Table 2
Results of analysis of gene polymorphisms.

Gene	Location in gene	dtSNP ID	Allele	Frequency		Genotype	Frequency	
				Autism	Control		Autism	Control
HOXD11	Promoter		G	0.979	1	GG	0.959	1
			T	0.021	0	GT	0.042	0
						TT	0	0
	Intron	rs84746	A	0.711	0.721	AA	0.571	0.561
			C	0.289	0.288	AC	0.230	0.371
						CC	0.133	0.067
	Exon 2	rs863678	G	0.541	0.567	GG	0.316	0.292
			T	0.459	0.443	GT	0.449	0.551
						TT	0.235	0.157
	Exon2	rs6745764	A	0.214	0.18	AA	0.031	0.011
			G	0.786	0.82	AG	0.367	0.337
						GG	0.602	0.652
HOXD12	Promoter		A	0.041	0.028	AA	0	0
			C	0.959	0.972	AC	0.082	0.056
						CC	0.918	0.944
	Promoter		G	0.929	0.955	GG	0.929	0.955
			T	0.071	0.045	GT	0.071	0.045
						TT	0	0
	Exon 1	rs847151	A	0.041	0.028	AA	0	0
			G	0.959	0.972	AG	0.082	0.056
						GG	0.918	0.944
HOXD13	Promoter	rs847196	C	0.893	0.938	CC	0.786	0.876
			G	0.107	0.061	CG	0.214	0.124
						GG	0	0
	Promoter		A	0.082	0.107	AA	0	0
			T	0.918	0.893	AT	0.163	0.213
						TT	0.837	0.787
	Exon 1		C	0.985	0.989	CC	0.969	0.978
			T	0.015	0.011	CT	0.031	0.022
						TT	0	0
	Exon 1	rs2518053	A	0.408	0.455	AA	0.173	0.235
			G	0.592	0.545	AG	0.469	0.438
						GG	0.357	0.326
	Intron	rs847194	A	0.684	0.657	AA	0.459	0.404
			C	0.316	0.343	AC	0.449	0.506
						CC	0.092	0.09

SNP with no polymorphism detected in the present cases analyzed among the SNPs listed at the GenBank

HOXD11	Promoter	rs2736846	*HOXD13*	Exon 1	rs847195
	Intron	rs2736847		Exon 1	rs13392701
	Exon 2	rs12995279		Intron	rs847193
	Exon2	rs12995280		Intron	rs847192
HOXD12	Exon 1	rs2551807		Exon 2	rs28928892
	Exon2	rs2553776		Exon 2	rs28933082
				Exon 2	rs28928891

exon 1 of *HOXD12* (rs847151, G364A) showed a nearly complete linkage disequilibrium. Heterozygosity for both *HOXD12* -C226A and *HOXD12* G364A was observed in five healthy controls and eight autism patients. Furthermore, all of the five controls heterozygous for *HOXD12* -C226A and *HOXD12* G364A were homozygous for *HOXD11* -G112G. On the other hand, of the eight autism patients heterozygous for both *HOXD12* -C226A and *HOXD12* G364A, four were homozygous and four were heterozygous for *HOXD11* G-112T. Taken together, heterozygosity in all the three loci *HOXD11* G-112T, *HOXD12* -C226A, and *HOXD12* G364A was found in four autism patients but not in the healthy controls. Table 3 shows the relationships between the polymorphisms in these three loci for two cases: SDG and non-SDG with autism and SDG and non-SDG without autism. Of the four patients heterozygous for *HOXD11* G-112T, three in whom digit length was measured were classified into SDG with autism and the rest was unknown. The clinical type of ASD of the patients with having *HOXD11* heterogeneity was classified as autistic disorder in all cases. No patients

Please cite this article in press as: Sugie Y et al. Study of *HOXD* genes in autism particularly regarding the ratio of second to fourth digit length. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.005

Table 3
Frequency of *HOXD* gene polymorphisms between SDG and NSDG patients with or without autism.

Gene	dbSNP ID	Genotype	Autistic patients			Normal	Non-autistic	
			Total	SDG	NSDG	Control	SDG	NSDG
HOXD11		GG	94	8	17	89	8	8
		GT	4	3	0	0	0	0
HOXD12		CC	90	8	17	84	8	8
		AC	8	3	0	5	0	0
HOXD12	rs847151	GG	90	8	17	84	8	8
		AG	8	3	0	5	0	0

heterozygous for *HOXD11* G-112T were observed among the 16 non-autistic disease controls including the eight patients with SDG.

4. Discussion

In genetic research for autism, some studies have been conducted that focused mainly on language development skills (e.g., age at first word, age at first phrase, onset of first phrase >36 months, and nonverbal communication) skill. Other studies have focused on the establishment of motor language development, bladder and bowel control milestones, developmental regression, repetitive/stereotyped behavior, restricted behavior, interest, and activity [2–4,11–13].

Manning et al. [6] reported that 2D/4D is low in autism and Asperger syndrome. In Japan, Osawa et al. [7] reported a higher incidence of low 2D/4D in autism patients than in healthy children. From their report, we assumed that it is possible to consider a low 2D/4D as a specific feature in some autism patients. Such patients formed part of a group of subjects (SDG) for investigation in our study. It was assumed that SDG in autism may express one of the common features; hence, 2D/4D may be associated with one of the etiological genes of autism. Manning et al. [14] reported the findings of their 2D/4D measurement as follows: (1) there is a gender difference in 2D/4D measurements (2D/4D is lower in males than in females); (2) a low 2D/4D is observed across races and countries; (3) 2D/4D is closely related to fetal growth, sperm count, family size, myocardial infarction, and breast cancer; and (4) 2D/4D is related to sexual differentiation, the production of sex hormones in the fetal stage, and disease programming in the fetal stage. In addition, there is an inverse correlation between 2D/4D and testosterone concentration at the fetal stage, and 2D/4D correlates with the CAG repeat number in the androgen receptor gene [15].

A study of female twins conducted by Paul et al. [16] showed that the concordance rate of 2D/4D is higher in monozygotic twins than in dizygotic twins, that the heritability of 2D/4D is approximately 66%, and that the genetic contribution to 2D/4D in females may be more influential than the effects of prenatal environmental factors. Although it is uncertain whether these findings differ significantly between males and females in the absence of any report for males, it seems possible that 2D/4D is affected by both hereditary and secondary perinatal environmental factors.

One study showed that the mean 2D/4D did not change with gestational age from the 9th week to the 40th week [17]. In addition, there was a small increase in 2D/4D with age, which was lowest in the right hand [18]. This study indicates that 2D/4D is probably established in the uterus and that this ratio remains almost constant until adult life.

Because 2D/4D, an easily measurable physical feature, is already determined in utero and remains constant until adult life, it can be used regardless of age differences among subjects and is universal; moreover, its measurement is noninvasive. Therefore, 2D/4D is an excellent parameter for evaluating a group of autistic patients.

In genomic scans of families having more than one member with autism, the susceptibility loci for autism were investigated, and identified; these included 2q21–q33 [3,4]. In the candidate genes located here, the NRP2 gene is reported as one of the genes related to autism [19]. In addition, specific polymorphism has been found in distal-less 2 (DLX2) and cAMP guanine nucleotide exchange factor II (cAMP-GEFII) in a few cases of autism [20]. On the other hand, no significant correlation has been reported between autism and distal-less 1 (DLX1) [20,21] and DLX2 [20]. With regard to *HOXD* genes, Bacchlli et al. reported that there is no relationship between *HOXD1* and autism [20]. There has been no report on *HOXD11*, *HOXD12* or *HOXD13* to date.

It seems that these genes may be found to be significant in the development of autism when cases as a study subject have been carefully chosen and classified by the specific characteristics of presenting behavior or phenotypic clinical presentations.

The present study has limitations because it is a case-control study, rather than a family study, with a small number of subjects enrolled. However, in this study,

Please cite this article in press as: Sugie Y et al. Study of *HOXD* genes in autism particularly regarding the ratio of second to fourth digit length. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.005

HOXD11 SNP -112G/-112T heterozygosity was specifically observed in autism patients with low 2D/4D. On the basis of this result, we expect that the relationships between autism and the *HOXD* genes or other candidate genes located in 2q will be clarified by studying a larger population with low 2D/4D, that is, by studying patients heterozygous for -112G/-112T in the *HOXD11* promoter.

References

[1] International Molecular Genetic Study of Autism Consortium. A full genome screen for autism with evidence for linkage to a region on chromosome 7q. Hum Mol Genet 1998;7:571–8.

[2] Yang MS, Gill M. A review of gene linkage, association and expression studies in autism and an assessment of convergent evidence. Int J Dev Neurosci 2007;25:69–85.

[3] Buxbaum JD, Silverman JM, Smith CJ, Kilifarski M, Reichert J, Hollander E, et al. Evidence for a susceptibility gene for autism on chromosome 2 and for genetic heterogeneity. Am J Hum Genet 2001;68:1514–20.

[4] Shao Y, Wolpert CM, Raiford KL, Menold MM, Donnelly SL, Ravan SA, et al. Genomic screen and follow-up analysis for autistic disorder. Am J Med Genet 2002;114:99–105.

[5] Alarcón M, Cantor RM, Liu J, Gilliam Cthe Autism Genetic Resource Exchange Consortium, Geschwind H. Evidence for a language quantitative trait locus on chromosome 7q in multiplex autism families. Am J Med Genet 2002;70:60–71.

[6] Manning JT, Baron-Cohen S, Wheelwright S, Sanders G. The 2nd to 4th digit ratio and autism. Dev Med Child Neurol 2001;43:160–4.

[7] Osawa J, Sugie H, Fukuda T, Ito M, Sugie Y, Ohzeki T. Evaluation of the 2nd to 4th digit ratio in the patients with autism. No To Hattatsu (Tokyo) 2005;37:424–5, [in Japanese].

[8] Zákány J, Fromental-Ramain C, Warot X, Duboule D. Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. Proc Natl Acad Sci USA 1997;94:13695–700.

[9] Kuroiwa A. The role of homeobox genes on cartilage pattern formation. Jikken Igaku 2002;17:2441–8, [in Japanese].

[10] American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 4th ed. Washington, DC: American Psychiatric Association; 1994.

[11] Alarcón M, Yonan AL, Gilliam TC, Cantor RM, Geschwind DH. Quantitative genome scan and Ordered-Subsets Analysis of autism endophenotypes support language QTLs. Mol Psychiatry 2005;10:747–57.

[12] Freitag CM. The genetics of autistic disorders and its clinical relevance: a review of the literature. Mol Psychiatry 2007;12:2–22.

[13] Alarcón M, Abrahams BS, Stone JL, Duvall JA, Perederiy JV, Bomar JM, et al. Linkage, association, and gene-expression analyses identify *CNTNAP2* as an autism-susceptibility gene. Am J Hum Genet 2008;82:150–9.

[14] Manning JT, Churchill AJ, Peters M. The effects of sex, ethnicity, and sexual orientation on self-measured digit ratio (2D:4D). Arch Sex Behav 2007;36:223–33.

[15] Manning JT, Bundred PE, Flanagan BF. The ratio of 2nd to 4th digit length: a proxy for transactivation activity of the androgen receptor gene? Med Hypotheses 2002;59:334–6.

[16] Paul SN, Kato BS, Cherkas LF, Andrew T, Spector TD. Heritability of the second to fourth digit ratio (2d:4d): a twin study. Twin Res Hum Genet 2006;9:215–9.

[17] Malas MA, Dogan S, Evcil EH, Desdicioglu K. Fetal development of the hand, digits and digit ratio (2D:4D). Early Hum Dev 2006;82:469–75.

[18] Trivers R, Manning J, Jacobson AA. Longitudinal study of digit ratio (2D:4D) and other finger ratios in Jamaican children. Horm Behav 2006;49:150–6.

[19] Wu S, Yue W, Jia M, Ruan Y, Lu T, Gong X, et al. Association of the neuropilin-2 (NRP2) gene polymorphisms with autism in Chinese Han population. Am J Med Genet B Neuropsychiatr Genet 2007;144B:492–5.

[20] Bacchelli E, Blasi F, Biondolillo M, Lamb JA, Bonora E, Barnby G, et al. Screening of nine candidate genes for autism on chromosome 2q reveals rare nonsynonymous variants in the cAMP-GEFII gene. Mol Psychiatry 2003;8:916–24.

[21] Hamilton SP, Woo JM, Carlson EJ, Ghanem N, Ekker M, Rubenstein JL. Analysis of four DLX homeobox genes in autistic probands. BMC Genet 2005;6:52.

Please cite this article in press as: Sugie Y et al. Study of *HOXD* genes in autism particularly regarding the ratio of second to fourth digit length. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.005

Ther Adv Neurol Disord
(2010) 3(2) 127–135
DOI: 10.1177/1756285609359546

A preclinical trial of sialic acid metabolites on distal myopathy with rimmed vacuoles/ hereditary inclusion body myopathy, a sugar-deficient myopathy: a review

May Christine V. Malicdan, Satoru Noguchi and Ichizo Nishino

Abstract: Distal myopathy with rimmed vacuoles (DMRV), also called hereditary inclusion body myopathy (hIBM), is a moderately progressive hereditary muscle disorder affecting young adults. DMRV/hIBM is characterized clinically by muscle atrophy and weakness initially involving the distal muscles, and pathologically by the presence of small angular fibers, formation of rimmed vacuoles and deposition of various proteins in the muscle fibers. This disease is known to be caused by mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase gene, which encodes the essential enzyme in sialic acid biosynthesis, leading to a reduction of sialic acid levels in the serum and skeletal muscles of affected patients. As it is a metabolic disease, metabolite supplementation is theoretically one of the therapeutic options. In this review, recent animal models for DMRV/hIBM are briefly characterized followed by a focus on the administration of sialic acid metabolites as a reliable therapeutic option to DMRV/hIBM with the following points highlighted: the property of compounds, the pharmacokinetic metabolism *in vivo*, and the therapeutic effects on the DMRV/hIBM mouse model.

Keywords: sialic acid, GNE, muscular dystrophy, amyloid, therapy

Introduction

Distal myopathy with rimmed vacuoles (DMRV) or hereditary inclusion body myopathy (hIBM) is an autosomal recessive debilitating disorder affecting young adults with the age of onset ranging from 15 years to the late 30s [Nonaka *et al.* 2005; Nishino *et al.* 2002], and is due to mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (*GNE*) gene [Eisenberg *et al.* 2001]. The disease is characterized clinically by preferential involvement of the tibialis anterior and hamstring muscles and relative sparing of the quadriceps [Nishino *et al.* 2005; Nonaka *et al.* 2005; Argov and Yarom, 1984]. The course of the disease is gradually progressive, whereby patients usually become wheelchair-bound around 12 years after the onset of the disease. Findings in skeletal muscle biopsy include the presence of rimmed vacuoles, which are seen as clusters of autophagic vacuoles on electron microscopy, scattered atrophic fibers, and muscle fiber degeneration.

Despite the identification of the causative gene, the treatment for DMRV/hIBM has remained elusive as the pathomechanism of this disease has not been fully clarified. In addition, the lack of an appropriate model for understanding the disease and evaluating potential treatment options has contributed to the lag in development of a cure. In general, several strategies exist for the treatment of hereditary muscle disorders, such as gene therapy, cell therapy, and a pharmacological approach. Among these options, pharmacological treatment has been the most widely applied strategy to many muscular dystrophies and myopathies, as both the drug and study protocol can be flexibly designed based on the cause, pathogenesis, and symptoms of the disease.

This review focuses on a treatment option based on the notion of decreased sialic acid production in muscle cells owing to mutations in the *GNE* gene. Based on our recent findings on the preclinical trial of sialic acid metabolites to the

Correspondence to:
Ichizo Nishino
Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
nishino@ncnp.go.jp

May Christine V. Malicdan, Satoru Noguchi
Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

DMRV/hIBM mouse model, we review the properties of potential compounds taking into account the application *in vivo* of these compounds to mice. Likewise, we also discuss the phenotype of the mouse model and its response to therapy in order to clarify the reliability of sialic acid supplementation for DMRV/hIBM therapy in the future.

Distal myopathy with rimmed vacuoles/ hereditary inclusion body myopathy animal models

Several strategies based on genetic technology by manipulating the *GNE* gene have been attempted to generate animal models for DMRV/hIBM. Simple knock-out mice represent embryonic lethality by 9.5 dpc to suggest the importance of sialic acid in early embryogenesis [Schwarzkopf *et al.* 2002]. The same concept of the importance of sialic acid was recently demonstrated in knock-in mice carrying the p.M712T mutation, which is the most common *GNE* mutation among Jewish patients. The p.M712T mice showed a renal phenotype so severe that most homozygous mice could not survive beyond 3 days after birth (P3) [Galeano *et al.* 2007]. This renal phenotype is apparently caused by an anomaly in the morphogenesis of glomerular tissues due to the remarkable reduction in sialylation of podocalyxin, a major sialylated component of podocytes. In the M712T mice that were able to survive beyond P3, however, a phenotype pointing to skeletal muscle weakness or abnormalities in muscle pathology was not found. This result may suggest that the essential requirement of *GNE* activities for a certain level of sialic acid production is different between human and mice; in other words, the need for sialic acid at least during development might be higher in mice as compared with humans.

Our group adopted a different strategy to generate Gne$^{-/-}$h*GNE*D176VTg, a mouse model for DMRV/hIBM. This model harbored a transgene of p.D176V mutated human *GNE* cDNA but is knocked-out of endogenous Gne, creating a scenario in which only mutated GNE proteins are highly expressed and the endogenous *GNE* gene was disrupted [Malicdan *et al.* 2007a,b]. These mice were born at an almost Mendelian rate with a normal appearance (Figure 1(a)). As expected, blood and several organs including skeletal muscle exhibited hyposialylation. With age, these mice reproduced several myopathic phenotypes seen in the muscles of human DMRV/hIBM patients (Figure 1(b)–(d)). After 20 weeks of age, the DMRV/hIBM mice showed physiologic muscle weakness, seen as impaired motor performance of the mouse and reduced force generation of the skeletal muscle [Malicdan *et al.* 2008]. This reduction of the force can be attributed to muscle atrophy, as specific twitch and tetanic forces per cross-section area are maintained at normal values. The reduction in gross size of the skeletal muscle is accompanied by an increase in the number of small angular fibers on muscle cross-sections (Figure 1(c), white arrows). Serum creatine kinase is moderately elevated at this age. After 30 weeks of age, specific force generation in the gastrocnemius and tibialis anterior muscles was notably reduced, while in muscle pathology variation in muscle fiber size was more remarkable, and intracellular deposition of amyloid and other various proteins was noted in the gastrocnemius muscle. After 40 weeks, the muscle force generation increasingly worsened, as reflected by increased twitch/tetanic ratio, which could likely be due to the appearance of the characteristic rimmed vacuole (Figure 1(c), red arrows) and accumulation of autophagic vacuoles [Malicdan *et al.* 2007a,b] that can impair the contractile system of the muscle. With these results, the GNE$^{-/-}$hGNED176VTg mouse is the only existing pathogenic model for DMRV/hIBM at the moment.

Potential compounds for therapy of distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy

DMRV/hIBM is caused by mutations in the *GNE* gene, most of which are missense in the *GNE* gene. *GNE* encodes a critical enzyme, uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase/N-acetylmannosamine (ManNAc) kinase, for the biosynthesis of sialic acid in higher vertebrates including mammals (Figure 2, left panel). This enzyme catalyzes two steps in the sialic acid biosynthesis pathway: the epimerization of UDP-GlcNAc to ManNAc and the phosphorylation of ManNAc, the product of which is the substrate used to make sialic acid. Sialic acid production is determined by a negative feedback effect of the produced sialic acid on this UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE protein) step. The sialic acid product, cytidine monophosphate-neuraminate (CMP-NeuAc), binds the allosteric site of the GNE protein, inhibiting UDP-GlcNAc 2-epimerase activity. In principle, *GNE*

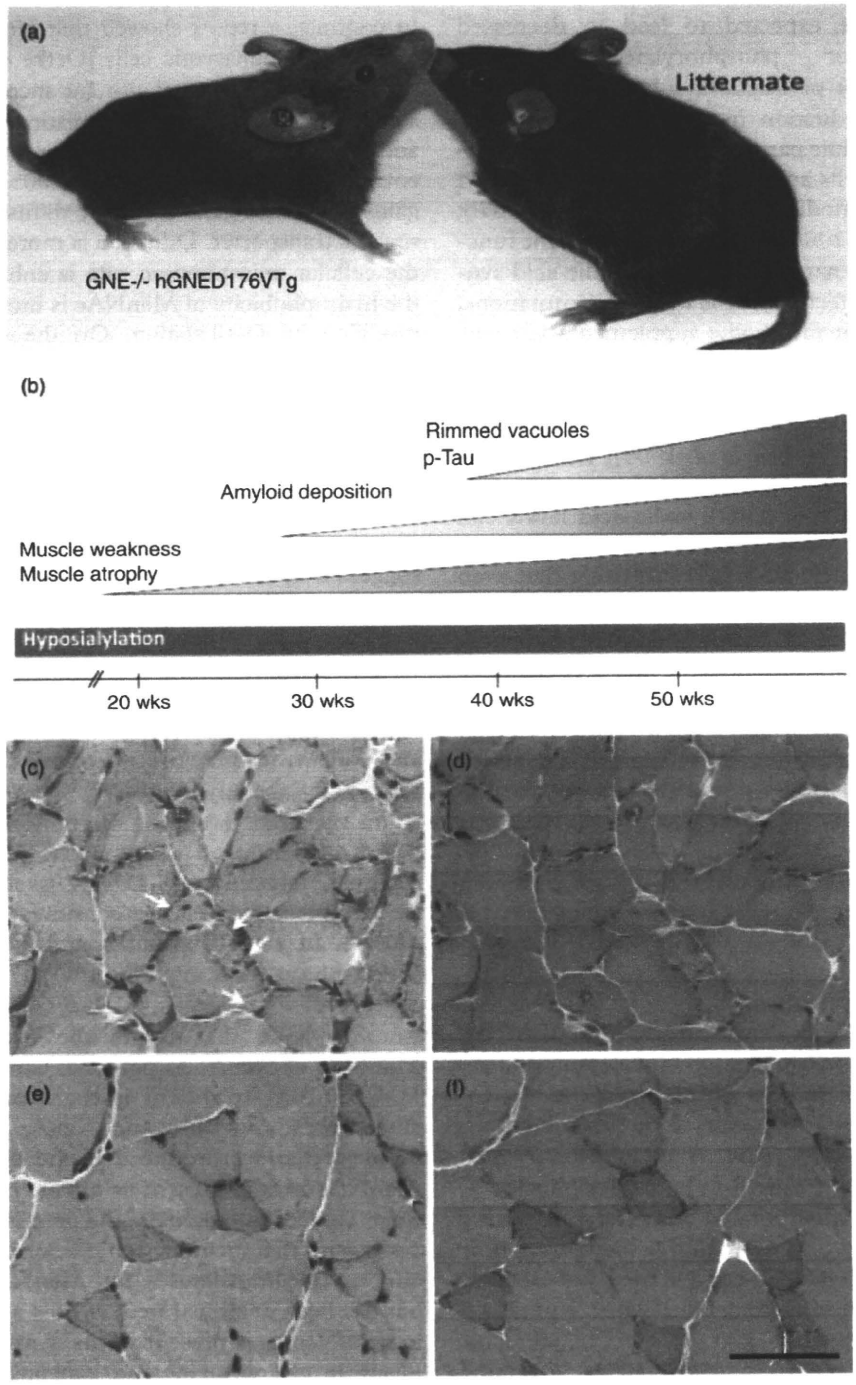

Figure 1. Overall phenotype of the distal myopathy with rimmed vacuoles (DMRV)/hereditary inclusion body myopathy (hIBM) mouse. (a) At birth, DMRV/hIBM mice appear normal, although slightly smaller than control littermates. (b) The onset of changes in muscle pathology are increasingly noted with age. Note that hyposialylation of serum and other organs is observed from birth. Typical fibers with rimmed vacuoles (red arrows) and small atrophic fibers (white arrows) are seen on hematoxylin and eosin (H&E) (c) and modified Gomori trichrome (mGT) (d). (e) H&E and (f) mGT-stained sections of muscle from age-matched control littermates. The characteristic features of DMRV/hIBM in muscle pathology are not seen in littermates. 187 × 295 mm (400 × 400 dpi).

mutations are expected to lead to decreased ManNAc or phosphorylated ManNAc (ManNAc-6P) production, subsequently resulting in the reduction in sialic acid production (Figure 2, middle panel), which was noted in skeletal muscle cells and serum in the DMRV/hIBM patients and model mice [Malicdan *et al.* 2007b; Noguchi *et al.* 2004]. On the other hand, the function of other enzymes involved in sialic acid synthesis is not affected in cells with *GNE* mutations, suggesting that metabolite supplementation may be an effective option in increasing sialic acid levels. In fact, several reports have shown that in cells with deficient *GNE* activity, such as BJA-B K20 and K6 [Keppler *et al.* 1999], Lec3 [Hong and Stanley, 2003] and *GNE* KO ES cells [Schwarzkopf *et al.* 2002], sialic acid levels can be recovered by supplementation either with ManNAc or sialic acid. It is surprising that even in *GNE* KO ES cells, which lack both UDP-GlcNAc 2-epimerase and ManNAc kinase activities, ManNAc treatment had a remarkable effect on the production of polysialic acid antigens on their surface membrane [Schwarzkopf *et al.* 2002]. These previous reports suggest that abundant GlcNAc 6-kinase activity contributes to phosphorylation of ManNAc in these cells [Hinderlich *et al.* 2001], although the pathogenesis of mutations in the ManNAc kinase domain of the GNE protein could not be explained in this concept.

ManNAc and NeuAc

As candidates for drugs for DMRV-hIBM, all metabolites downstream of GNE catalysis may be considered. In general, however, the nucleotide derivatives are thought to be rarely incorporated into cells and the phosphorylated compounds are believed to be dephosphorylated before being incorporated into the cell during their delivery. Theoretically, therefore, only ManNAc and NeuAc can be used for extrinsic administration to augment sialic acid synthesis.

There have been several trials that demonstrated manipulation of cellular sialic acid levels in normal cells and animals by administration of compounds extrinsically. In these trials, it was considered that ManNAc is the preferred molecule to increase the cellular sialic acid level, and that NeuAc may not be efficiently incorporated into cells due to its acidity from the outside, although NeuAc recycles within cells. However, this suggestion was made based on the finding of hyper-sialylation in normal cells and animals [Hirschberg *et al.* 1976]. In contrast, a report showed that NeuAc can be taken up by eukaryotic cells [Oetke *et al.* 2001], supporting its potential use for increasing sialic acid levels intracellularly. Interestingly, ManNAc and NeuAc reportedly use different routes for entry into cells [Bardor *et al.* 2005]. ManNAc gains entry into cells either by diffusion or via a specific transporter. Diffusion is more probable as the cellular incorporation rate is enhanced when the hydrophobicity of ManNAc is increased when modified by O-alkylation. On the other hand, NeuAc is incorporated by micropinocytosis and is subsequently transported from the endosomes to the lysosomes, and finally into the cytosol by the specific transporter, sialin.

The ability of cells to incorporate both ManNAc and NeuAc to possibly a comparable degree is also suggested by our results in DMRV cells, whereby we demonstrated that the addition of ManNAc and NeuAc in the medium of primary cells from DMRV patients recovered sialylation of the cells to a similar level [Noguchi *et al.* 2004]. These results support the notion that both ManNAc and NeuAc, in principle, are equally useful compounds for therapy. However, this does not discount the need to choose carefully the molecule based on the target tissues and their status. For example, targeting the DMRV skeletal muscle for treatment requires the consideration that the muscles are actually atrophic, and that the endocytic pathway itself might be affected.

Another issue that favors the use of either ManNAc or NeuAc as potential compounds for DMRV/hIBM treatment is the source of such compounds. ManNAc and NeuAc are natural monosaccharides produced in the animal body. ManNAc is only present as a free molecule at a trace amount and actually the presence of glycoconjugates that include ManNAc residues or specific glycosyltransferases for ManNAc residues has not been demonstrated, at least among vertebrates. NeuAc is present virtually as a glycoconjugate in glycoproteins and gangliosides, and is almost never a free molecule within cells. While both compounds are naturally occurring, it has been demonstrated that large-scale production for drug synthesis by pharmaceutical companies is possible [Yamaguchi *et al.* 2006].

Route of administration of sialic acid metabolites

Previous reports have suggested that extrinsically administered sialic acid is rapidly excreted

to urine. Uptake, metabolism and excretion of orally and intravenously administered radioisotope-labeled NeuAc and sialyllactose were examined in mice and rats. In 20-day-old mice, 90% of orally administered NeuAc was absorbed from the intestine at 4 h [Nöule and Schauer, 1981] but between 60 and 90% of NeuAc was excreted in the urine within 6 h. Only small amount of NeuAc (less than 6%) was incorporated into tissues, and was subsequently metabolized to ManNAc and pyruvate. These data imply that the small amount of sialic acid in food cannot be directly used for the synthesis of glycoconjugates for the purpose of increasing the levels of sialic acid in the tissues. In intravenous injection of NeuAc into rats, 90% was excreted in the urine within 10 min. Oral administration of sialyllactose resulted in the longer retention of sialic acid in tissues, however almost all were excreted within 24 h in which a half amount was metabolized to NeuAc. Sialyllactose injected intravenously into rats was rapidly excreted similarly to NeuAc.

Continuous administration of NeuAc orally and intraperitoneally for 8 days was examined in 14-day-old rat pups and resulted in a more remarkable incorporation of NeuAc into brain gangliosides and glycoproteins [Carlson and House, 1986]. This experiment suggested that orally administered NeuAc might be more significantly incorporated depending on the timing in animal development, and if the same dose was administered in several aliquots, then exogenous NeuAc might be more efficiently utilized as a substrate for sialylation of membrane gangliosides and glycoproteins. Further, when compared with the study carried out in older mammals, these experiments suggest that older animals did not show significant incorporation of sialic acid, at least after acute dosing. These likewise imply the need for a frequent and prolonged administration of metabolites in order to increase the sialic acid in the tissues.

We also analyzed the pharmacokinetics of NeuAc and ManNAc in blood and urine after intragastric and intraperitoneal administration in adult mice [Malicdan *et al.* 2009]. After a single intraperitoneal injection of NeuAc, the sialic acid level in the blood is notably increased within minutes, but 90% is found in the urine within 5–30 min and almost all is excreted within 4 h. After giving a single dose by an intragastric route, the levels in the blood are half as compared with the intraperitoneal route, but the excretion rate is slower as 70% is found in the urine within 30–60 min. Although after 120 min, the NeuAc levels in blood by both routes returned to a constant level, administration by an intragastric route resulted in a higher level of NeuAc even in the constant state. A similar pattern of excretion was seen after a single dose of ManNAc was given. These results suggest again that NeuAc and ManNAc are rapidly excreted and the intragastric route may be more advantageous in increasing the blood levels of sialic acid. For the treatment of DMRV/hIBM mice, we used the intragastric route by adding the compounds to drinking water (1-day dose of compounds dissolved in water, the amount of which was adjusted to what the mouse can drink within the day). By this method, the daily dose was divided into small aliquots and the frequency of drug intake is actually increased as mice drink 11.13 ± 1.28 times a day [Ritskes-Hoitinga *et al.* 2004].

Treatment of distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy mice

In designing a protocol for preclinical therapeutic trials, several factors are considered, including the property of the drug and the progression of diseases. In addition, the purpose of the treatment can either be aimed at prevention, arrest of disease progression, or cure from the diseased condition. The purpose of treatment is relevant in diseases that have a defined onset and progression, thus the goal of therapy would largely be affected by the starting time of the treatment. As DMRV/hIBM is a disease affecting young adults, maximum efficacy of treatment with sialic acid and its metabolites may be expected in preventing the onset of disease. In our recent paper, we examined the effect of sialic acid compounds in preventing the development of a myopathic phenotype in the DMRV/hIBM mouse model [Malicdan *et al.* 2009].

The study protocol that we used involved administering ManNAc to the mice from a preclinical age (5–6 weeks) continuously until the mice reached the age when all myopathic symptoms are found (54–57 weeks). ManNAc was added to drinking water and given in three doses: 20 mg (low dose), 200 mg (medium dose), and 2000 mg (high dose)/kg body weight of mice in a day. During the treatment period, survival rate was remarkably improved as compared with control-treated mice at all three doses. At the

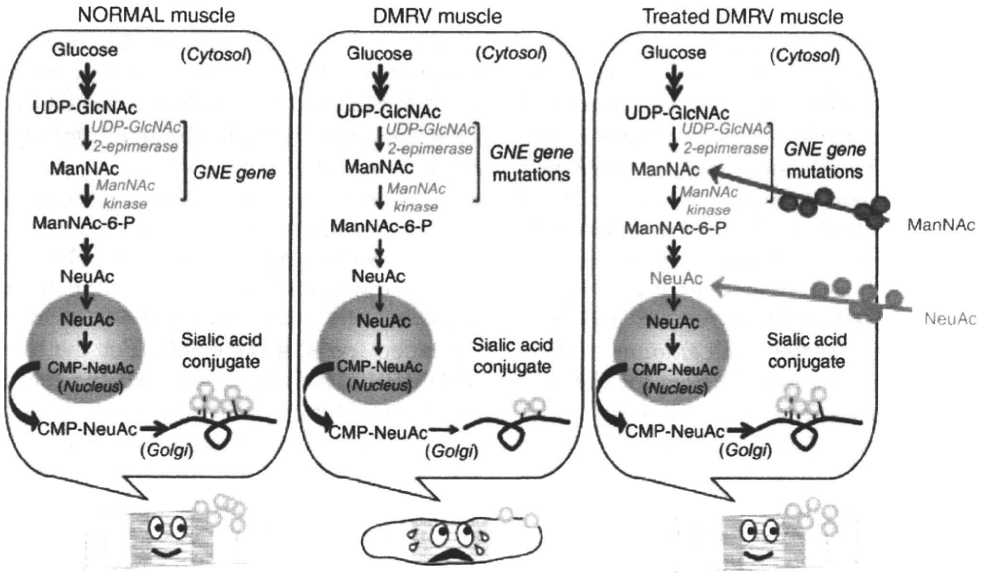

Figure 2. Incorporation of exogenous metabolites into sialic acid biosynthesis. **Left panel** shows the sialic acid biosynthetic pathway initiated within the cytosol, which starts with a series of steps whereby glucose is sequentially converted to UDP-GlcNAc. The succeeding essential steps involve the bifunctional enzymes encoded by *GNE: UDP-GlcNAc 2-epimerase* which epimerizes UDP-GlcNac into ManNAc, and *ManNAc kinase* which catalyzes the phosphorylation of ManNAc into ManNAc-6-P. The next two steps in the pathway eventually convert ManNAc-6-P into NeuAc. Activation of free NeuAc into CMP-NeuAc occurs in the nucleus. CMP-NeuAc is later transported to the Golgi apparatus. Sialic acids are ultimately transferred to oligosaccharide chains of gangliosides or sialoglycoproteins become sialylated (sialic acid conjugates). **Right panel** depicts a situation whereby the activity of the bifunctional enzyme is reduced because of mutations in *GNE*, resulting into decreased sialic acid production (and sialylation) of gangliosides or sialoglycoproteins, and reduced functional status of skeletal muscles in DMRV/hIBM. **Middle panel** shows the steps whereby exogenous sialic acids are incorporated into the pathway, improving sialylation status of sialic acid conjugates, and contributing to the improvement of skeletal muscle function in DMRV/hIBM.

end of the treatment, the phenotypes of DMRV/hIBM mouse were evaluated and compared with control-treated DMRV/hIBM mice and non-affected littermates. At all doses, serum creatine kinase activity, motor performance of mice, physiological contractile properties of isolated skeletal muscles as well as muscle pathology were notably improved to a level almost similar to that of the non-affected littermates. Intracellular protein deposits and rimmed vacuoles were rarely seen in the skeletal muscles of mice treated with ManNAc. Sialic acid levels in the blood and tissues were elevated, and more importantly, the levels of sialic acid in the muscle were recovered to an almost normal level after treatment [Malicdan *et al.* 2009], providing evidence that prophylactic oral administration of ManNAc to DMRV/hIBM mice was remarkably effective. Figure 2 (middle panel) is a simplified schema depicting the purported decrease of sialic acid levels on the glycoproteins and glycolipids in the muscle, which may contribute to the generation of symptoms in DMRV/hIBM by an unidentified mechanism. Figure 2 (right panel) also shows the incorporation of exogenously administered sialic acid metabolites into the sialic acid biosynthetic pathway. Once incorporated, sialic acids are supposedly utilized by glycoproteins and glycolipids in the muscle, preventing muscle weakness, or at least enhancing its physiologic function. We could not see, however, any correlation of the improvement of phenotype to the doses of administered ManNAc, except in the survival rate of the mice and sialic acid levels in the blood. This absence of dose response could potentially be explained by the following concepts: (a) administered ManNAc is effective in improving phenotypes completely only with the lowest dose used in the study, that is, 20 mg/kg bodyweight/day; (b) when large amounts of ManNAc are given, excess amounts of administered ManNAc will be excreted rapidly in the urine and only small aliquots of ManNAc will be retained in the blood for a certain time; and

(c) skeletal muscle has a maximum limit for actually incorporating ManNAc, or there might be other systems that can regulate the amounts of cellular ManNAc (e.g. by GlcNAc 2-epimerase, which is the catabolic enzyme). Nonetheless, these results suggest the need for a detailed analysis on the incorporation and metabolism of chronically administered ManNAc in living animals.

We also examined the effect of oral NeuAc and sialyllactose together with ManNAc using the minimum dose used in ManNAc treatment (20 mg/kg bodyweight/day) on the phenotypes of DMRV/hIBM mice starting at the preclinical age of 10–20 weeks. Treatment was also continued up to 54–57 weeks of age. Similar beneficial effects on motor performance, force generation of skeletal muscles, and muscle pathology were obtained after the treatment, as compared with the ManNAc trial. When the different compounds were compared as regards efficacy, we did not find any conspicuous differences, suggesting that these are equally good options for treatment.

One of the characteristic features in DMRV/hIBM is the accumulation of numerous autophagic vacuoles in the myofibers in which the transport and function of lysosomes may also be affected. As NeuAc is known to be incorporated into cells via the macropinocytotic/lysosomal pathway, one might question how NeuAc could actually be incorporated; however, from our data, treatment with NeuAc and sialyllactose improved sialic acid levels in various tissues with almost the same efficacy as with the ManNAc treatment. As the compounds were given at a preclinical stage, the accumulation of autophagic vacuoles, which is a phenomenon at a later stage, was prevented by the prophylactic treatment of sialic acid compounds. These issues on the preference and efficacy of the compounds at different stages of disease can only be answered by further studies that will focus on the systematic treatment in various and later ages of DMRV/hIBM mice.

Toxicology of the compounds

Long-time administration of low dose ManNAc and sialic acid metabolites was tolerated well in DMRV/hIBM mice [Malicdan *et al.* 2009]. In a previous paper where *N*-acyl-mannosamines and their O-acetylated derivatives were administered for 2 weeks to normal mice, no significant difference was seen in mice given the drug or phosphate-buffered saline alone, at least in terms of morphology of the investigated organs, histochemical data on various kinds of metabolic enzymes, and the expression of several markers of blood cells [Gagiannis *et al.* 2007]. Interestingly, however, O-acetylated ManNAc showed cytotoxicity of cultured cells in higher concentrations [Schwartz *et al.* 1983]. This could imply that the rapid excretion rate of ManNAc in rodents may actually prevent the toxic effects of the compounds.

Can reduction in sialic acid cause myopathy?

Whether decreased sialic acid production causes myopathy has been controversial. Although we and others demonstrated that hyposialylation does exist in DMRV/hIBM cells, others reported that there is no overall hyposialylation in the myoblasts and lymphoblastoid cell line from DMRV/hIBM patients [Salama *et al.* 2005; Hinderlich *et al.* 2004]. In addition, *GNE*-gene product has been proposed to have functions and roles outside sialic acid biosynthesis [Amsili *et al.* 2007; Wang *et al.* 2006]. We also do not completely discount that there might be other factors that could contribute to the pathogenesis of DMRV/hIBM. Nevertheless, the fact that increasing sialylation status, at least in skeletal muscles, can prevent the development of the myopathic phenotype in the majority of sialic acid metabolite-treated DMRV/hIBM mice clearly suggests that hyposialylation is one of the key factors in the pathomechanism of DMRV/hIBM (Figure 2, right panel). Addressing this metabolic impairment is a logical option for therapy, before a definitive cure is developed. Several issues still need to be clarified, however, including the role of sialic acid in muscle biology and the mechanism by which perturbation of sialic acid levels could lead to muscle atrophy, weakness, and degeneration.

Based on the concept of reduced sialylation in DMRV/hIBM, Sparks *et al.* tried *in vivo* sialic acid administration in human patients [Sparks *et al.* 2007]. This was an open-label study on the efficacy of intravenous immunoglobulin on four patients with DMRV/hIBM. The basis for this study was that 1 g immunoglobulin contains 8 μmol sialic acid and thus could be used to deliver sialic acid into patients' cells. After a few doses of intravenous immunoglobulin, patients subjectively experienced a mild improvement in muscle strength. However, for a robust effect to be seen, a placebo-controlled study may be needed.

Final remarks

Our recent preclinical trial on the effect of sialic acid metabolites has shown promising results for preventing the onset of myopathy in the DMRV/hIBM mouse model. We have shown that these sialic acid metabolites were equally effective and well tolerated, thus may be considered for therapeutic trial in DMRV/hIBM patients. It is important to point out, however, that the study design involved the treatment of mice before they developed obvious myopathic symptoms. This prophylactic treatment and its study design may, therefore, need careful interpretation, as we usually do not encounter pre-symptomatic patients. Notwithstanding the complex issues surrounding therapeutic trials in humans, we hope that the next step should include a careful evaluation of these metabolites in preparation for a formal clinical trial. Even though our results on sialic acid administration to DMRV mice are encouraging, further steps are needed to define precisely the metabolism and incorporation of ManNAc and sialic acid. In addition, the application of the present strategy to DMRV mice at different stages of the disease may benefit translation into clinical trial in the future. Another important issue is the fact that DMRV/hIBM is a rare disease. For a clinical trial to be set in motion there is clearly a need for international collaboration among clinicians and scientists working on this disabling myopathy.

Acknowledgments

This study is supported partly by: the Research on Psychiatric and Neurological Diseases and Mental Health from the Japanese Health Sciences Foundation; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); the Research Grant (19A–7) for Nervous and Mental Disorders from the Ministry of Health Labor and Welfare; the Kato Memorial Trust for Nambyo Research; and the Neuromuscular Disease Foundation.

Conflicts of interest statement

The authors have declared that there is no conflict of interest.

References

Amsili, S., Shlomai, Z., Levitzki, R., Krause, S., Lochmuller, H., Ben-Bassat, H. *et al.* (2007) Characterization of hereditary inclusion body myopathy myoblasts: possible primary impairment of apoptotic events. *Cell Death Differ* 14: 1916–1924.

Argov, Z. and Yarom, R. (1984) 'Rimmed vacuole myopathy' sparing the quadriceps. A unique disorder in Iranian Jews. *J Neurol Sci* 64: 33–43.

Bardor, M., Nguyen, D.H., Diaz, S. and Varki, A. (2005) Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells. *J Biol Chem* 280: 4228–4237.

Carlson, S.E. and House, S.G. (1986) Oral and intraperitoneal administration of *N*-acetylneuraminic acid: effect in rat cerebral and cerebellar *N*-acetylneuraminic acid. *J Nutr* 116: 881–886.

Eisenberg, I., Avidan, N., Potikha, T., Hochner, H., Chen, M., Olender, T. *et al.* (2001) The UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. *Nat Genet* 29: 83–87.

Gagiannis, D., Gossrau, R., Reutter, W., Zimmermann-Kordmann, M. and Horstkorte, R. (2007) Engineering the sialic acid in organs of mice using N-propanoylmannosamine. *Biochim Biophys Acta* 1770: 297–306.

Galeano, B., Klootwijk, R., Manoli, I., Sun, M., Ciccone, C., Darvish, D. *et al.* (2007) Mutation in the key enzyme of sialic acid biosynthesis causes severe glomerular proteinuria and is rescued by *N*-acetylmannosamine. *J Clin Invest* 117: 1585–1594.

Hinderlich, S., Berger, M., Keppler, O.T., Pawlita, M. and Reutter, W. (2001) Biosynthesis of N-acetylneuraminic acid in cells lacking UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase. *Biol Chem* 382: 291–297.

Hinderlich, S., Salama, I., Eisenberg, I., Potikha, T., Mantey, L.R., Yarema, K.J. *et al.* (2004) The homozygous M712T mutation of UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase results in reduced enzyme activities but not in altered overall cellular sialylation in hereditary inclusion body myopathy. *FEBS Lett* 566: 105–109.

Hirschberg, C.B., Goodman, S.R. and Green, C. (1976) Sialic acid uptake by fibroblasts. *Biochemistry* 15: 3591–3599.

Hong, Y. and Stanley, P. (2003) Lec3 Chinese hamster ovary mutants lack UDP-N-acetylglucosamine 2-epimerase activity because of mutations in the epimerase domain of the Gne gene. *J Biol Chem* 278: 53045–53054.

Keppler, O.T., Hinderlich, S., Langner, J., Schwartz-Albiez, R., Reutter, W. and Pawlita, M. (1999) UDP-GlcNAc 2-epimerase: a regulator of cell surface sialylation. *Science* 284: 1372–1376.

Malicdan, M.C., Noguchi, S., Hayashi, Y.K., Nonaka, I. and Nishino, I. (2009) Prophylactic treatment with sialic acid metabolites preclude

the development of a myopathic phenotype in the DMRV-hIBM mouse model. *Nat Med* 15: 690–695.

Malicdan, M.C., Noguchi, S., Hayashi, Y.K. and Nishino, I. (2008) Muscle weakness correlates with muscle atrophy and precedes the development of inclusion body or rimmed vacuoles in the mouse model of DMRV/hIBM. *Physiol Genomics* 17: 106–115.

Malicdan, M.C., Noguchi, S. and Nishino, I. (2007a) Autophagy in a mouse model of distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy. *Autophagy* 3: 396–398.

Malicdan, M.C., Noguchi, S., Nonaka, I., Hayashi, Y.K. and Nishino, I. (2007b) A Gne knockout mouse expressing human GNE D176V mutation develops features similar to distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy. *Hum Mol Genet* 16: 2669–2682.

Nishino, I., Malicdan, M.C., Murayama, K., Nonaka, I., Hayashi, Y.K. and Noguchi, S. (2005) Molecular pathomechanism of distal myopathy with rimmed vacuoles. *Acta Myol* 24: 80–83.

Nishino, I., Noguchi, S., Murayama, K., Driss, A., Sugie, K., Oya, Y. *et al.* (2002) Distal myopathy with rimmed vacuoles is allelic to hereditary inclusion body myopathy. *Neurology* 59: 1689–1693.

Noguchi, S., Keira, Y., Murayama, K., Ogawa, M., Fujita, M., Kawahara, G. *et al.* (2004) Reduction of UDP-N-acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase activity and sialylation in distal myopathy with rimmed vacuoles. *J Biol Chem* 279: 11402–11407.

Nonaka, I., Noguchi, S. and Nishino, I. (2005) Distal myopathy with rimmed vacuoles and hereditary inclusion body myopathy. *Curr Neurol Neurosci Rep* 5: 61–65.

Nöule, U. and Schauer, R. (1981) Uptake, metabolism and excretion of orally and intravenously administered, 14C- and 3H-labeled N-acetylneuraminic acid mixture in the mouse and rat. *Hoppe Seylers Z Physiol Chem* 362: 1495–1506.

Oetke, C., Hinderlich, S., Brossmer, R., Reutter, W., Pawlita, M. and Keppler, O.T. (2001) Evidence for efficient uptake and incorporation of sialic acid by eukaryotic cells. *Eur J Biochem* 268: 4553–4561.

Ritskes-Hoitinga, M. (2004) Nutrition of laboratory mice. In: Hedrich, H. and Bullock, G. (eds). *The laboratory mouse*. Elsevier Academic Press: London.

Salama, I., Hinderlich, S., Shlomai, Z., Eisenberg, I., Krause, S., Yarema, K. *et al.* (2005) No overall hypo-sialylation in hereditary inclusion body myopathy myoblasts carrying the homozygous M712T GNE mutation. *Biochem Biophys Res Commun* 328: 221–226.

Schwartz, E.L., Hadfield, A.F., Brown, A.E. and Sartorelli, A.C. (1983) Modification of sialic acid metabolism of murine erythroleukemia cells by analogs of N-acetylmannosamine. *Biochem Biophys Acta* 762: 489–497.

Schwarzkopf, M., Knobeloch, K.P., Rohde, E., Hinderlich, S., Wiechens, N., Lucka, L. *et al.* (2002) Sialylation is essential for early development in mice. *Proc Natl Acad Sci U S A* 99: 5267–5270.

Sparks, S., Rakocevic, G., Joe, G., Manoli, I., Shrader, J., Harris-Love, M. *et al.* (2007) Intravenous immune globulin in hereditary inclusion body myopathy: a pilot study. *BMC Neurol* 7: 3.

Yamaguchi, S., Ohnishi, J., Maru, I. and Ohta, Y. (2006) Simple and large-scale production of N-acetylneuraminic acid and N-acetyl-D-mannosamine. *Trends Glycosci Glycotechnol* 18: 245–252.

Wang, Z., Sun, Z., Li, A.V. and Yarema, K.J. (2006) Roles for GNE outside of sialic acid biosynthesis: modulation of sialyltransferase and BiP expression, GM3 and GD3 biosynthesis, proliferation and apoptosis, and ERK1/2 phosphorylation. *J Biol Chem* 281: 27016–27028.

Acta Neuropathol (2010) 119:481–486
DOI 10.1007/s00401-010-0660-7

ORIGINAL PAPER

Congenital myotonic dystrophy can show congenital fiber type disproportion pathology

Kayo Tominaga · Yukiko K. Hayashi · Kanako Goto · Narihiro Minami · Satoru Noguchi · Ikuya Nonaka · Tetsuro Miki · Ichizo Nishino

Received: 19 November 2009 / Revised: 15 February 2010 / Accepted: 15 February 2010 / Published online: 24 February 2010

Abstract Congenital myotonic dystrophy (CDM) is associated with markedly expanded CTG repeats in *DMPK*. The presence of numerous immature fibers with peripheral halo is a characteristic feature of CDM muscles together with hypotrophy of type 1 fibers. Smaller type 1 fibers with no structural abnormality are a definitive criterion of congenital fiber type disproportion (CFTD). Nonetheless, we recently came across a patient who was genetically confirmed as CDM, but had been earlier diagnosed as CFTD when he was an infant. In this study, we performed clinical, pathological, and genetic analyses in infantile patients pathologically diagnosed as CFTD to evaluate CDM patients indistinguishable from CFTD. We examined CTG repeat expansion in *DMPK* in 28 infantile patients pathologically diagnosed as CFTD. Mutation screening of *ACTA1* and *TPM3* was performed, and we compared clinical and pathological findings of 20 CDM patients with those of the other cohorts. We identified four (14%) patients with CTG expansion in *DMPK*. *ACTA1* mutation was identified in four (14%), and *TPM3* mutation was found in two (7%) patients. Fiber size disproportion was more prominent in patients with *ACTA1* or *TPM3* mutations as compared to CFTD patients with CTG expansion. A further three patients among 20 CDM patients showed pathological findings similar to CFTD. From our results, CDM should be excluded in CFTD patients.

Keywords Congenital myotonic dystrophy (CDM) · Congenital fiber type disproportion (CFTD) · *DMPK* · CTG expansion · *ACTA1* · *TPM3*

Electronic supplementary material The online version of this article (doi:10.1007/s00401-010-0660-7) contains supplementary material, which is available to authorized users.

K. Tominaga · Y. K. Hayashi · K. Goto · N. Minami · S. Noguchi · I. Nonaka · I. Nishino (✉)
Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan
e-mail: nishino@ncnp.go.jp

T. Miki
Proteo-Medicine Research Center, Ehime University, Toon, Ehime 791-0295, Japan

K. Tominaga · T. Miki
Department of Geriatric Medicine, Ehime University Graduate School of Medicine, Toon, Ehime 791-0295, Japan

Introduction

Congenital myotonic dystrophy (CDM; OMIM 160900) is caused by marked expansion of trinucleotide (CTG) repeat in the 3′ untranslated region of the dystrophia myotonica protein kinase gene (*DMPK*; OMIM 605377) on chromosome 19q [1, 5, 9]. The CTG repeat in normal individuals varies from 5 to 35, whereas it expands to more than 1,000 repeats in CDM [7]. Typically, the mothers of CDM patients show clinical features of myotonic dystrophy which makes the diagnosis of CDM easier. Clinically, CDM patients show hypotrophy at birth, tented upper lip, facial muscle weakness, and neonatal respiratory insufficiency. Mental retardation becomes evident in later life. On muscle pathology, the presence of numerous immature fibers with peripheral halo is a characteristic feature together with increased number of fibers with centrally placed nuclei and hypotrophy of type 1 fibers, mimicking myotubular myopathy [6].

Smaller sized type 1 fibers as compared to type 2 fibers are a characteristic pathological feature of congenital fiber type disproportion (CFTD; OMIM 255310). CFTD is a

congenital myopathy defined by type 1 fiber hypotrophy of 12% or more than type 2 fibers, and with the absence of structural abnormalities within myofibers [2]. Type 1 fiber predominance is also commonly seen. Clinically, CFTD patients show hypotonia, facial muscle weakness, and severe respiratory insufficiency at birth. Long face, high-arched palate, and joint contractures are often seen. CFTD is a genetically heterogeneous disorder and mutations in the genes for tropomyosin 3 (*TPM3*; OMIM 191030), α-skeletal muscle actin 1 (*ACTA1*; OMIM 102610), and selenoprotein N1 (*SEPN1*; OMIM 606210) have been identified [3, 4, 8]. Reportedly *TPM3* mutations are the most common ones and observed approximately in 20–25% of the CFTD patients [4]. *ACTA1* mutations were identified in 6% of CFTD [8], and only one family was reported having an *SEPN1* mutation [3].

Although the muscle pathology features of CDM seem to be well defined, our experience with one CDM patient who was previously diagnosed as CFTD made us hypothesize that CDM may have features other than the presently defined ones, both in terms of muscle pathology and clinical characteristics. In this study, we looked for CDM patients among patients who presented with CFTD. We also performed clinical and pathological analysis to find out whether patients with CDM can be distinguished from CFTD.

Materials and methods

Patients

All clinical materials used in this study were obtained for diagnostic purposes and with informed consent. This work was approved by the Ethical Committee of National Center of Neurology and Psychiatry (NCNP). In this study, we chose muscle specimens from patients younger than 1 year of age. From the muscle repository of NCNP, there were 28 unrelated patients who were pathologically diagnosed as CFTD. Twenty CDM patients, who had symptomatic family members and whose diagnosis was genetically confirmed, were also used for comparison.

Histochemistry

Biopsied skeletal muscles were frozen with isopentane cooled in liquid nitrogen. Serial frozen sections of 10 μm thickness were stained with hematoxylin and eosin (H & E), modified Gomori-trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), and ATPases (pH 10.6, pH 4.6 and pH 4.3). For each muscle specimen, the mean fiber diameter was determined by obtaining the shortest anteroposterior diameter of 100 each of type 1 and type 2 (A + B) fibers using ATPase stains. The myofiber diameter was used to calculate the fiber size disproportion (FSD). FSD was computed as: difference of type 2 fiber diameter (mean) and type 1 fiber diameter (mean) divided by type 2 fiber diameter (mean) × 100%.

Genetic analyses

Genomic DNA was extracted from peripheral lymphocytes or frozen muscle specimens using standard protocol. To examine CTG repeat expansion in *DMPK*, triplet repeat primed PCR was performed as described previously [12]. The presence of the expanded CTG repeats was examined by Gene Mapper using ABI PRISM 310 automated sequencer (Applied Biosystems Japan Co., Ltd, Japan). To know the approximate number of triplet repeats, we performed Southern blotting analysis using PCR-amplified CTG repeats because of the limited amounts of muscle specimens [10]. The primer sequences used in this study are F: 5′-CGAACGGGGCTCGAAGGGTCCTTGTAGC CG-3′, and R: 5′-TCTTTCTTTACCAGACACTAGGG-3′.

The PCR products were electrophoresed with 1% of Seakem HGT agarose gel (Cambrex Bio Science Rockland Inc., ME, USA), transferred to Hybond-XL (GE Healthcare, UK) for overnight, hybridized with ^{32}P-labeled probes of $(CTG)_{10}$ oligonucleotide at 65°C for overnight, and detected using BAS2500 (Fuji Film, Japan). By using genomic DNA from a CDM patient with known CTG repeat number, we confirmed that this PCR-based method can detect the corresponding size of the CTG repeats using genomic DNA. For mutation screening of *ACTA1* and *TPM3*, all exons and their flanking intronic regions were amplified by PCR and directly sequenced by an ABI PRISM 3100 automated sequencer (Applied Biosystems). Primer sequences are listed in the Supplemental Table.

Statistical analyses

All data are presented as means ± SD. Comparisons among groups were done by using Student's *t* test and analysis of variance (ANOVA) as appropriate. Statistical significance was considered when p value was less than 0.05.

Results

Genetic analyses

By using triplet repeat primed PCR, expanded CTG repeats in *DMPK* were detected in 4 of 28 (14%) unrelated patients who were pathologically diagnosed as CFTD (Figs. 1, 2a). This diagnosis of CDM was further confirmed by Southern

blotting analysis, wherein all four patients had more than 1,000 CTG repeats (Fig. 2b). We also identified three heterozygous *ACTA1* mutations (p.Gly48Cys, p.Leu221-Pro, and p.Pro332Ser) in four unrelated CFTD patients. Two mutations of p.Leu221Pro and p.Pro332Ser have already been reported [8], whereas the p.Gly48Cys mutation observed in two patients was a novel one. The Gly48 is a highly conserved amino acid among several species. Two unrelated CFTD patients had the same heterozygous mutation p.Arg168Cys in *TPM3*, which was previously reported in CFTD patients [4].

Clinical findings

We compared the clinical findings among 4 CFTD patients with CTG expansion and 6 CFTD patients with *ACTA1* or *TPM3* mutations, and compared the clinical features with 20 patients genetically confirmed as CDM (Table 1). In terms of family history, none of the four CFTD patients with CTG expansion had a positive family history. This is in stark contrast with the typical picture in CDM patients, as all of them had at least one symptomatic family member. Hydramnios and premature delivery were seen in more than 50% of the CFTD patients with CTG expansion and CDM, but none of CFTD patients with *ACTA1* or *TPM3* mutations. Hypotonia and respiratory insufficiency at birth were seen in all groups except for two patients with *TPM3* mutation.

Muscle pathological findings

As muscle pathology can have drastic changes according to the gestational age of infantile patients, we adjusted the age by setting the full-term day (37 weeks of gestation) as putative birthday. After adjustment, the age at biopsy of the CDM patients ranged from −7 to 43 weeks, and those of the four CFTD patients with CTG expansion were from 21 to 42 weeks.

Congenital fiber type disproportion is defined as a congenital myopathy wherein FSD is higher than 12%, but with no associated structural abnormalities within the myofibers [2]. In this study, FSD in CDM, CFTD with CTG expansion, CFTD with *ACTA1* mutation, and CFTD with *TPM3* mutation was calculated to be 7.2 ± 6.8% (mean ± SD), 23.0 ± 5.0, 47.5 ± 4.0, and 52.0 ± 9.9%, respectively (Fig. 3). FSD was significantly ($p < 0.05$) higher in CFTD with *ACTA1* or *TPM3* mutations as compared to the CFTD patients with CTG expansion and CDM.

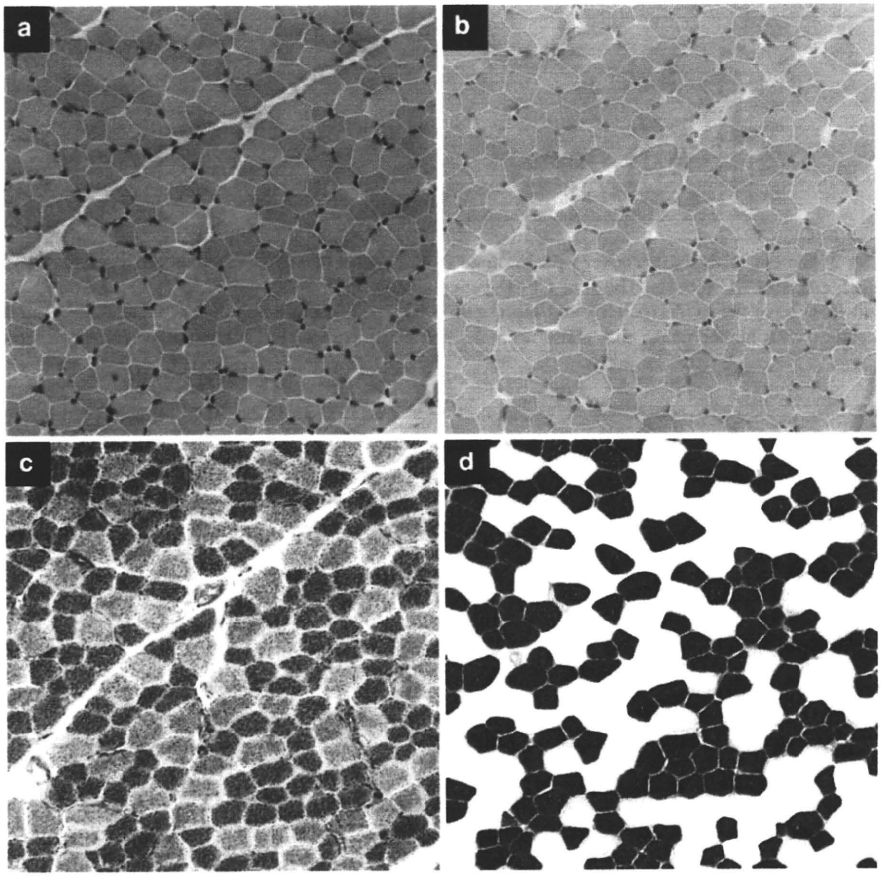

Fig. 1 Muscle pathology of a 42-week-old CFTD patient with CTG expansion. **a** Hematoxylin and eosin, **b** modified Gomori trichrome, **c** NADH-TR, and **d** ATPase (pH 4.4) stain. Type 1 fiber atrophy (FSD [(mean type 2 fiber diameter) − (mean type 1 fiber diameter)/mean type 2 fiber diameter × 100] = 26%), type 1 fiber predominance (65%), and only 1% of type 2C fibers with no peripheral halo is seen. *Bar* 50 μm

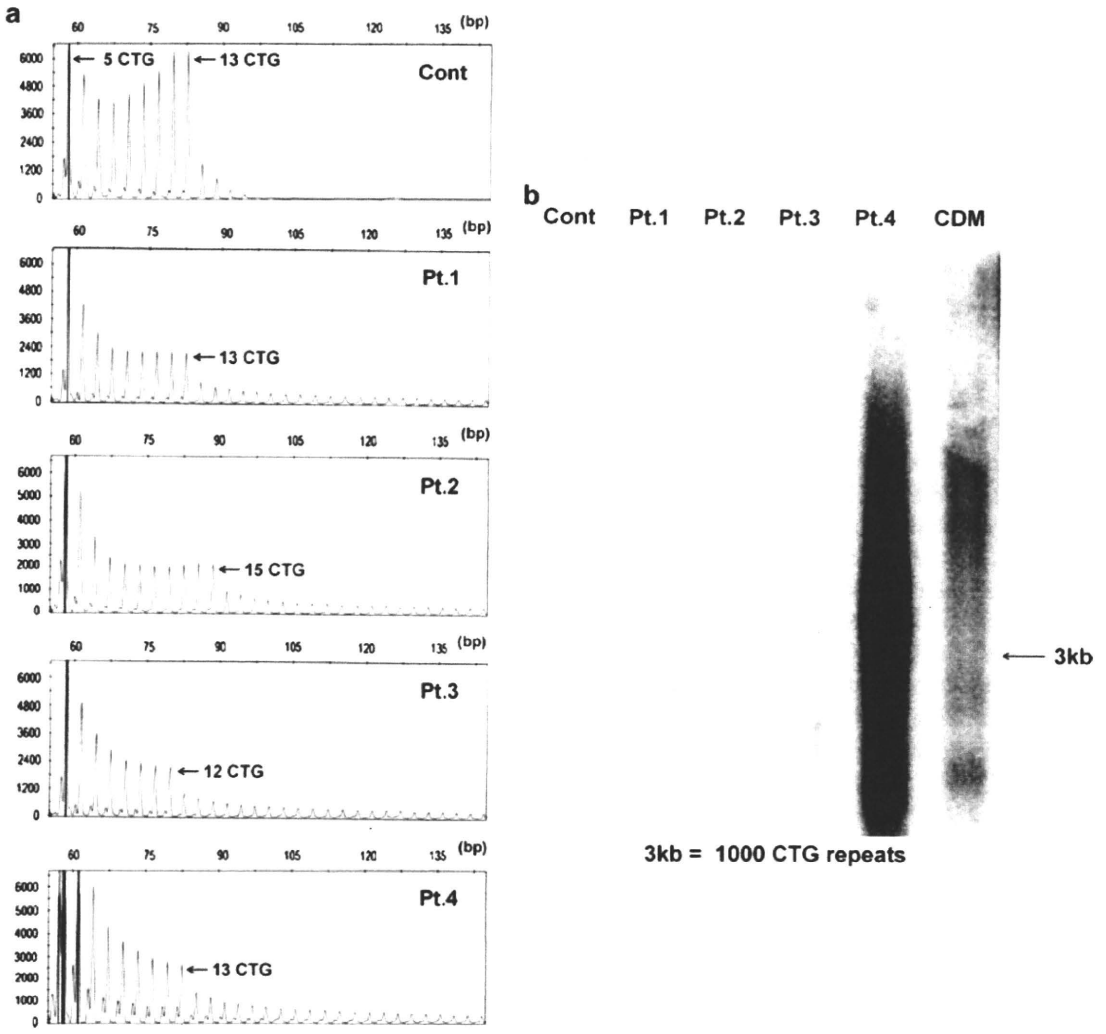

Fig. 2 Genetic analyses. **a** Triplet repeat primed PCR. Control (*Cont*) has 5 and 13 CTG repeats. The four CFTD patients (Pt.1, Pt.2, Pt.3, and Pt.4) have the ladder pattern that represents a large CTG allele together with higher peaks that show normal-sized allele (*arrows*). **b** Southern blotting analysis using PCR products. Four CFTD patients (Pt.1, Pt.2, Pt.3, and Pt.4) and one genetically confirmed CDM showed smear band larger than 3 kb corresponding to 1,000 CTG repeats, whereas a control (*Cont*) has no detectable band

Table 1 Clinical summary of the patients

Pathological diagnosis	CDM	CFTD	CFTD	CFTD
Gene mutation	CTG expansions in *DMPK*	CTG expansions in *DMPK*	*ACTA1*	*TPM3*
Number of patients	20	4	4	2
Hydramnios	65% (13/20)	50% (2/4)	0% (0/4)	0% (0/2)
Premature delivery (<37w)	50% (10/20)	50% (2/4)	0% (0/4)	0% (0/2)
Hypotonia at birth	100% (20/20)	100% (4/4)	100% (4/4)	0% (0/2)
Respiratory insufficiency at birth	95% (19/20)	75% (3/4)	75% (3/4)	0% (0/2)
Symptoms seen in family	100% (20/20)	0% (0/4)	0% (0/4)	0% (0/2)

In addition to FSD, we also checked other features in pathology that define either CFTD or CDM. Type 1 fiber predominance is a notable pathological finding observed in CFTD, and all our CFTD patients, including those with CTG expansion, showed type 1 fiber predominance. The mean composition of type 1 fibers in CDM, CFTD with

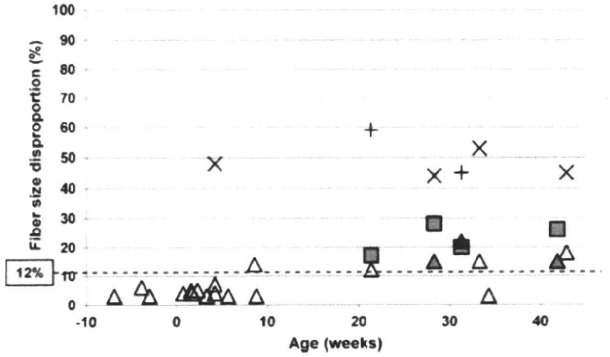

Fig. 3 Fiber size disproportion (*FSD*) of each patient. CFTD with CTG expansion (*filled square*; $N = 4$), CDM (*open triangle*; $N = 17$), CDM with similar pathological findings to CFTD (*filled triangle*; $N = 3$), CFTD with *ACTA1* mutations (*multi symbol*; $N = 4$), and CFTD with *TPM3* mutations (*plus*; $N = 2$). *Dot line* at 12% of FSD is the lowest FSD by the definition of CFTD

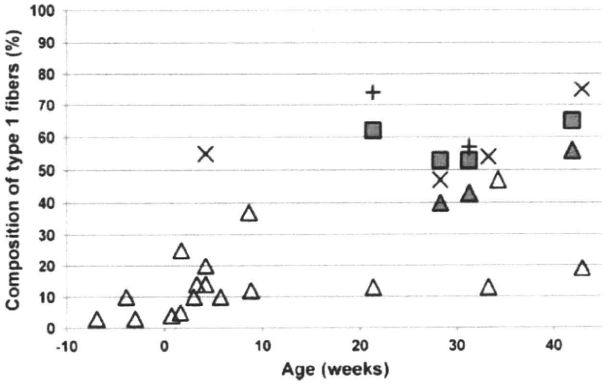

Fig. 4 Composition of type 1 fibers in each patient. *Filled square* CFTD with CTG expansion, *open triangle* CDM, *filled triangle* CDM with similar pathological findings to CFTD, *multi symbol* CFTD with *ACTA1* mutations, and *plus* CFTD with *TPM3* mutations

CTG expansion, and CFTD with *ACTA1* or *TPM3* mutations was 19.6 ± 16.3, 58.2 ± 6.2, 57.8 ± 2.0, and 65.5 ± 12.0%, respectively (Fig. 4). On the other hand, the presence of numerous immature type 2C fibers with peripheral halo is a characteristic finding in CDM. A markedly increased number of type 2C fibers were actually observed in CDM especially in patients younger than 10 weeks of adjusted age (Fig. 5). The frequency of type 2C fibers was inversely correlated to age of patients, while the number of type 1 fibers was directly proportional to age of patients. In other words, type 2C fibers were increased among younger age, while type 1 fiber predominance is seen more among older patients. Peripheral halo was observed in 14 of 20 (70%) CDM patients even in a 43-week-old patient. In CFTD patients with CTG expansion, type 2C fibers accounted for less than 20% and in CFTD with *ACTA1* or *TPM3* mutations, only a few type 2C fibers were seen. No peripheral halo was seen in either group. The increased number of fibers with internally located nuclei is another characteristic pathological finding of myotonic dystrophy. In our series, fibers containing internal nuclei were variably increased up to 26% in CDM patients, whereas less than 2% of fibers contained internal nuclei in the CFTD patients with CTG expansion or mutation in *ACTA1* or *TPM3*. The number of the fibers with internal nuclei is relatively correlated to the number of immature fibers in CDM, which may reflect immaturity of the fibers as described previously [6, 11].

Of the 20 CDM patients, 3 showed pathological findings similar to CFTD with CTG expansion. The ages of these three patients were 29, 32 and 42 weeks, respectively. FSD was 15–21%, with less than 20% of type 2C fibers and no peripheral halo. In these patients, the clinical diagnosis of CDM was made based upon the presence of the symptomatic family member.

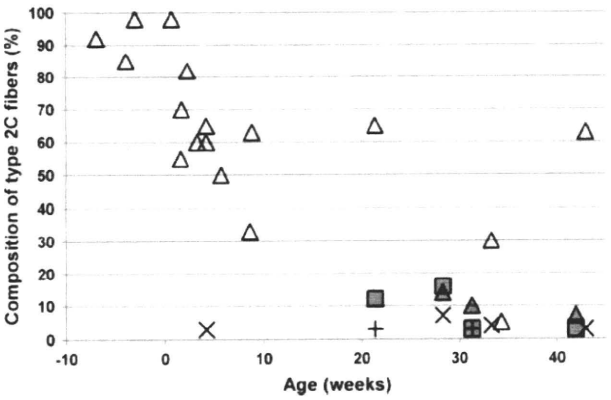

Fig. 5 Composition of type 2C fibers in each patient. *Filled square* CFTD with CTG expansion, *open triangle* CDM, *filled triangle* CDM with similar pathological findings to CFTD, *multi symbol* CFTD with *ACTA1* mutations, and *plus* CFTD with *TPM3* mutations

Discussion

In this study, we identified 4 of 28 patients (14%) who have CTG expansion in *DMPK* but were pathologically diagnosed as CFTD. Clinical symptoms of CFTD and CDM are quite similar during neonatal stage, including hypotonia and respiratory insufficiency. However, most of CDM patients are readily diagnosed by the presence of symptomatic family members, typically the mother. In fact, all CDM patients in our series had symptomatic family members and 75% of the mothers had the diagnosis of myotonic dystrophy. In contrast, no notable clinical symptoms were recorded in the mother of the CFTD patients with CTG expansion, and we could not examine the repeat size of the mothers. No marked difference in the size of CTG repeats was seen between CFTD patients with CTG expansion and CDM.

Among the CDM patients we examined, three patients showed pathological findings similar to those observed in CFTD with CTG expansion. They showed a small number of type 2C fibers, no peripheral halo, and hypotrophy of type 1 fibers (FSD >15%). The diagnosis of CDM was done from the typical clinical symptoms of myotonic dystrophy observed in the family member. Interestingly the ages of these three patients were over 29 weeks. Consistently, the ages of the patients who have CFTD with CTG expansion ranged from 21 to 42 weeks. These results suggest that CFTD pathology may be seen in this age range of CDM patients.

We identified four patients with mutations in *ACTA1* and two in *TPM3*. FSD in these patients was over 45% and significantly higher than that observed in CFTD with CTG expansion. This finding is also consistent with a previous report of CFTD patients with *TPM3* mutations whose muscle showed higher than 50% of FSD [4]. From these results, CDM should be considered for the patients whose muscle shows CFTD with FSD lower than 40%. In our series, only 4 (14%) and 2 (7%) of 28 patients had the mutations respectively in *ACTA1* and *TPM3*, leaving 18 (65%) patients still genetically uncharacterized and suggesting that defects in these genes may not be the major causes of CFTD in Japan. Further studies are necessary to elucidate such causes.

Acknowledgments We thank Dr. May Christine V. Malicdan (National Institute of Neuroscience, NCNP) for reviewing the manuscript. This study was supported by: the a Grant-in-Aid for Scientific Research and a Grain-in-aid for Exploratory Research from Japan Society for the Promotion of Science; by Research on Psychiatric and Neurological Diseases and Mental Health of Health Labour Sciences Research Grant and the Research Grant (20B-12, 20B-13) for Nervous and Mental Disorders from the Ministry of Health, Labour, and Welfare; by Research on Health Sciences focusing on Drug Innovation from the Japanese Health Sciences Foundation; and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

1. Brook JD, McCurrach ME, Harley HG et al (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell 69:385
2. Clarke NF, North KN (2003) Congenital fiber type disproportion—30 years on. J Neuropathol Exp Neurol 62:977–989
3. Clarke NF, Kidson W, Quijano-Roy S et al (2006) SEPN1: associated with congenital fiber-type disproportion and insulin resistance. Ann Neurol 59:546–552
4. Clarke NF, Kolski H, Dye DE et al (2008) Mutations in TPM3 are a common cause of congenital fiber type disproportion. Ann Neurol 63:329–337
5. Fu YH, Pizzuti A, Fenwick RG Jr et al (1992) An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 255:1256–1258
6. Harper PS, Monckton DG (2004) Myotonic dystrophy. In: Engel AG, Franzini-Armstrong C (eds) Myology, 3rd edn. McGraw-Hill, New York, pp 1039–1076
7. The International Myotonic Dystrophy Consortium (IDMC) (2000) New nomenclature and DNA testing guidelines for myotonic dystrophy type 1 (DM1). Neurology 54:1218–1221
8. Laing NG, Clarke NF, Dye DE et al (2004) Actin mutations are one cause of congenital fibre type disproportion. Ann Neurol 56:689–694
9. Mahadevan M, Tsilfidis C, Sabourin L et al (1992) Myotonic dystrophy mutation: an unstable CTG repeat in the 3′ untranslated region of the gene. Science 255:1253–1255
10. Surh LC, Mahadevan M, Korneluk RG (1998) Analysis of trinucleotide repeats in myotonic dystrophy. In: Dracopoli NC, Haines JL, Korf BR, Morton CC et al (eds) Current protocols in human genetics, vol 2. Wiley, New York, unit 9.6.1-13
11. Tanabe Y, Nonaka I (1987) Congenital myotonic dystrophy. Changes in muscle pathology with ageing. J Neurol Sci 77:59–68
12. Warner JP, Barron LH, Goudie D et al (1996) A general method for the detection of large CAG repeat expansions by fluorescent PCR. J Med Genet 33:1022–1026

Curr Neurol Neurosci Rep (2011) 11:97–103
DOI 10.1007/s11910-010-0154-y

Lipid Storage Myopathy

Wen-Chen Liang · Ichizo Nishino

Published online: 3 November 2010

Abstract Lipid storage myopathy (LSM) is pathologically characterized by prominent lipid accumulation in muscle fibers due to lipid dysmetabolism. Although extensive molecular studies have been performed, there are only four types of genetically diagnosable LSMs: primary carnitine deficiency (PCD), multiple acyl-coenzyme A dehydrogenase deficiency (MADD), neutral lipid storage disease with ichthyosis, and neutral lipid storage disease with myopathy. Making an accurate diagnosis, by specific laboratory tests including genetic analyses, is important for LSM as some of the patients are treatable: individuals with PCD show dramatic improvement with high-dose oral L-carnitine supplementation and increasing evidence indicates that MADD due to *ETFDH* mutations is riboflavin responsive.

Keywords Lipid storage myopathy · Primary carnitine deficiency · Multiple acyl-coenzyme A dehydrogenase deficiency · Neutrolipid storage disease

Introduction

Lipid is defined as a hydrophobic biomolecule and consists of mainly two types of molecules: fatty acid and its derivatives including triglycerides, and sterol-containing metabolites such as cholesterol. To date, all known muscle lipid disorders are associated with dysmetabolism of fatty acid and its derivatives. Fatty acids are eventually catabolized through the β-oxidation cycle in the mitochondrial matrix and adenosine triphosphate (ATP) is produced. Short- and medium-chain fatty acids can enter cells and then mitochondria by diffusion. In contrast, long-chain fatty acids that are richer sources of ATP require specific transport mechanisms, namely fatty acid transporters at the plasma membrane and carnitine palmitoyltransferase system at the mitochondrial membranes. Not surprisingly, muscle lipid disorders are due to a defect in intracellular triglyceride (TG) catabolism, transport of long-chain fatty acids and carnitine, or fatty acid β-oxidation.

In infantile cases, clinical manifestations are often similar in all types of lipid dysmetabolism, including hypotonia, hypoketotic hypoglycemic encephalopathy, hepatomegaly, and cardiomyopathy. In late-onset cases, muscle lipid disorders usually show two different phenotypes [1]: recurrent rhabdomyolysis or progressive muscle weakness. Recurrent rhabdomyolysis has been associated with defects of intramitochondrial fatty acid transport and β-oxidation, such as deficiencies of carnitine palmitoyltransferase II, mitochondrial trifunctional protein and very-long-chain acyl-coenzyme A (CoA) dehydrogenase. Interestingly, muscle pathology usually shows only nonspecific findings with a variable degree of necrotic and regenerating changes in muscle fibers reflecting recent episodes of rhabdomyolysis and lipid droplets are not usually increased. In contrast, the muscle weakness phenotype is usually associated with increased lipid droplets in muscle fibers. Importantly, there are only four types of genetically diagnosable lipid storage myopathy (LSM): primary carnitine deficiency (PCD), multiple acyl-CoA dehydrogenase deficiency (MADD), neutral lipid storage disease with

W.-C. Liang · I. Nishino (✉)
Department of Neuromuscular Research,
National Institute of Neuroscience,
National Center of Neurology and Psychiatry (NCNP),
4-1-1 Ogawahigashi-cho, Kodaira,
Tokyo 187-8502, Japan
e-mail: nishino@ncnp.go.jp

W.-C. Liang
Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung Medical University,
No 100, Tz-You 1st Rd.,
Kaohsiung 807, Taiwan
e-mail: wen.chen.liang@gmail.com

ichthyosis (NLSDI), and neutral lipid storage disease with myopathy (NLSDM) (Table 1). In this review, we will cover clinical, pathologic, and molecular aspects of these four LSMs.

Primary Carnitine Deficiency

PCD is an autosomal-recessive disorder, caused by the mutations in *SLC22A5*, which encodes a carnitine/organic cation transporter OCTN2 [2]. Many different mutations, including frameshift, nonsense, and missense mutations, have been reported in PCD patients. The mutations causing premature stop codons may lead to absent OCTN2 activity, whereas missense mutations result in different residual OCTN2 activity of carnitine transport. In addition, mutations in the promoter of *SLC22A5* have also been linked to Crohn's disease, an inflammatory bowel disease with autoimmune dysregulation [3].

As OCTN2 is a sodium-dependent carnitine transporter, which transfers carnitine across the plasma membrane using the sodium electrochemical gradient and because carnitine is essential for the transfer of long-chain fatty acids from the cytoplasm to the mitochondrial matrix for following oxidation (Fig. 1), defects in OCTN2 result in cytoplasmic accumulation of long-chain fatty acid, and consequently triglycerides as lipid droplets, as well as defective ATP synthesis by β-oxidation.

As mentioned earlier, infantile patients principally present with hypotonia, Reye-like syndrome, and cardiomyopathy. However, cardiomyopathy may develop in isolation or with a milder metabolic presentation during childhood or even older age [4]. Muscle weakness can also be seen. Conversely, some individuals can be asymptomatic for the whole life [5, 6], clearly demonstrating the wide variability of clinical severity in PCD. Other uncommon clinical symptoms of PCD include ventricular fibrillation and peripheral neuropathy [7, 8]. Interestingly, heterozygotes can develop cardiac hypertrophy but it is still controversial whether heterozygous mutations are directly responsible for the cardiomyopathy [9]. There is no clear correlation between genotype and phenotype, in either clinical or biochemical aspects, suggesting that the wide phenotypic variability may be related to epigenetic or exogenous factors that exacerbate carnitine deficiency [10]. Common blood tests may reveal increased levels of hepatic enzymes and creatine kinase (CK).

Muscle pathology is characterized by increased lipid droplets in both number and size in muscle fibers, especially in type 1 fibers, in addition to fiber size variation. On electron microscopy, lipid droplets are often present next to mitochondria.

Mitochondria are usually enlarged but cristae are normally aligned and no inclusions are seen in contrast to so-called mitochondrial encephalomyopathy, which show abnormal mitochondrial cristae and inclusions [11].

Measurement of free carnitine and all acylcarnitine species is essential for the diagnosis. Typically, both are extremely low in PCD. Heterozygous individuals have half-normal carnitine transport activity in fibroblasts and borderline carnitine levels in plasma [12]. Secondary carnitine deficiency should be carefully excluded, which may show decreased free carnitine level but specific species of acylcarnitine are usually elevated. In rare occasions, plasma carnitine level can be normal in PCD. Under such conditions, assessment of carnitine transport in fibroblasts may be useful to confirm the diagnosis. However, because in vitro functional measurements of OCTN2 transport activity are not widely available, screening for mutations in *SLC22A5* is an alternative way to establish the diagnosis of PCD.

PCD patients usually respond very well to high-dose L-carnitine supplementation (100–400 mg/kg/d). Early carnitine therapy has been believed to prevent the occurrence of cardiomyopathy and other irreversible organ damage [10]. In recent years, activation of peroxisome proliferator-activated receptor-α (PPAR-α) has been demonstrated to cause an upregulation of OCTN2, leading to an increase of intracellular carnitine concentration in animal models [13, 14]. In addition, a study has shown that mutant OCTN2 is retained in the cytoplasm and not localized in the plasma membrane, probably resulting in the degradation in endoplasmic reticulum (ER) [15]. Accordingly, PPAR-α agonists and the drugs reducing the efficiency of protein degradation in the ER or capable of binding OCTN2 may be potential candidates for treating PCD patients in the future.

Multiple Acyl-CoA Dehydrogenase Deficiency

MADD, also known as glutaric aciduria type II, is caused by defects in electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) (also called ETF-ubiquinone oxidoreductase). ETF, ETFDH, and most mitochondrial enzymes associated with electron transfer system as well are flavoproteins, which contain flavin adenine dinucleotide prosthetic groups. In the process of fatty acid β-oxidation in mitochondrial matrix, high-energy electrons, produced at several acyl-CoA dehydrogenases, are transferred to respiratory chain via ETF, which then transfers electrons to ETFDH located in the inner mitochondrial membrane. ETFDH subsequently passes the electrons to ubiquinone in the respiratory chain (Fig. 1). Therefore, a defect in ETF or ETFDH can theoretically affect all acyl-CoA dehydro-