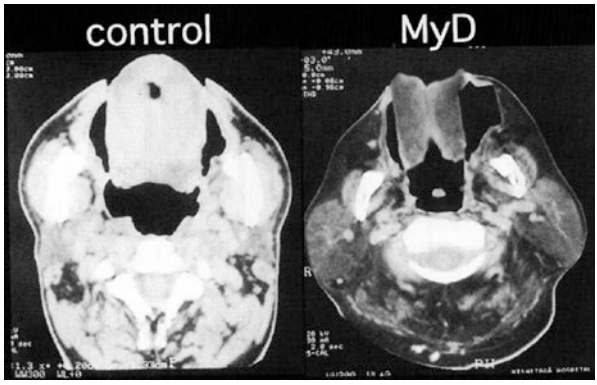


Fig. 11.5 CT of deglutition-related muscles (control and DM1) ref. [22]. Muscle atrophy in the tongue, masseter, and medial pterygoid muscle in DM1 was more severe compared with control muscle

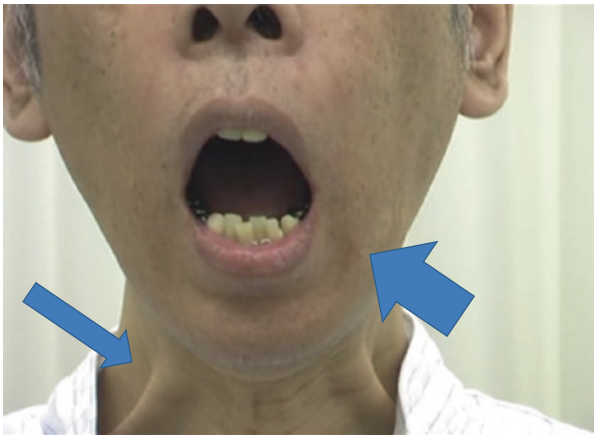


Fig. 11.6 Myotonia during mouth opening. Myotonia is apparent in the sternocleidomastoid and perioral muscle

In addition, myotonic phenomena are seen in the circumferential musculature of the buccal capsule, along with muscle weakness of the *labia oris* (Fig. 11.6).

Velopharyngeal insufficiency, time lag of soft palate elevation, and a disorder of food transport to the pharynx are also evident [27].

11.2.1.3 Pharyngeal Stage

Pharyngeal muscle weakness results in decreased strength in pharyngeal peristalsis epiglottis dysraphicus a delayed deglutition reflex [17], residue in pharynx and aspiration result [27, 28] (Fig. 11.7). Because there is a large amount of subclinical aspiration without awareness (silent aspiration), the risk of liquid aspiration is high. Clinical severity of dysphagia is related to the muscular impairment rating scale (MIRS) in DM1 [29]. Pharyngeal muscle power is weak, and the cough reflex is decreased, so participation of muscle weakness is bigger than myotonic in the pharyngeal stage [14]. The threshold value of the cough reflex is high under the cough provocation test by citric acid inhalation, and this is related to silent aspiration [30].

Compared with healthy individuals, bolus transit time is longer, the beginning of hyoid movement is delayed, and the late initiation of dilatation of the upper esophageal sphincter (UES) is observed by manometory [27].

11.2.1.4 Esophageal Stage

In pathological examination, esophageal smooth muscle lesions and striated muscle lesions are found to the same degree. Decreased static pressure of the UES and degradation of esophagus peristalsis and gastroesophageal reflux are also present

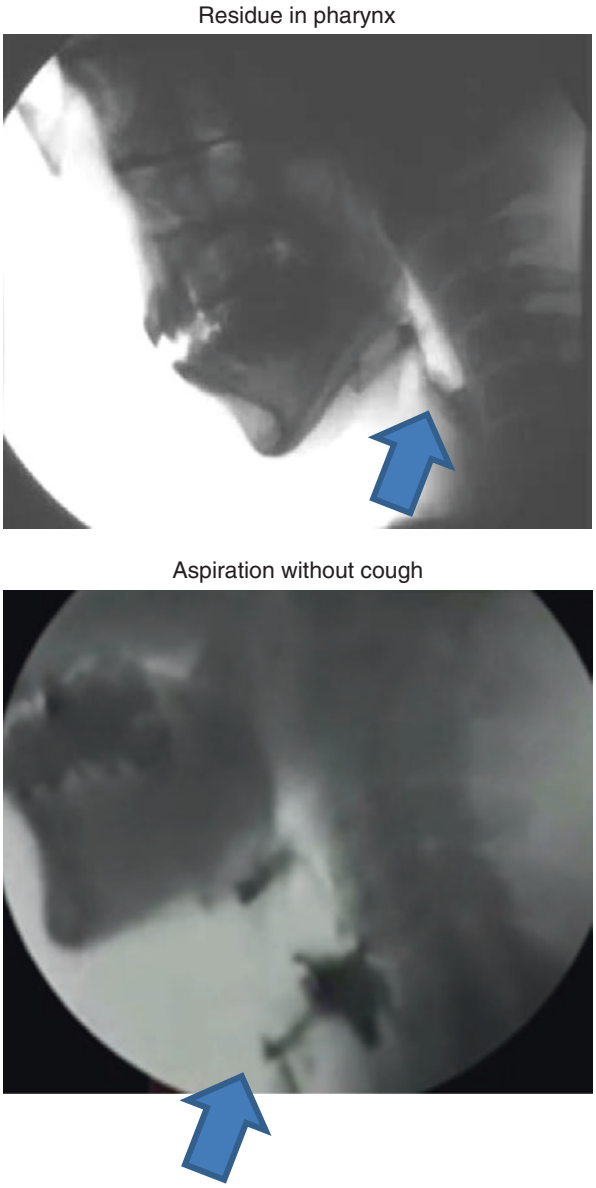


Fig. 11.7 VF findings in pharyngeal stage in DM1. Residue in the pyriform sinus and aspiration without cough

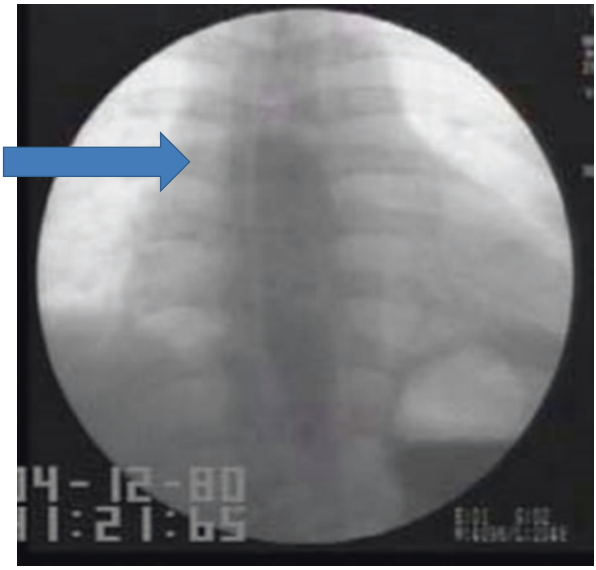


Fig. 11.8 VF findings in the esophageal stage in DM1. Degradation of esophagus peristalsis

[31]. VF evidence shows lumen expansion of the superior esophagus, and residue is frequently present in the esophagus (Fig. 11.8). As a whole, food transit time from the oral cavity to the esophagus is prolonged.

11.2.1.5 Eating Disorder: Disorder of Feeding Behavior

A myotonic phenomenon impairs the use of tableware, and the neck falls down due to cervical muscle weakness. This myotonic phenomenon is worsened by lowered temperature [32].

11.2.1.6 Respiratory Disturbance

Restrictive respiratory disturbance during meal results from respiratory muscle weakness and dysfunctional respiratory-swallowing control by the central nervous system (Fig. 11.9). Due to respiratory muscle weakness and disorder of the respiratory center, respiratory insufficiency induces pneumonia via aspiration. Some patients show SpO_2 degradation during meals. In such cases, such reductions may be due to either aspiration or deficiencies in respiratory-swallowing muscle cooperation.

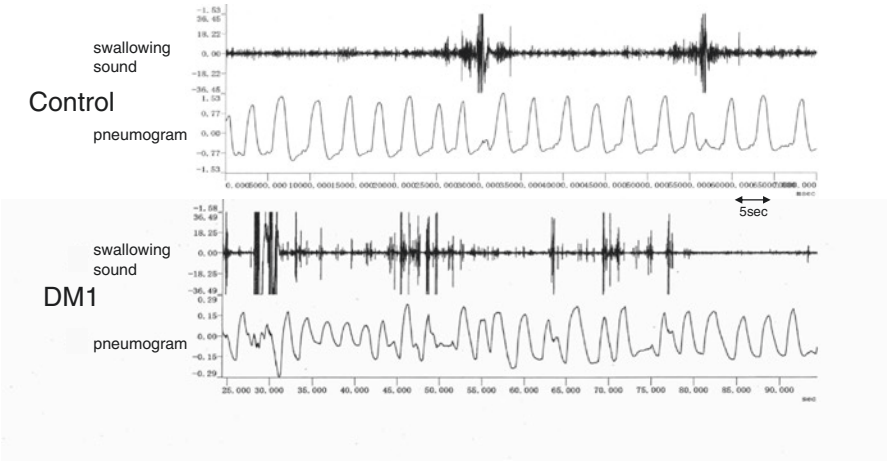


Fig. 11.9 Disturbed respiratory-swallowing coordination

11.2.2 Management of Eating Dysphagia [33]

11.2.2.1 Anticipatory Stage

DM patients show poor awareness of dysphagia, and the risk of aspiration in self-feeding patients is considerably high. As a result, patients need to be watched and managed during eating. Caregivers often did not consider this as a problematic symptom. They instead highlighted more debilitating symptoms like fatigue or weakness, but sufficient observation is required to allow suitable aspiration risk management [19]. Family guidance is also important. Regardless of subjective symptoms, periodical follow-up is needed.

11.2.2.2 Oral Preparatory Stage and Oral Propulsive Stage

One report described a corrective oral surgery was effective for occlusal deficiency/abnormal occlusion.

In DM1 patients, myotonic phenomena were shown to be reduced with hyperthermia treatment (Fig. 11.10) and worsened under cold conditions [33, 34]. In our study, we applied perioral exercise with a hot pack to the DM1 patients and achieved amelioration of excursion [35] (Figs. 11.11 and 11.12). Hyperthermia treatment such as a hot pack on the masseter muscle appears effective and induces easier

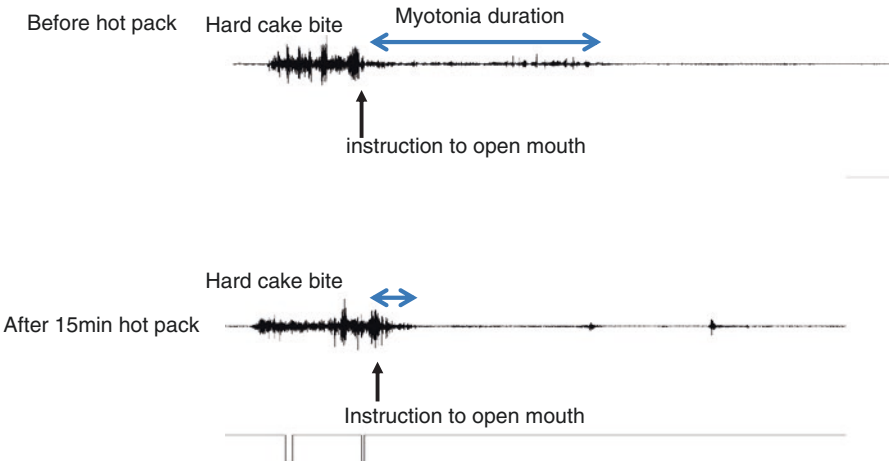


Fig. 11.10 Myotonic discharge in masseter muscle with hot pack. Myotonic discharge was shortened after 15 min of using a hot pack just after biting into a hard cake

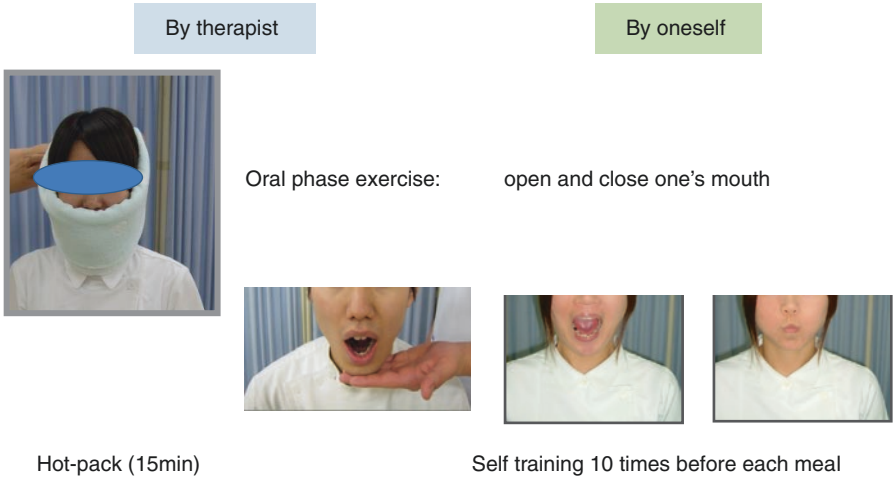


Fig. 11.11 Perioral exercise for the mouth with hot pack

opening of the mouth, as in a study of Duchenne muscular dystrophy [36]. In addition, a warm-up phenomenon [32] (Fig. 11.13) is seen in which a buccal capsule circumferential muscular myotonia phenomenon is reduced with repeated opening and closing of the mouth [34]. We think that mild preprandial buccal circumference exercises are effective. Lip strengthening is also effective [35, 37].

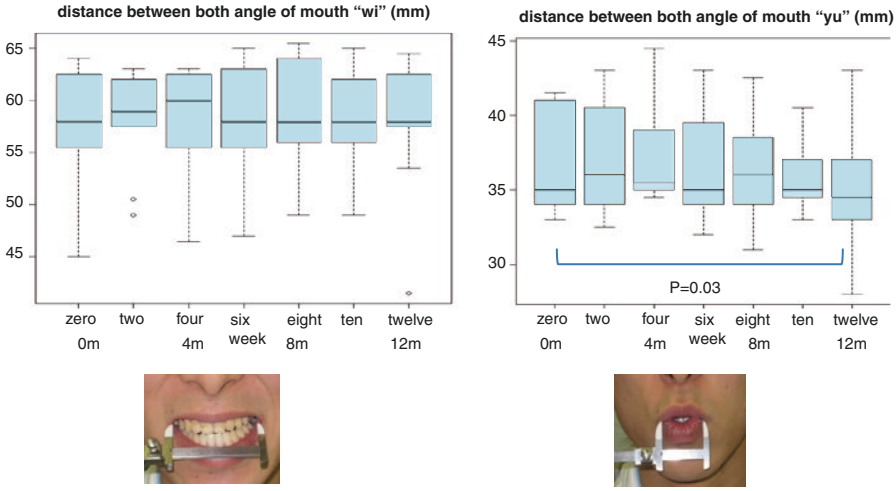


Fig. 11.12 Distance between angle of the mouth (mm) pronouncing "e" [maximum]/"u" [minimum], after perioral exercise with hot pack. Distance in "u" was significantly shortened after 6 months of perioral exercise with hot pack (ref. [35])

Fig. 11.13 Warm-up phenomena ref. [32]. Myotonic discharge was shortened by repeated percussion

11.2.2.3 Pharyngeal Stage

Dilatation of the diameter of UES using balloon catheter [9] is effective for UES dysfunction [38] (Fig. 11.14, Table 11.1). By catheter drawing method, we air the balloon and expanded in small quantities after inserting the tip of the balloon catheter in the esophagus and just pull up it to oral cavity.

11.2.2.4 Esophageal Stage

In primary care of gastroesophageal reflux disease, proton pump inhibitor improves the symptoms and heals esophagus mucous membrane disorder. Esophageal myotomy and cardioplasty have been reported for esophageal achalasia in DM1 [39].

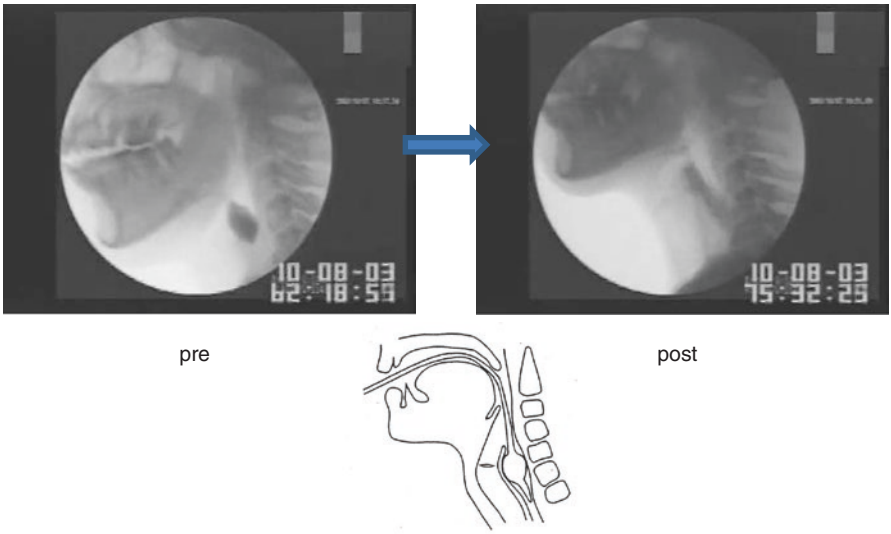


Fig. 11.14 Effect of balloon dilatation therapy ref. [38]. Patients underwent dilation by filling a 12- to 14-F Foley balloon catheter with 2 mL of air. After balloon dilatation therapy, diameter of UES opening was elongated

Table 11.1 Effect of balloon dilatation therapy in DM1 ref. [38]

	Maximum diameter of UES post/ pre-balloon dilatation therapy (mm)	Pharyngeal transit time	
		Pre(s)	Post(s)
40 y.o.	1.2	1.48	0.8
57 y.o.	1.5	6.66	2.12

Diameter of the UES was dilated and pharyngeal transit time was shortened just after balloon dilation

11.2.2.5 Physical Exercises

Exercises are effective to prevent disuse symptoms in swallowing muscle maintenance. Interventions to prevent neck descent and maintain positioning help with eating behavior.

11.2.2.6 Respiratory Insufficiency

When drop in SpO_2 is revealed during meals, even in the early stage of respiratory insufficiency, a noninvasive ventilation (NIV) is desirable while eating. Tracheal cannulation with tracheostomy disturbs laryngeal elevation, and the patient cannot cough material. As a result, aspiration sometimes becomes more apparent after tracheostomy. As for care after tracheostomy, as well as from the tracheostomy

aperture, we need suction of secretions from the buccal capsule and pharynx to prevent aspiration pneumonitis.

11.2.2.7 Percutaneous Endoscopic Gastrostomy (PEG)

During PEG construction, we need salivary aspiration at the time of construction and keep watching for aggravation of respiratory insufficiency, under respiratory monitoring. Under NIV, PEG construction under open gastrostomy was reported in patients with neuromuscular disease in respiratory failure [40].

Dysphagia in DM1 was reported to significantly impact adherence to pharmacotherapies [41]. Evidence for the management of dysphagia in DM1 remains insufficient. However, as exemplified in the following case, we experience improvements in swallowing function and reductions in risk with various interventions, even for progressive disease.

Case

The patient was a male in his 20s. Because of serious pneumonia despite his young age, he was introduced to a neurologist for differential diagnosis of neuromuscular disease. Myotonic dystrophy was diagnosed. The patient had been aware of grip myotonia and dysphagia since 15 years old. In VF, residue in the pharynx and little penetration were found (Fig. 11.15).

We started deglutition rehabilitation. The intervention was the use of a hot pack and stretching of the masseter and cervical muscles. We instructed the patient in hot pack and excursion training before meals at home (Figs. 11.16 and 11.17). Since rehabilitation, the patient has not developed any pneumonia or dysphagia. Residue in the pharynx was decreased according to VF after 6 months (Fig. 11.15).

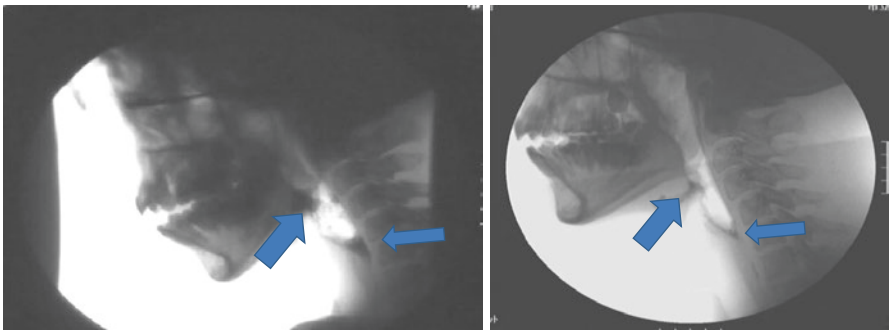
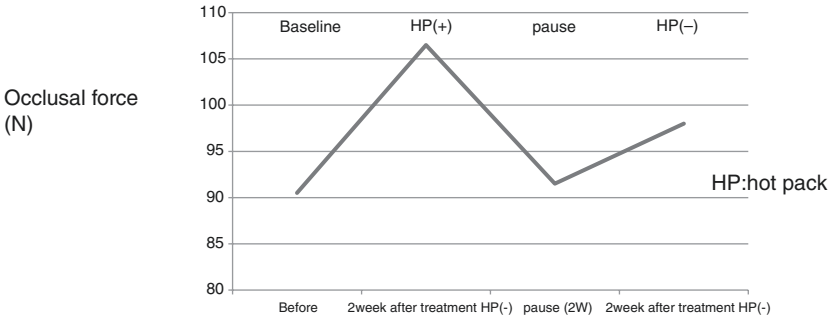


Fig. 11.15 Case report: effect of treatment with hot pack (VF). Residue in pharynx decreased after hot pack treatment



Distance between angle of mouth (mm)	Baseline	2W after treatment HP(+)	pause (2W)	2W after treatment HP(–)
“e” [maximum] (mm)	5.65	6.1	5.6	5.95
"u” [minimum] (mm)	3.6	3.35	3.65	3.45
mouth opening (mm)	4.875	5.25	5.1	5.2

Fig. 11.16 Case report: effect of treatment with hot pack. Occlusal force was increased after treatment, but during pause, occlusal force and mouth movement decreased

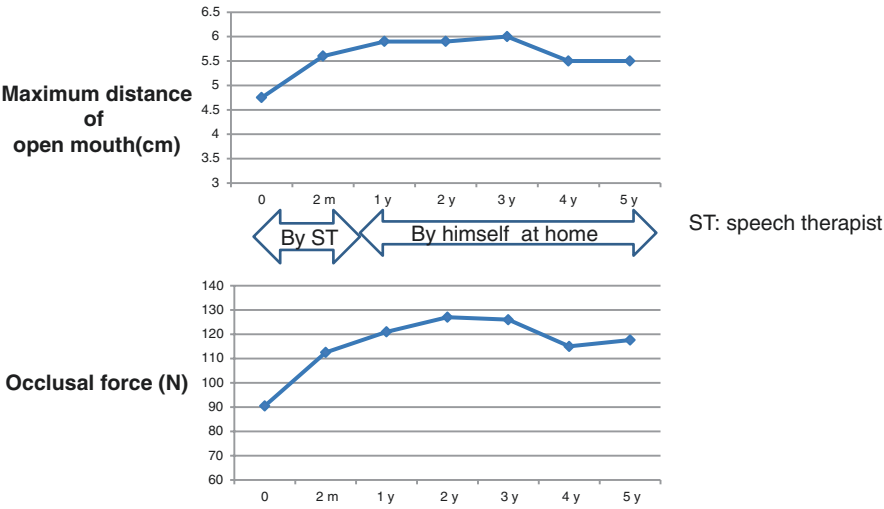


Fig. 11.17 Case report: effect of treatment with hot pack. Maximum distance of mouth opening and occlusal force was increased after 6 months of treatment

Table 11.2 Profile of deglutition-related muscles in DM1/DM2 ref. [45]

	DM1	DM2
Disturbance in muscle		
Muscle weakness	Distal>proxmaldist	Proximal>distal
Facial muscle	+++	+
Ptosis	+++	+
Temporal muscle	+++	+
Sternocleidomastoid	+++	++
Cervical muscle	++	++
Finger muscle	+++	+
Iliopsoas muscle	+	+++
Myotonia	Distal	Proximal>distal
Myalgia	+	++
Pseudohypertrophy	−	+
Myotonia	+++	++
Increased CK	++	++

11.3 Dysphagia in DM2

Little is known about swallowing function in myotonic dystrophy type 2 (DM2) [42]. One report described a frequency of 52% for dysphagia in DM2 [43]. In DM2, dysphagia appears for liquids in 38% and for solid foods in 41% [43]. DM2 dysphagia is more severe in older patients. However, dysphagia is generally mild, and does not lead to weight loss or aspiration pneumonia [44].Other deglutition-related muscles (facial and cervical muscles) show weakness milder than that in DM1 [45] (Table 11.2). Further clinical research into DM is thus needed.

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Chapter 12
Disease Modeling and Drug Development with DM1 Patient-Derived iPS Cells

Toshiyuki Araki, Masayoshi Kamon, and Hidetoshi Sakurai

Abstract Since generation of induced pluripotent stem cells (iPSCs) was first reported in human in 2007, application of the technology to generate iPSCs has been applied to basic research on pathogenic mechanism of human diseases as well as development of regeneration therapy. For the former application, iPSCs generated from cell/tissue samples obtained from patients, particularly of inherited disorders, have been used for modeling diseases in cellular levels and drug screening by using the disease model developed with iPSCs generated from patient samples. Among a number of genetically inherited disorders, type 1 myotonic dystrophy (DM1) is well suitable for disease modeling studies using iPSCs derived from patients' cells/tissues. In this chapter, previous research applications of iPSCs generated from DM1 patients' cells/tissues are reviewed, and potentials of DM1 patient-derived iPSCs as a powerful tool for DM1 pathogenesis research and drug development against DM1 are discussed.

Keywords Induced pluripotent stem cells (iPSCs) · Trinucleotide repeat · RNA toxicity · Repeat instability · Dystrophia myotonica protein kinase (DMPK) · Mismatch repair · Muscleblind-like 1 (MBNL1)

12.1 Introduction

Generation of induced pluripotent stem cells (iPSCs) was first established in rodents [1] and subsequently extended in human in 2007 by the teams of Yamanaka using a combination of transcription factors, Oct4, Sox2, Klf4, and Myc, which are relevant for immaturity and proliferative phenotypes characteristic for pluripotent

M. Kamon · T. Araki (✉)
Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan
e-mail: taraki@ncnp.go.jp

H. Sakurai
Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan

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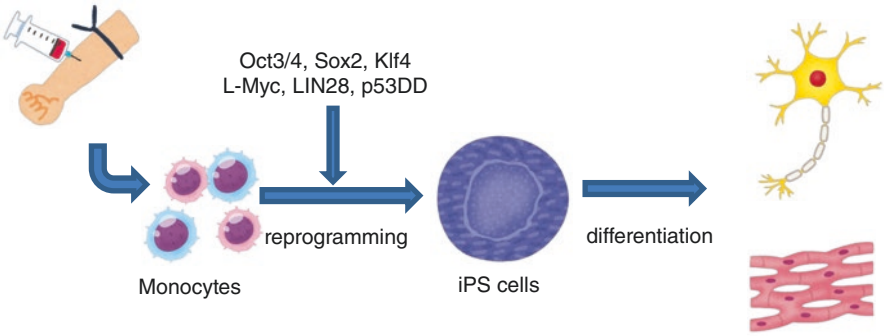

Fig. 12.1 Generation of iPS cells from patient-derived cells. iPSCs are generated from monocytes from the peripheral blood or skin fibroblasts of patients by expressing Yamanaka four factors [2, 69–72]. We also express LIN28 [73] and a dominant negative form of p53 (p53DD) [69] in addition to the four factors for increased efficiency. The established iPSC clones are then checked for their quality (incorporation of plasmid DNA fragments used for reprogramming in the iPSC genome, immaturity and multipotency of the established cells, microbial infections, etc.), prior to the use in differentiation experiments to generate target cell types. The iPSC clones that we generated in "The Program for Intractable Diseases Research utilizing Disease-specific iPS cells" (Research Center Network for Realization of Regenerative Medicine; Japan Agency for Medical Research and Development) have been deposited to the iPSC bank of RIKEN BioResource Research Center and available for research and drug development purposes. See the website of the cell engineering division/cell bank of RIKEN BioResource Research Center (http://cell.brc.riken.jp/en/) for details and availability

stem cells [2]. iPSC technology has great potential to pioneer a new medical field. iPSCs have been generated, not only from normal cells but also from cells obtained from disease patients [3] (Fig. 12.1). Patient-derived iPSCs are expected to be used to model aspects of human diseases to help elucidate the pathogenesis and to screen chemical compounds which can normalize the pathological phenotype [4–9].

Modeling human diseases by patient-derived iPSCs may be regarded more useful in certain cases than others. Firstly, monogenic disorders (diseases caused by a single gene mutation) are better candidates to be modeled using patient-derived iPSCs than polygenic or sporadic disorders, since the disease-associated mutation should cause some pathogenic changes in the cells differentiated from iPSCs [5]. In addition, the iPSCs with the mutation corrected by genome editing technology should serve as a control cells without disease-associated phenotypes [9, 10]. This is a great practical advantage in performing experiments, because phenotypic differences between iPSCs established from two different control subjects are sometimes larger than those between control and patient-derived iPSCs. Secondly, cells differentiated from patient-derived iPSCs can be invariable human cells with disease mutation when biopsy samples of the tissues of interest are difficult to obtain. Easily recognizable examples include disease of the brain or heart. Compared to these, skeletal muscle tissue samples may be easier to obtain as biopsy samples from patients. However, since terminally differentiated skeletal muscle fibers do

not proliferate and the possible passage numbers of myoblasts are known to be limited [11], patient-derived biopsy samples are not suitable for drug screening requiring a large number of cells. On the other hand, patient-derived iPSCs, which allows unlimited number of passages, can be valuable cell resource for such purposes as modeling diseases for drug discovery. Thirdly, because of the pluripotent nature of stem cells, patient-derived iPSCs can be differentiated into cells of different tissue/organ types, and therefore, suitable for modeling systemic diseases or diseases affecting multi-organs.

Among a variety of inherited intractable skeletal muscle diseases, two types of myotonic dystrophy (dystrophia myotonica types 1 and type 2; DM1 and DM2) are the most common muscular dystrophy in adults [12, 13]. Both DM1 and DM2 are inherited in an autosomal dominant manner, with highly variable clinical presentations affecting multiple systems including the skeletal, cardiac and smooth muscles, the brain, the lens, endocrine, and immunological functions [14, 15]. While the mutations of both DM1 and DM2 are transcribed into mRNAs, they do not affect sequences of the proteins encoded by the genes but cause RNA toxicity. The diagnosis of DM1 is established in a proband with identification of a heterozygous pathogenic variant in dystrophia myotonica protein kinase (DMPK) by molecular genetic testing; the size of the CTG trinucleotide repeat in the noncoding region of DMPK is expanded to more than 50 in DM1 pathologic allele, while the repeat size ranging 5–34 in normal [12]. The number of CTG trinucleotide repeat is known to be correlated with the severity of the disease [16, 17] and the congenital form of DM1 that is associated with large (usually >1000) (CTG) expansions. The disease-associated CTG trinucleotide repeat tends to expand through successive generations, which provides a molecular basis for the observed increase in severity and earlier age of onset within a family. These genetic and clinical characteristics match well with the criteria when disease modeling employing patient-derived iPSCs is particularly useful (mentioned above). Therefore, we obtained peripheral blood cells or skin fibroblasts from DM1 patients, established iPSC from the patient cells, and initiated disease modeling studies to understand pathogenic mechanism of DM1 and to develop novel therapeutic strategies against it.

12.2 Differentiation of Human iPSCs to Myotubes for Disease Modeling Research

Efficient generation of functional muscle cells *in vitro* from stem cells is a key to the application of patient-derived iPSCs on muscle disease modeling. Myogenic differentiation of stem cells, particularly in human, usually showed poor reproducibility and yielded mixed populations with low percentages of the muscle cell population [18–20]. To develop an efficient and rapid method for myogenic differentiation of human iPSCs, we employed a method to express myogenic differentiation 1

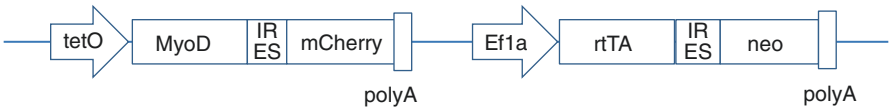

Fig. 12.2 Myogenic differentiation by expressing MyoD1 using PiggyBac system A schema for the expression vector we used for MyoD1 expression to differentiate iPSCs into myotubes. This construct is designed to give neo resistance to the transfected cells and to express MyoD together with mCherry in response to doxycycline administration

(MyoD1) gene in an inducible manner in human iPSCs [21]. We chose to use this method among a variety of protocols for myogenic differentiation of iPSCs for DM1 experiments, because we planned to use the DM1 patient-derived iPSCs for disease modeling in culture and possible drug screening, but not for transplantation or *in vivo* experiments requiring high-efficiency fusion with native myofibers for development of regeneration therapy. Among various different means of overexpression of MyoD1 [22–27], we constructed a self-contained, drug-inducible expression vector, based on the PiggyBac transposon, to achieve increased efficiency of transgene incorporation into human iPSCs [28] (Fig. 12.2). This vector constitutively expresses the neomycin (G418) resistance gene along with the rtTA transactivator element, which mediates doxycycline-dependent activation of cDNA cassettes controlled by TetO promoter. Activation of gene expression in response to doxycycline can be indirectly monitored by coincident mCherry activation. After transfection of this Tet-MyoD1 vector together with PB transposase, followed by selection of Tet-MyoD1-transduced cells using G418 for 5 days, we were able to select clones with appropriate levels of MyoD1 expression, based on robust and uniform mCherry expression. We found that, with the progression of differentiation by the induction of exogenous MyoD1 expression by doxycycline, endogenous MyoD1 started to be expressed to promote myogenic differentiation, and switching the culture media from iPSC medium to differentiation medium also help promote the differentiation. With optimized protocol we have established, we were able to observe ~90% differentiation rate by evaluation using immunohistochemistry for myosin heavy chain, a marker for mature muscle cells. The differentiated muscle cells derived from MyoD1-transduced human iPSCs showed gene expression profiles similar to those of mature myocytes *in vivo*, and they were able to fuse to form multinuclear myotubes and show contraction by electrical stimulation. These results suggested that our method of myogenic differentiation of human iPSCs by MyoD1 gene expression is reliable to be applied for modeling muscle diseases using patient-derived cell samples.

There are other methods/protocols for myogenic differentiation of human stem cells reported after our publication. The methods may be categorized into overexpression of key transcription factors for myogenic differentiation and the sequential recapitulation of key stages of paraxial mesoderm development and its differentiation into muscle cells. The former method includes expression of Pax7 [29] and

MyoD expression in combination with histone demethylase JMJD3 expression [30] or BAF60C [31]. The latter includes the dual modulation of Wnt and bone morphogenetic protein (BMP) pathway signaling [32] or application of small molecules identified by screenings [33]. While the methods to mimic developmental differentiation may be able to generate myofibers closed to native ones and are suitable for the development of regeneration therapy, they require complexed procedures with much longer period of time. In that sense, myogenic differentiation by overexpression of a single transcription factor, MyoD, is still a well-recommendable method for most research purposes.

12.3 Instability of Expanded Trinucleotide Repeat Observed in DM1 Patient-Derived iPSCs

Mitotic instability of extended CTG repeat in 3′ untranslated region of DMPK gene is a characteristic genetic feature of DM1 [12, 34]. Such instability often leads to somatic mosaicism for the size of the CTG expansion [35]. DMPK alleles of CTG length greater than 34 repeats are unstable and may expand in length during meiosis, which causes anticipation, the occurrence of increasing disease severity and decreasing age of onset in successive generations. While the somatic mosaicism and intergenerational instability tend to expand the repeat, there are rare cases of repeat contraction during transmission from parents to child [36, 37]. Repeat instability is also observed age-dependently, which suggests active repeat expansion in postmitotic cells [38, 39]. From these observations, it is estimated that instability arises not only with DNA replication (genome duplication) but also as a result of genome-maintenance repair.

By using DM1 patient-derived iPSCs, a previous report showed repeat expansion as the number of passaging as iPSCs increases, but not after differentiation into embryoid body or neurosphere [40]. However, the repeat size was assessed by estimation of PCR amplicon containing the repeat on ethidium bromide-stained agarose gel in this report, meaning that the size estimation did not reflect variation of repeat size distribution in different cells. Therefore, to examine if repeat instability is observed in DM1 patient-derived iPSCs in our study, we aimed to analyze cellular distribution of repeat size in DM1 patient-derived iPSCs as well as in cells differentiated from them. For this purpose, we established multiple iPS clones from DM1 patients and differentiate the iPSCs of different passage numbers into different target tissues, including skeletal muscle, cardiac muscle, and skeletal muscle cells. We analyzed CTG repeat numbers by small-pool PCR [41], in which PCR amplification of a trinucleotide repeat is performed in multiple small pools of input DNA containing ~0.5–200 genome equivalents, which is sufficient to generate ~1 trinucleotide repeat-containing amplicon. The PCR products were resolved by agarose gel

electrophoresis, and the repeat size was estimated by sensitive detection of the repeat-containing amplified DNAs by Southern blot hybridization [42]. This method allows the detailed quantification of the degree of repeat-length variation in a given sample, including the detection of common variants and those alleles present only in a small subset of cells. We found by using this method that the mean size of the CTG repeat of DM1 patient-derived iPSCs expands and the variance of the repeat size in the iPSC population increases as the number of passages of the iPSCs increases. On the other hand, mean repeat size and variance of the repeat size of the differentiated skeletal muscle cells, cardiac muscle cells, and neurons were not significantly different from those in originating iPSCs [42] (Fig. 12.3).

Different mechanisms of repeat expansion in mitotic cells have been reported mainly in the context of DNA replication, recombination, transcription, and repair [43, 44]. While these mechanisms have been demonstrated using model systems

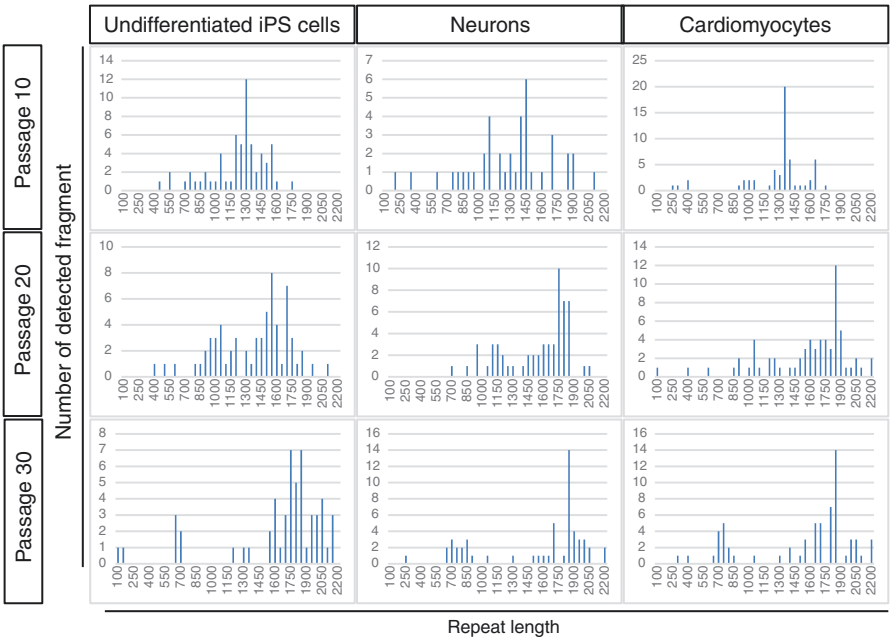

Fig. 12.3 Representative CTG repeat profiles of DM1 patient-derived iPS cells. DM1 patient-derived iPS cells were established, passaged for indicated number of times, and differentiated into neurons or cardiomyocytes using previously described protocols [74, 75]. Genomic DNAs extracted from iPSCs and differentiated cells at indicated passage points were subject to analysis of CTG repeat size by small-pool PCR. Presented histograms show representative profiles of CTG repeat number frequency distribution in indicated conditions identified by the small-pool PCR analysis (modified from data reported previously [42]). Note that the repeat length of the highest frequency of the undifferentiated iPSCs increased with the increased passage numbers, representing the increase of mean repeat length of the DM1 patient-derived iPSC population. On the other hand, differentiation of iPSCs into neuron and cardiomyocytes did not seem to affect the repeat size-frequency profile

such as yeast, the proposed mechanisms do not seem to explain iPSC-specific instability of the CTG repeat that we observed. Immature nature of iPSCs suggests that DM1 iPSC-specific instability may resemble germline repeat instability observed in DM1 patients. Previous reports have shown that CTG repeat instability in germline tissues depends on mismatch repair proteins, including MSH2, MSH3, and PMS2 [45–49]. Expression and functional significance of these proteins will need to be explored in DM1 patient-derived iPSCs. Modification to genomic DNA may also play a role in DM1 iPSC-specific instability. To explain the DM1 patient-derived iPSC-specific changes in genome DNA, our data from an assay for transposase-accessible chromatin using sequencing may be suggestive [42]. We found that the chromatin status in cardiac muscle cells was closed at SIX5/DMPK locus, whereas it was open in control. Difference in chromatin status or epigenetic changes may influence context of DNA replication and/or recombination to affect stability of the repeat sequence.

While we and others observed the repeat instability only in iPSCs [40, 42], we cannot exclude the possibility that this could be due to technical limitations. By the current technology of stem cell differentiation in culture, skeletal myotubes cannot be maintained for many days because of technical reasons. It is possible that long-term culturing of DM1 patient-derived myotubes may show effects on CTG repeat expansion. With regard to cardiomyocytes, maturation of differentiated cell has not been technically established thus far [50]. If phenotypic maturation of cardiomyocytes differentiated from human iPSCs becomes technically possible and the cardiomyocytes can be maintained for a long period of time in culture, CTG expansion which we did not see in our report could be observed. Nonetheless, DM1 iPSC has been the only culture model of repeat instability. It is possible that the mechanistic background of the observed DM1 iPSC-specific instability is not precisely modeling the pathological instability in patients, but it is likely that understanding the mechanism of the repeat instability in DM1 iPSCs will give us clues to understand the pathological instability in patients.

12.4 Application of DM1 Patient-Derived iPSCs for Drug Development

Currently there are no treatments available to patients with DM1. After definition of DM1as a microsatellite expansion disorder [51–55], many different approaches have been reported and are under development to ameliorate RNA toxicity. One type of approach is to decrease the expression level of trinucleotide repeat-containing mRNA [56]. Downregulation of mRNA for DMPK can ameliorate RNA toxicity, while it may also inhibit normal DMPK function. However, it is possible to think that certain degree of DMPK mRNA downregulation could decrease the RNA toxicity while maintaining its normal function [57]. One strategy to achieve this could be to degrade toxic RNAs or inhibit DMPK sequestration by antisense oligonucleotide [58]. Another recent approach may be to employ genome editing

strategy to inhibit transcription or destabilize DMPK mRNA [59]. The other therapeutic approach is to modulate toxic RNAs with small molecule compounds [60]. Previous reports have shown that pentamidine and its derivatives have been shown to decrease the production of toxic RNAs in DM1 mice [61, 62]. There are certain types of compounds, such as aminoglycoside derivatives [63], and erythromycin in a more recent report [64] demonstrated to block the binding between toxic RNAs and muscleblind-like 1 (MBNL1), an alternative splicing factor that binds to the expanded CUG repeat and decreases the levels of abnormal splicings.

Small molecule compounds offer the advantage of oral formulation, but to screen a large number of compounds, high-throughput assay systems need to be developed for efficient screening. Different model systems have been employed to screen compounds to modulate toxic RNAs. One type of experimental disease modeling is to inhibit molecular interaction with toxic RNA by using an *in vitro* system. This includes *in vitro* screenings to visualize inhibition of binding between a CUG repeat sequence and MBNL1 protein by chemiluminescence, fluorescence resonance energy transfer (FRET) system, or competitive electrophoretic mobility shift assay [65]. Another type of modeling method is to use cultured cells expressing a pathologically long CUG repeat-bearing gene. This includes HeLa DM1 cell model expressing 960 interrupted CUG repeats [66]. Generation of immortalized myoblast cell lines derived from DM1 patient fibroblasts by constitutive overexpression of human telomerase reverse transcriptase (hTERT) and inducible overexpression of the MyoD1 was also reported [67]. However, these previous attempts to identify compounds to modulate toxic RNAs have not been fruitful. Although identification of some bioactive compounds has been reported previously, they have not been proven to be useful *in vivo* or clinically, because the identified compound is not specific enough or the compound is not safe for application clinically [61]. One possible explanation of the failure to obtain truly active compounds is that the model systems employed to screen compounds were not optimal. For instance, regarding the *in vitro* system to screen compounds that can inhibit binding of CUG repeat sequence and MBNL1, three-dimensional structure of the artificial trinucleotide repeat mRNA may not properly recapitulate pathological context. Regarding the cellular models overexpressing long CUG repeat mRNA, fibroblasts and myoblasts may not be a good type of cells for examining abnormal splicing events, because these are not the cell types manifesting pathological phenotypes due to miss-splicing. The compounds identified from these screening under suboptimal conditions may tend to show activity only in artificial conditions employed for experimental screenings, but not *in vivo*.

DM1 patient-derived iPSCs and cells differentiated from the iPSCs can make good model cell system for screening compounds based on these observations. Abnormal splicing observed in patient tissues have been demonstrated in these cells [42]. Therefore, possible strategies for drug screening using these cells include the use of a previously described fluorescent reporter system to detect changes in mRNA splicing of a key gene for disease phenotype (Fig. 12.4). Because formation of ribonuclear foci that sequester MBNL1 is observed in DM1 patient-derived

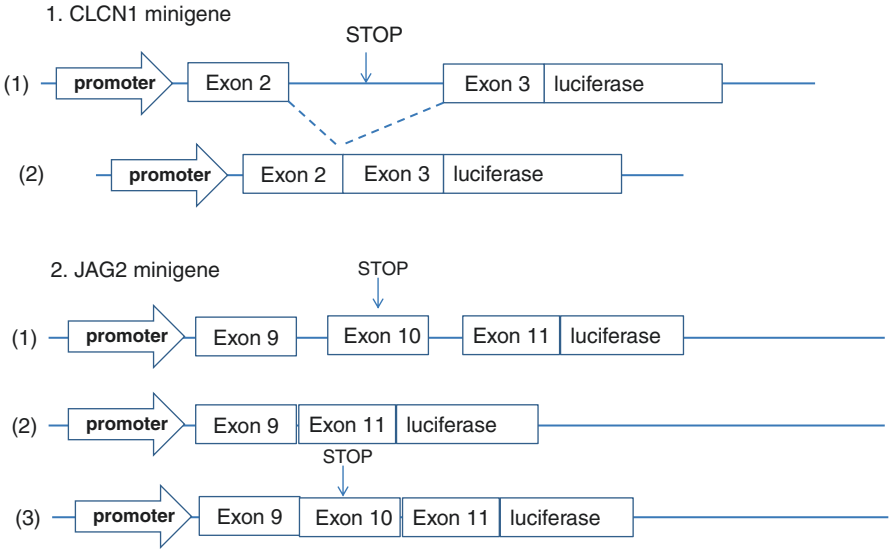

Fig. 12.4 Candidate constructs for reporter assays to screen compounds that normalize pathogenic splicing observed in DM1. High-throughput screening for compounds that normalize splicing changes caused by extended CUG repeat requires reporter constructs. Here are some candidate reporter constructs to be used with DM1 patient-derived iPSCs. In experiments with the "CLCN1 minigene" construct [67], normal excision of intron between exon 2 and 3 of CLCN1 gene (from (1) to (2)) is expected to be disturbed in DM1 patient-derived cells, which results in the loss of luciferase expression in the cells transfected with this construct. In experiments with the "JAG2 minigene," exon 10 which contains a stop codon in frame is expected to be spliced out (from (1) to (2)) in wild-type cells, and GFP fused to exon 11 should be expressed, but in DM1 patient-derived cells, exon 10 is expected to be included (from (1) to (3)), and GFP is not expressed [76]. Expression of mCherry should provide transcription efficiency control in the experiment

iPSCs, the effect of small molecule compounds on RNA foci formation is also of choice. While screening of compounds using DM1 patient-derived iPSCs may have not been reported as scientific papers yet, there are numbers of reports from pharmaceutical industry appeared in academic meetings. Thus, usefulness of DM1 patient-derived iPSCs for drug discovery may hopefully be proven shortly.

12.5 Future Directions

Application of iPS cell technology has been explored for development of regeneration therapy as well as basic research involving disease modeling in a cellular level. DM1 patient-derived iPSCs has mainly been used for the latter purposes. Interesting possibilities in the future use of DM1 patient-derived iPSCs include mechanistic analysis of repeat expansion observed in the DM1 patient-derived iPSCs and possibly screening of compounds to inhibit the repeat expansion. Another possibility

may be to try experimental gene therapies on DM1 patient-derived iPSCs. Recent progress of genome editing has been applied to gene therapy of genetic disorders [10]. With regard to genomic abnormality of DM1, it has been shown that normalization of the CTG repeat size or deletion of the repeat sequence is possible by artificial induction of trans-splicing [68] or genome editing technology [59]. While animal models need to be used to show whether the systemic normalization of the genetic change is possible by genome editing, the "proof of principle" of the therapy can be examined in a cellular model, and DM1 patient-derived iPSCs are presumably the best model for this purpose.

In addition to DM1 and DM2, there are other genetic disorders caused by expanded numbers of trinucleotide repeats in genome, and it is estimated that mechanism of repeat expansion in genome may be largely shared among some of such disorders [43]. Application of DM1 patient-derived iPSCs on disease modeling may hopefully lead to understanding of pathogenic mechanism and development of therapeutics against not only DM1 itself but also other genetic disorders caused by trinucleotide repeat expansion.

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Chapter 13
Therapeutic Development in Myotonic Dystrophy

Masayuki Nakamori

Abstract Myotonic dystrophy (DM) is the most common form of muscular dystrophy in adults, caused by unstable genomic expansions of CTG or CCTG repeats. The mutant RNA transcripts containing expanded repeats cause a toxic gain-of-function by perturbing splicing factors in the nucleus, resulting in misregulation of alternative pre-mRNA splicing. Recent advances in basic and translational research and pharmacological approaches provide clues for therapeutic intervention in DM. Here, we review the therapeutic approaches for targeting the toxic RNA with antisense oligonucleotides and small molecules.

Keywords Splicing · MBNL · Antisense oligonucleotides · Small molecule

13.1 Introduction

Occurring in 1 of every 8000 people, myotonic dystrophy (DM) is one of the most common muscle diseases in adults and is an intractable disease with an autosomal dominant genetic form [1]. DM patients exhibit various symptoms such as myotonia, progressive muscle weakness/muscle atrophy, cardiac conduction defects, cognitive dysfunction, cataracts, and endocrine dysfunction. Many patients are in a chronic state of being confined to bed due to progressive muscle weakness and end up developing aspiration pneumonia, dysphagia, respiratory failure, or fatal arrhythmias. Although methods for the medical management of respiratory muscle weakness and cardiac conduction defects are progressing, there is still no curative treatment for DM. However, recent research has revealed the pathomechanism of DM, and this has advanced the development of therapeutics. In this article, we outline the latest therapeutic developments to treat DM and potentially reach a cure.

M. Nakamori
Department of Neurology, Osaka University Graduate School of Medicine, Osaka, Japan
e-mail: mnakamor@neurol.med.osaka-u.ac.jp

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13.2 Therapeutic Strategy for DM

As detailed in other chapters, DM is one of the "repeat" expansion disorders caused by the abnormal elongation of simple tandem repeat sequences, where DM1 has an abnormal expansion of CTG repeats, and DM2 has an abnormal expansion of CCTG repeats [1]. The expanded repeats in DM1 and DM2 are in noncoding regions of the genome, and the abnormal RNAs transcribed from these causative genes are thought to be the basis of the pathology [2]. Abnormal RNAs with expanded CUG repeats for DM1 and elongated CCUG repeats for DM2 assume a repeat hairpin structure and form aggregates called foci in the nucleus of the cell. Proteins that regulate pre-mRNA splicing, such as *muscleblind-like* (MBNL) proteins (MBNL1, MBNL2, and MBNL3) and CELF1, which are capable of binding RNA containing CUG or CCUG sequences, are affected by these abnormal RNAs. This consequently leads to the misregulation of alternative splicing of their target exons. Such abnormalities have been identified in more than 100 genes for patients with DM, including a skeletal muscle-specific chloride channel (causing myotonia), an insulin receptor (causing glucose intolerance), proteins involved in intramuscular calcium homeostasis, muscle cytoskeletal proteins (possibly causing progressive muscle wasting), and a myocardial sodium channel (possibly causing cardiac conduction disorders) [3–10]. Thus, it has become apparent that the pathology of DM stems from an extensive failure of the splicing regulatory mechanism. The production and accumulation of abnormal RNA is now a major target for the development of DM therapeutics. Indeed, some therapies have already entered into clinical trials, and the direction of these therapeutic strategies and the possibilities for clinical application are outlined below (Fig. 13.1).

13.2.1 Splicing Correction

Because splicing misregulation is the molecular basis of DM, correction of individual mis-splicing events is the most direct way to alleviate the symptoms, and recent advances in antisense oligonucleotides (ASO) technology has enabled this strategy. ASOs are short, synthetic, modified nucleic acids that bind complementary RNA to regulate its function [11]. ASOs modulate splicing events by hiding specific sites essential for exon inclusion from the splicing machinery, and ASOs blocking target splice sites can skip the target exon(s) and restore normal splicing. This approach has been applied as a treatment for Duchenne muscular dystrophy (DMD). The exon skipping achieved by ASOs restores dystrophin expression in DMD patients and is currently evaluated by clinical trials [12]. In a mouse model of DM1, morpholino ASOs targeting the 3′ splice site of skeletal muscle-specific chloride channels (*Clcn1*) exon 7a reversed the defects in alternative splicing [13]. Briefly, the morpholino ASOs blocked the inclusion of exon 7a, restored *Clcn1* mRNA to normal levels, increased the expression of CLCN1 protein in sarcolemma, stabilized

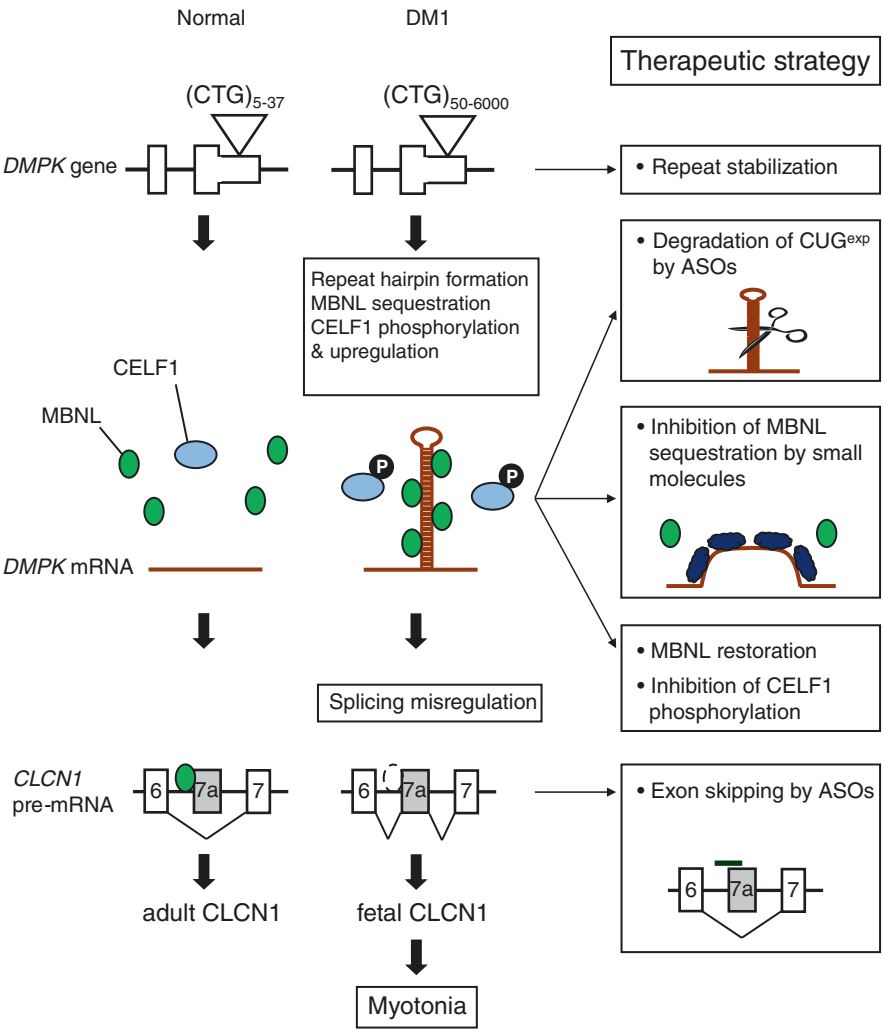

Fig. 13.1 Schematic illustration of the RNA-mediated disease mechanism (*left*) and possible therapeutic strategies (*right*) in DM1. Expanded CUG repeats in the mutant *DMPK* mRNA form hairpin structures and nuclear foci, sequester MBNL1 in the nucleus, and phosphorylate and stabilize CELF1. Loss of functional MBNL1 and upregulation of CELF1 cause misregulation of alternative splicing. Mis-splicing of *CLCN1* exon 7a induces a frameshift and premature termination codon in exon 7, resulting in loss of functional CLCN1 protein on the sarcolemma and myotonia in DM1. Therapeutic strategies are currently being developed for DM, including (1) stabilization of expanded repeats, (2) degradation of the toxic RNA by ASOs, (3) neutralization of CUG^{exp} toxicity by preventing MBNL sequestration with small molecules, (4) modulation of MBNL or CELF1, and (5) induction of exon skipping of individual targets. Figure modified from ref. [1]

the membrane potential, and thereby alleviated myotonia. These findings suggest that ASO-induced exon skipping is a powerful tool for correcting mis-splicing in DM; however, there are several hurdles to overcome in the application of this approach in DM patients: (1) alternative splicing of *CLCN1* is more complicated in humans than in mice and (2) mis-splicing events responsible for other symptoms, especially muscle wasting, are not known.

13.2.2 Modulation of Alternative Splicing Factors

In DM, MBNL1 sequestration leads to multiple mis-splicing events, such as that involving *CLCN1* [14]. One therapeutic approach is the restoration of MBNL1 protein in the nucleoplasm. Overexpression of MBNL1 by adeno-associated viral gene delivery in skeletal muscle of mice expressing CUG^{exp} reversed MBNL1-dependent mis-splicing of exons and rescued myotonia [15]. However, the MBNL1 overexpression in a DM1 mouse model did not improve muscle pathology. In addition, Mbnl1 knockout mice neither reproduce the developmental features of CDM nor display the severe muscle wasting that occurs in adult-onset DM1 [14]. Thus, as MBNL1 sequestration does not provide a unifying explanation for the disease process, simultaneous modulation of other splicing factors, such as MBNL2 and CELF1, may be required for successful treatment of DM.

In addition to MBNL1 sequestration, increased steady-state levels of CELF1 protein is another important molecular event involved in DM1 pathogenesis [16]. Overexpression of CELF1 in the mouse heart and skeletal muscle results in DM1-associated splicing changes and disease phenotypes [17–19]. Activation of the PKC signaling pathway is suggested to lead to the hyperphosphorylation and stabilization of CELF1 [20]. A recent report showed that PKC inhibitors reduced CELF1 phosphorylation and its steady-state levels, reversed misregulation of splicing events associated with CELF1, and ameliorated the cardiac conduction defects and contraction abnormalities in a heart-specific DM1 mouse model [21]. Although this approach may be promising for the treatment of cardiac symptoms in DM, there remain two important unanswered questions: (1) why is CELF1 not consistently increased in DM2 despite cardiac involvement similar to DM1, and (2) how do expanded CUG RNAs activate the PKC signaling pathway?

13.2.3 Novel Treatment for DM Using ASOs: Degradation of Abnormal RNA

In diseases such as DM, where abnormal RNA is the underlying pathology, ASOs that directly control abnormal RNA expression may be the optimal therapeutic approach. Previously, treatments that introduced short hairpin RNA and short interfering RNA (which suppress the target RNA using RNA interference) through viral

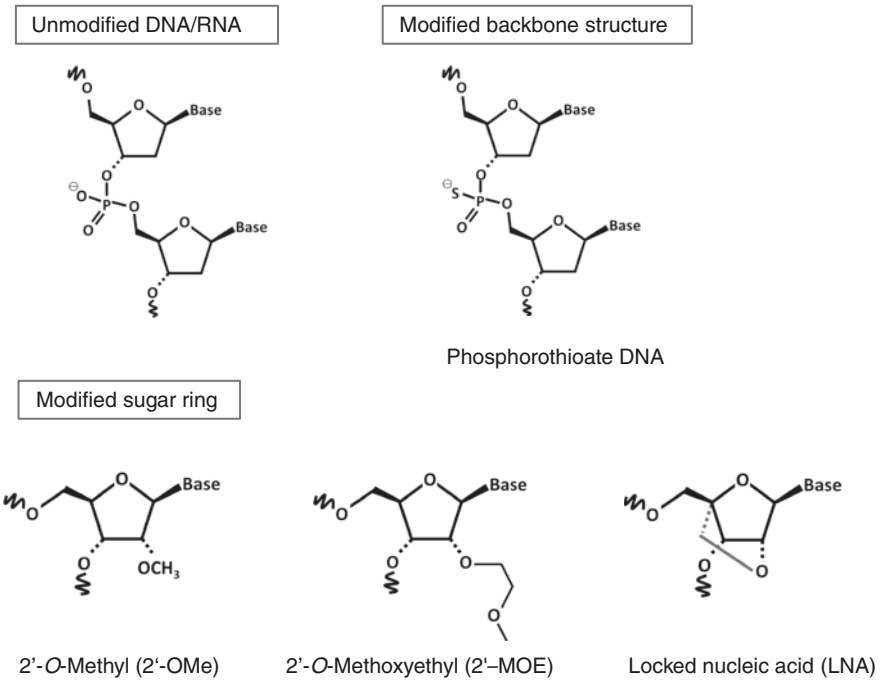

Fig. 13.2 Chemical structures of antisense oligonucleotide (ASO) and schematic of unmodified DNA/RNA base pair, backbone modifications, and 2′-sugar modifications. Different backbone and 2′-sugar modifications that can be applied to increase nuclease resistance and RNA-binding affinity of the ASO

vectors have been examined [22, 23]. In practice, however, RNA-based medicines have poor stability inside the cell, which has limited their therapeutic potential. Dramatic advancements in nucleic acid modification technology, such as phosphorothioate, 2′-*O*-methyl, and locked nucleic acid (Fig. 13.2), in recent years, have led to the development of ASOs, which have greater stability in vivo. Moreover, these molecules possess higher target-binding abilities, higher tissue transferability, and reduced toxicity, thereby making the use of ASOs a potentially promising therapeutic approach. In particular, ASOs having a gapmer structure, which has the ability to directly break down the target mRNA through an RNA degradation enzyme (i.e., RNase H), is expected to see early clinical applications (Fig. 13.3). A gapmer is an ASO that has several modified nucleic acids at both ends to increase target-binding ability and resistance to enzyme degradation. More specifically, the gapmer possesses a sequence of regular DNA in the "gap" between these modified flanking regions. When the DNA binds to the mRNA of interest, RNase H cleaves the RNA side of the DNA:RNA hybrid structure. After the target mRNA is cleaved and degraded, the gapmer ASO remains in its original form and contributes to the cleavage of another mRNA target. Thus, a small amount of the gapmer achieves long-term target mRNA suppression.

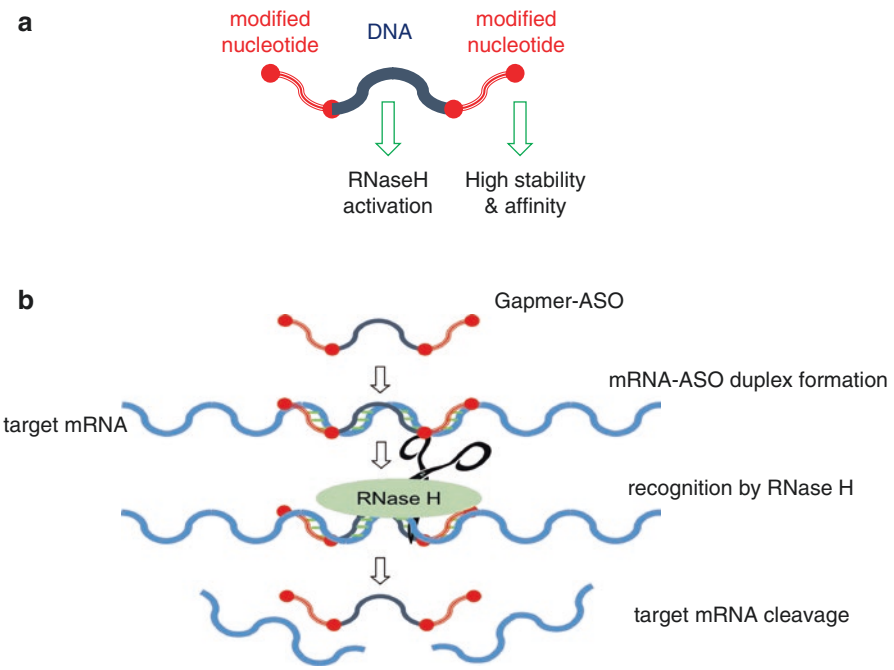

Fig. 13.3 (**a**) A gapmer design includes two chemically modified nucleotide sequences at the ends, flanking a central stretch of DNA sequence. The central gap can activate RNase H, whereas the flanking sequence provides high stability and affinity. (**b**) When gapmer ASOs bind to the target RNA, RNase H recognizes the heteroduplex and cleaves the RNA strand. Because RNase H releases the intact ASO upon cleavage, it conveniently allows for a single ASO to cleave target RNA many times, thus further increasing its potency

We first demonstrated the effect of a gapmer ASO in both cell and animal models by targeting the abnormally expanded CUG repeat portion of *DMPK* in DM1 [24]. However, since gapmer ASOs targeting such CUG repeats bind and suppress not only the abnormal mRNA of DM1 but also the normal mRNA derived from other genes having CUG repeating sequences, research is now focused on discovering other gapmer ASOs that target sequences other than CUG repeats. A mouse model for DM1 (HSA^{LR}) that possessed a transgene containing CUG repeats inserted in the 3′ untranslated region of the human actin gene has been widely used for treatment studies [25]. In addition to splicing abnormalities similar to those present in DM1 patients, this mouse model exhibits myotonia and muscle degeneration. By subcutaneously administering 25 mg/kg of gapmer ASO (targeting non-CUG repeat sequences) twice weekly for 4 weeks to HSA^{LR} mice, Thornton's group observed a significant decrease in abnormal RNAs and an improvement in splicing in the skeletal muscles of the mice [26]. In addition, myotonic discharge had decreased and almost disappeared according to a needle electromyogram. Even more surprisingly, due to the extraordinary stability of the gapmer ASO, the effects of suppressing abnormal RNA and improving splicing continued for 1 year after administration,

with suppressed myotonia and decreased muscle degeneration confirming its sustained effect over the long term. Furthermore, with the cooperation of Ionis Pharmaceuticals (formerly Isis Pharmaceuticals), a pharmaceutical company leading the world in nucleic acid medicine development, the effect and safety of such gapmer ASOs were demonstrated in monkey models [27]. Subsequently, a phase 1/2a trial (ClinicalTrials.gov Identifier: NCT02312011) was conducted in the USA as "IONIS-DMPK-2.5 Rx"; unfortunately, the trial has been put on hold after not showing the presumed effect. Future studies will likely focus on the development of ASOs with greater tissue transferability.

13.2.4 Novel Treatment of DM Using Small Molecules: Suppressing the Aggregation of Splicing Regulatory Elements

Although CELF1 shows activation and increased expression in DM1, it does not necessarily increase in DM2, and its involvement in the disease pathology is yet to be conclusively determined. In contrast, there is little doubt that MBNL is deeply involved in the pathology of DM, as knockout mice exhibit symptoms similar to DM and their symptoms are improved by the restoration of the gene [14, 15]. As mentioned above, MBNL binds to RNAs with CUG/CCUG repeats and becomes sequestrated by abnormal RNA in DM. To prevent the sequestration of MBNL, a therapeutic approach by small molecules, with even greater binding affinity for the CUG/CCUG repeats, has been proposed. Out of the 26 small molecules with an affinity to RNAs with CUG repeats, Berglund's group found that the antibiotic pentamidine has the effect of suppressing the sequestration of MBNL in vitro [28]. It has also been reported that pentamidine suppresses foci formation in DM1 model cells, and that its intraperitoneal administration to HSA^{LR} mice reduces splicing abnormalities. Pentamidine is a drug widely used in the treatment of *pneumocystis carinii* pneumonia; however, even when ten times the standard human dosage was administered to HSA^{LR} mice, the splicing of the skeletal muscle-specific chloride channel did not normalize, and myotonia did not disappear. Toxicity was also observed with these high-dose administrations. In addition, kanamycin derivatives, Hoechst 33,258 derivatives, and benzoquinoline derivatives have the effect of improving splicing in HSA^{LR} mice and suppressing the sequestration of MBNL, but the effects were minor, and their toxicity was significant [29–31]. Thus, these compounds are far from ready for clinical applications. Among the therapeutic approaches using these small molecules (summarized in Table 13.1), Berglund group developed pentamidine derivatives and identified a substance that normalizes splicing abnormalities, improves myotonia, and has reduced toxicity when administered to the DM1 mouse model [32, 33]. Even though these small molecules show promise in treating DM, they require safety testing over many years as new drugs that have never been administered to humans, so drug approvals are still 5–10 years away.

Table 13.1 Small molecules effective in DM1 model mice

Small molecules	Classification	Mechanism of action	Reference
Pentamidine[a]	Antibiotics	Reduction of toxic RNA	[28]
ABP-1	Polypeptide	Prevention of MBNL sequestration	[40]
4 K-2-DR9	Kanamycin derivative	Prevention of MBNL sequestration	[30]
H-1	Hoechst 33258 derivative	Prevention of MBNL sequestration	[29]
LO-11	Lead compound from dynamic combinatorial chemistry	Prevention of MBNL sequestration	[31]
TDZD-8	GSK3β inhibitor	Correction of cyclin D3 expression	[41]
Manumycin	Farnesyltransferase inhibitor	Non-specific splicing alteration	[42]
Heptamidine	Pentamidine derivative	Reduction of toxic RNA	[32]
Furamidine	Pentamidine derivative	Reduction of toxic RNA	[33]
Actinomycin D[a]	Antitumor drug	Reduction of toxic RNA	[34]
Erythromycin[a]	Antibiotics	Prevention of MBNL sequestration	[35]
Harmine	Alkaroid	Non-specific splicing alteration, increase in MBNL	[43]
Dihydroberberine	Alkaroid	Non-specific splicing alteration	[43]
Phenylbutazone	NSAIDs	Prevention of MBNL sequestration, increase in MBNL	[44]

[a]FDA/EMA/PMDA-approved drug

In addition to developing highly effective new drugs, we looked for existing drugs that showed beneficial effects in DM (also known as a drug repositioning strategy). First, we found that actinomycin D, which is used in childhood cancers such as Wilms tumors, has selective transcriptional repression of abnormal RNA [34]. Administering this compound to HSA^{LR} mice with a dose equivalent to the usual dose administered to children, we confirmed a decrease in abnormal RNA. That being said, in practice, it is difficult to administer this agent, an anticancer drug, to DM patients over a long period of time. In order to identify existing drugs that are even safer, we then screened 20 FDA/EMA/PMDA-approved drugs with RNA-binding ability and found that erythromycin, a macrolide antibiotic, has a high affinity for CUG repeats and inhibits MBNL sequestration [35]. Erythromycin is used not only as an antibiotic but is also taken over the long term for chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD). Moreover, erythromycin's high safety and tolerability have been established. Research into its therapeutic efficacy using a DM1 mouse model indicated that these mice showed significant improvement in splicing and myotonia with the systemic administration of a dose equivalent to the normal dose administered to humans as an antibiotic. The same was true when erythromycin was administered orally at a dose typically used

to treat COPD. This means that existing drugs already on the market may act on DM at doses that are already being used for the treatment of other diseases, and in the future, there are expectations for their early clinical application. Given that these small molecules inhibit MBNL sequestration via different mechanisms from the ASOs that break down abnormal RNAs, a combinatorial therapy of the two may produce greater efficacy.

13.2.5 Treatments Targeting Repeat Instability

The length of the CTG repeats that are expanded in DM1 is not constant, increasing with age, which contributes to the progression of symptoms [2]. Traditionally, repeat elongation has been thought to involve the DNA replication machinery, but in recent years, it became evident that elongation is greatly affected by the transcription machinery [36]. This is consistent with the fact that the repeat length is long in muscle tissue where the transcription of *DMPK* is active [37]. The instability of CTG repeats is often overlooked as a therapeutic target; however, neglecting these repeats, which grow year by year, may increase the load of abnormal RNAs and lead to decreased efficacy of other therapeutics (i.e., ASOs and small molecules). Although there have been compounds reported to stabilize the CTG repeats, they also had extensive effects on the DNA replication and repair machinery, which made them highly toxic and difficult to use in a clinical context [38]. However, in investigations using DM1 model cells and model mice, we found that ASOs that suppress abnormal RNAs also stabilize the repeats, thereby demonstrating their dual effect [24]. Such therapeutic approaches are expected to suppress the progression of DM, and in the future, a treatment that would shorten the repeat region might be developed.

13.3 Challenges in Developing Novel Treatments

As mentioned above, ASOs and small molecules are progressing as therapeutics against DM, but their effect(s) must be demonstrated in clinical trials before use in clinical practice. A sensitive and highly reliable index is important for the accurate determination of the effect of these investigational drugs in clinical trials. A recent study showed that several mis-splicing events may be a good biomarker for disease severity and therapeutic response in DM [9]. Clinical outcome measures, such as Myotonic Dystrophy Health Index (MDHI), may be also useful in this regard [39]. In order to alleviate the burden on patients, it is also desirable to establish biomarkers from blood-derived components that do not require muscle biopsy. In addition, in order to deliver therapeutic drugs as soon as possible to patients all over the world, it is necessary to steadily advance the establishment of a system to conduct international joint clinical trials and promote patient enrollment.

13.4 Conclusion

The pathogenesis of DM, which is a splicing disorder caused by abnormal RNA, has been elucidated, and there is rapid progression in the development of therapeutic agents, such as ASOs and small molecules. If the effectiveness of these therapeutic agents is demonstrated by future trials, a curative treatment for DM could be made possible. For DM patients suffering from various progressive symptoms, that day cannot come soon enough.

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