Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy

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Abstract

Hearing loss (HL) is one of the most common sensory impairments and etiologically and genetically heterogeneous disorders in humans. Muscular dystrophies (MDs) are neuromuscular disorders characterized by progressive degeneration of skeletal muscle accompanied by non-muscular symptoms. Aberrant glycosylation of α-dystroglycan causes at least eighteen subtypes of MD, now categorized as MD-dystroglycanopathy (MD-DG), with a wide spectrum of non-muscular symptoms. Despite a growing number of MD-DG subtypes and increasing evidence regarding their molecular pathogenesis, no comprehensive study has investigated sensorineural HL (SNHL) in MD-DG. Here, we found that two mouse models of MD-DG, Large<sup>mus−<sup>mod</sup></sup> and POMGnT1-KO mice, exhibited congenital, non-progressive, and mild-to-moderate SNHL in auditory brainstem response (ABR) accompanied by extended latency of wave I. Profoundly abnormal myelination was found at the peripheral segment of the cochlear nerve, which is rich in the glycosylated α-dystroglycan–laminin complex and demarcated by “the glial dome.” In addition, patients with Fukuyama congenital MD, a type of MD-DG, also had latent SNHL with extended latency of wave I in ABR. Collectively, these findings indicate that hearing impairment associated with impaired Schwann cell-mediated myelination at the peripheral segment of the cochlear nerve is a notable symptom of MD-DG.

Author summary

Hearing loss (HL) is one of the most common sensory impairments and heterogeneous disorders in humans. Up to 60% of HL cases are caused by genetic factors, and approximately 30% of genetic HL cases are syndromic. Although 400–700 genetic syndromes are associated with sensorineural HL (SNHL), caused due to problems in the nerve pathways from the cochlea to the brain, only about 45 genes are known to be associated with syndromic HL. Muscular dystrophies (MDs) are neuromuscular disorders characterized by progressive degeneration of skeletal muscle accompanied by non-muscular symptoms. MD-dystroglycanopathy (MD-DG), caused by aberrant glycosylation of α-dystroglycan, is an MD subtype with a wide spectrum of non-muscular symptoms. Despite a growing number of MD-DG subtypes (at least 18), no comprehensive study has investigated SNHL in MD-DG. Here, we found that hearing impairment was associated with abnormal myelination of the peripheral segment of the cochlear nerve caused by impaired dystrophin–dystroglycan complex in two mouse models (type 3 and 6) of MD-DG and in patients (type 4) with MD-DG. This is the first comprehensive study investigating SNHL in MD-DG. Our findings may provide new insights into understanding the pathogenic characteristics and mechanisms underlying inherited syndromic hearing impairment.

Introduction

Hearing loss (HL) is one of the most common sensory impairments and one of the most etiologically and genetically heterogeneous disorders in humans [1]. Congenital HL affects around 1 in 1000 live births, and an additional 1 in 1000 children will suffer from HL before adulthood [2]. Up to 60% of HL cases are caused by genetic factors [1], and approximately 70% and 30% of genetic HL cases are non-syndromic and syndromic, respectively [1, 3, 4]. Based on the site of the lesion electrophysiologic tests, HL is categorized into conductive, sensorineural, mixed, and central HL, as well as auditory neuropathy [1, 5]. In the past two decades, extensive studies on the genetics of hereditary HL have been conducted, implicating 121 causative genes in non-syndromic sensorineural HL (SNHL) at around 150 SNHL loci (http://hereditaryhearingloss.org/) [4]. Although 400–700 genetic syndromes are accompanied by SNHL [3, 4], only about 45 genes are known to be associated with syndromic HL [3]. Syndromic HL often exhibits inconsistencies in the severity of HL and the age of onset; these inconsistencies exist both between and within families [1].

Muscular dystrophies (MDs) are a heterogeneous group of genetic and neuromuscular disorders characterized by progressive degeneration of skeletal muscles accompanied by various non-muscular symptoms. They are caused by mutations in a large variety of genes, which encode proteins of the contractile apparatus, structural proteins, and post-translational modification enzymes; currently, more than fifty causative genes have been reported [6–8]. MDs are categorized based on factors such as causative genes, inheritance patterns, and clinical presentations. Duchenne MD is the most common form of MD, resulting from loss of functional dystrophin, a cytoplasmic actin-binding protein (Fig 1A). Several forms of MDs result at least partially from defects in the dystrophin–dystroglycan complex [8, 9], which serves to link the intracellular actin cytoskeleton to the extracellular matrix (basal lamina). Dystroglycan is a single gene (dystrophin-associated glycoprotein 1: DAG1) product that is processed into two subunits: β-dystroglycan (β-DG), a transmembrane protein that interacts with dystrophin in the cytoplasm, and α-dystroglycan (α-DG), a highly glycosylated...
protein that interacts with both β-DG and multiple components of the basal lamina, such as laminin (Fig 1A). Notably, proper glycosylation of α-DG is essential for its binding to the basal lamina.

A growing number of causative genes for congenital MD (CMD) with abnormal α-DG glycosylation, including FUKUTIN, POMGNT1, POMGNT2, FUKUTIN RELATED PROTEIN (FKRP), and LARGE, have been recognized, and to date, at least eighteen have been reported [8] (Fig 1B). Genetic abnormalities in FUKUTIN cause Fukuyama CMD, which is the second most common form of MD in Japan [10]. As aberrant α-DG glycosylation is a common biochemical feature, the diseases are called α-dystroglycanopathy or MD-dystroglycanopathy (MD-DG). However, MD-DG is clinically heterogeneous and exhibit a wide spectrum of symptoms, from combined brain and eye anomalies to only muscular phenotypes [11]. Most of the MD-DG causative genes encode enzymes that synthesize unique sugar chains of α-DG, namely CoreM1 and CoreM3 [8] (Fig 1B). LARGE is a bifunctional glycosyltransferase that synthesizes a repeating unit consisting of a gluconic acid and a xylose, namely matriglycan, which provides the laminin-binding moiety on the terminal portion of CoreM3. FUKUTIN is an enzyme involving the synthesis of the tandem ribitol-phosphate structure, which serves as a fundamental unit required for LARGE-dependent matriglycan formation. POMGNT1 functions not only in the synthesis of CoreM1 but also plays important roles in regulating FUKUTIN function. Thus, a lack of either FUKUTIN or POMGNT1 consequently affects LARGE-dependent laminin-binding activity [9, 12].

SNHL has been reported in specific types of MD, such as facioscapulohumeral MD (FSHD) and myotonic dystrophy (DM: classified into DM1 and DM2). FSHDs (FSHD1 and FSHD2) are caused by aberrant expressions of DUX4 [13], and about 20% of FSHD1 patients show SNHL at high frequencies [13, 14]. A transgenic mouse model of FSHD has been generated [15], and the auditory brainstem response (ABR) tests show SNHL at frequencies greater than 8 kHz [16]; however, the underlying mechanism of SNHL has not been evaluated even in the FSHD transgenic mice. DM1 is caused by an expanded CTG repeat in the 3’ untranslated region of the gene encoding myotonic dystrophy protein kinase, and a high prevalence of SNHL (68%) has been reported in DM1 patients [17]. Furthermore, dysfunction of outer hair cells (OHCs) is implicated in SNHL in DM1 patients [18, 19], although how OHC function is impaired has not been clarified. Additionally, it remains unclear whether SNHL is present in Dmdmdx mice, a model of Duchenne MD (19, 20). Finally, although Largemd mice were proposed as a model of FSHD in the 1990s [20], Largemd mice were later proven to be a model of MD-DG type 6 [21]. Thus, molecular mechanisms underlying SNHL in FSHD and DM are ambiguous, and hearing function in MD-DG remains unclear despite the growing categories of MD-DG.

In the present study, we examined hearing function in Largemd and POMGnt1-KO mice, models of MD-DG, and patients with Fukuyama CMD, a type of MD-DG. We found hearing impairment in both MD-DG mouse models and in Fukuyama CMD patients. In the mouse models, profoundly abnormal myelination was observed at the peripheral segment of the cochlear nerve located at Rosenthal’s canal (RC) and the osseous spiral lamina (OSL), where the cochlear nerve is myelinated by Schwann cells. Our findings indicate that MD-DG is associated with congenital, non-progressive retrocochlear hearing impairment.

Results

HL in Large-deficient and POMGnt1-KO mice

To evaluate hearing function in MD-DG, we firstly measured ABRs to click and tone-burst stimuli (8, 16, and 24 kHz) in 2-3-, 5-, 8-, and 10-week-old Large-deficient (Largemd/md) mice. ABR threshold was significantly higher in Largemd/md mice regardless of the age and sound frequency, except for 24 kHz in those aged 5 weeks, than in control (WT) and heterozygous Large-deficient (Largemd/md) mice (Fig 2A). The hearing impairment in Largemd/md mice was non-progressive. In Largemd/md mice, the wave I latency, but not latencies between wave I and wave V (I-V) or III-V, was significantly delayed at the ages of 3 and 8 weeks, and the amplitude of wave I was significantly reduced at the age of 8 weeks compared with control mice (Fig 2B). Distortion product otoacoustic emission (DPOAE), which is used to estimate OHC function in the cochlea [22], was impaired in Largemd/md mice aged 2–9 weeks compared with control mice (S1A Fig). Subsequently, we examined the morphology of the cochlea in Largemd/md mice. Hematoxylin and eosin (HE) and phalloidin staining revealed no apparent morphological abnormality or HC loss in the cochlea in Largemd/md mice at the age of 8 weeks (Fig 2C).
**POMGnTI-KO** mice, another MD-DG mouse model also showed non-progressive ABR impairment at all frequencies compared with control and heterozygous *POMGnTI-KO* mice, but the impairment was milder in *POMGnTI-KO* mice than in *Large<sup>myd<sub>myd</sub></sup>* mice (Fig 3A). In addition, the wave I latency was significantly delayed in *POMGnTI-KO* mice at the age of 4 weeks compared with control mice, but no difference was detected between *POMGnTI-KO* and control mice at the age of 10 weeks (Fig 3B). No abnormality was observed in DPOAE in *POMGnTI-KO* mice at the age of 6 weeks (S1B Fig). In addition, no apparent morphological abnormality or HC loss was observed in *POMGnTI-KO* mice (Fig 3C). These findings suggest that: 1) hearing function is impaired in both these mouse models of MD-DG; 2) the hearing impairment is already present and non-progressive at 2–4 weeks after birth, when hearing function has matured [23, 24]; and 3) the impairment degree is more in *Large<sup>myd<sub>myd</sub></sup>* mice (moderate) than in *POMGnTI-KO* mice (mild).

**Decreased levels of α-DG glycosylation, laminin, and myelin basic protein (MBP) at the RC and OSL in *Large<sup>myd<sub>myd</sub></sup>* and *POMGnTI-KO* mice**

In the rodent inner ear, strong immunoreactivity of α-DG has been observed in the perineural basal lamina of the peripheral, but not central, segment of the cochlear nerve, which is clearly demarcated by “the glial dome” [25, 26]. The glial dome is located at the level of the basal turn of the cochlea and is the transitional zone of Schwann cells (peripheral) and oligodendrocytes (central) [27, 28]. Notably, α-DG is strongly positive at the RC and OSL, where cell bodies of spiral ganglion neurons (SGNs) are located and peripheral axons of SGNs (bipolar neurons) pass to reach hair cells (HCs). Moreover, greater degrees of glycosylated α-DG in the cochlea than in the cerebellum and brain have been demonstrated in rodents [25].

To detect the affected lesion in the cochlea in *Large<sup>myd<sub>myd</sub></sup>* and *POMGnTI-KO* mice, we performed immunostaining using antibodies detecting the glycosylated form of α-DG, core α-DG protein, β-DG, pan-laminin, or laminin α2. Although immunoreactivities of core α-DG and β-DG did not differ between control and *Large<sup>myd<sub>myd</sub></sup>* mice, those of glycosylated α-DG, pan-laminin, and laminin α2 significantly decreased at the peripheral portion of the cochlear nerve (distal from the glial dome), especially at the RC and OSL, in *Large<sup>myd<sub>myd</sub></sup>* mice compared with control mice (Fig 4A–4C). Immunoreactivity of glycosylated α-DG, but not core α-DG or β-DG, was also decreased in *POMGnTI-KO* mice compared with control mice (Fig 5A and 5B). Furthermore, the immunoreactivity of laminin α2 was mildly but significantly decreased (Fig 5A and 5B). We were unable to show the decreased levels of glycosylated α-DG or laminin α2 by immunoblotting because of the limitation of the antibodies. However, we detected decreased levels of pan-laminin by immunoblotting using lysates obtained from the spiral ganglion (SG), which is located in the RC, in P5-P7 *Large<sup>myd<sub>myd</sub></sup>* mice (Fig 5C). In *POMGnTI-KO* mice, the pan-laminin levels also tended to be decreased compared with the controls, but in a limited sample number (Fig 5D, n = 1 in WT, n = 2 in heterozygous *POMGnTI-KO*, and n = 2 in *POMGnTI-KO*).

![Fig 2](image1.png)
**Fig 2**
Moderately impaired hearing in *Large<sup>myd<sub>myd</sub></sup>* mice.

![Fig 3](image2.png)
**Fig 3**
Mildly impaired hearing in *POMGnTI-KO* mice.

![Fig 4](image3.png)
**Fig 4**
Decreased glycosylated α-DG and laminin levels distal to the glial dome in *Large<sup>myd<sub>myd</sub></sup>* mice.
Subsequently, to investigate the underlying molecular mechanism of the lesions, we examined the myelination of the cochlear nerve at the RC and OSL using immunohistochemistry with an antibody against MBP, a myelination marker. Immunoreactivity of MBP was decreased at the RC and OSL in Large<sup>m</sup> mice (Fig 6A). The decreased levels of MBP were further confirmed by immunoblotting using lysates obtained from the 5G of P5-P7 Large<sup>m</sup> mice (Fig 6B). Decreased immunoreactivity and protein levels of MBP were also detected in POMGnTI-KO mice (Fig 6C and 6D), but the decreased levels were larger in Large<sup>m</sup> mice than in POMGnTI-KO mice. The expression levels of 2, 3-cyclic nucleotide-3-phosphodiesterase (CNPase), another myelination marker, were also decreased in the Large<sup>m</sup> mice (S2 Fig).

No HL in Dmd<sup>mdx/mdx</sup> mice

HL has been never reported in patients with Duchenne MD or Becker MD [29]. To confirm this finding, we examined HL in Dmd<sup>mdx/mdx</sup> mice, a model of Duchenne MD. ABR and DPOAE of Dmd<sup>mdx/mdx</sup> mice were comparable with those of control mice (S3A Fig). No differences were detected in immunoreactivities of core and glycosylated α-DG proteins (S3B Fig) or MBP between control and Dmd<sup>mdx/mdx</sup> mice (S3C Fig). These findings strongly suggest that HL observed in two mouse models of MD-DG is a notable phenotype/symptom of MD-DG.

Abnormal myelination at the peripheral segment of the cochlear nerve in both Large<sup>m</sup> and POMGnTI-KO mice

In peripheral nerves, α-DG is expressed in the outer (abaxonal) membranes of the Schwann cells to bind the basal lamina [30–32]. Transmission electronic microscopy (TEM) analysis was used to examine myelination of the peripheral segment of the cochlear nerve, which is projected by Schwann cells [27], in Large<sup>m</sup> mice at the age of 2, 6, and 10 weeks. Large<sup>m</sup> mice showed various abnormalities, such as naked axons, axons with disrupted myelin, and axons with secondary changes (vacuoles and/or aggregates) (Figs 7A and S4A and S4B). The percentage of axons with abnormal myelination (naked axons and axons with disrupted myelin) at the OSL was decreased in Large<sup>m</sup> mice aged 6 and 10 weeks compared with that in mice aged 2 weeks and those aged 2 and 6 weeks, respectively; however, this number was significantly higher in Large<sup>m</sup> mice than in age-matched control mice aged 2–10 weeks (Fig 7B). The number of axons with secondary changes was significantly higher in Large<sup>m</sup> mice than in controls 6 and 10, but not 2 weeks old (S4B Fig). In addition, diameters of myelinated axons in the transverse section at the OSL were more broadly distributed in Large<sup>m</sup> mice than in control mice aged 6 weeks (Fig 7C), but it was not significantly different between Large<sup>m</sup> mice aged 6 weeks and those aged 10 weeks (S5A Fig). No apparent difference was observed in the percentage of myelinated axons of the cochlear nerve proximal to the glial dome in Large<sup>m</sup> mice aged 5 weeks (S5B Fig). However, the percentage of axons with abnormal myelination was mildly higher in Large<sup>m</sup> than in control mice (S5B Fig), suggesting that lesions proximal to the glial dome is milder than those distal to the glial dome. No significant difference in total myelination proximal to the glial dome between Large<sup>m</sup> and control mice was confirmed by MBP immunostaining (S5C Fig). Although the difference was smaller in POMGnTI-KO mice than in Large<sup>m</sup> mice, the percentage of axons with abnormal myelination at the OSL was significantly higher in POMGnTI-KO mice than in control mice at the age of 10 weeks (Figs 7D and 7E and S4C and S4D). These findings suggest that the main lesion in MD-DG model mice is localized at the peripheral cochlear nerves distal to the glial dome.
Furthermore, MBP immunoreactivity at the corpus callosum was examined to determine the myelination status in the brain. MBP immunoreactivity was mildly decreased in Large<sup>myld/myld</sup> mice compared with control mice at the age of 8 weeks (S6A Fig). This finding was confirmed by MBP immunoblotting using whole-brain lysates from P7 mice (S6B Fig).

**Fukuyama CMD patients showed delayed latency of wave I in ABR**

Based on the findings obtained from the two mouse models of MD-DG, we hypothesized that MD-DG patients have the primary lesion at the peripheral segment of the cochlear nerve located at the RC and OSL, thus leading to retrocochlear SNHL. To test our hypothesis, we attempted to analyze auditory function in patients with Fukuyama CMD, which is the most frequent MD-DG in Japan. None of the Fukuyama CMD patients analyzed had complaint of deafness (Table 1). Because of mild to moderate intellectual impairment, a subjective audiometric examination was not applicable, and we performed the ABR analysis instead.

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<th>Table 1</th>
<th>Clinical characteristics of Fukuyama CMD patients evaluated in the present study.</th>
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ABR response to 40-dB SPL stimuli were detected in 17 ears among 18 ears in 9 Fukuyama CMD patients, which was recognized by the presence of wave V. Only a 15-month-old boy with homozygous SINE-VNTR-Alu (SVA) mutation (Case 1 in S1 Table) showed no recognizable ABR waveform in his left ear with 60-dB SPL stimuli. However, we could not discriminate whether the undetectable ABR was caused by conductive HL or SNHL; thus, this ear was excluded from the following analysis. In addition, ABRs to 40-dB SPL stimuli were detected in all 18 ears in 9 normal healthy volunteers (S2 Table).

Next, we analyzed the amplitude and latency of ABR waves above the thresholds to examine the possibility of latent hearing impairment. The latency of wave I at 60 dB (2.03 ± 0.32 ms) was significantly delayed in the ears in Fukuyama CMD patients with recognizable ABR compared with normal controls (1.62 ± 0.15 ms, \( P < 0.0001 \)) (Fig 8A and S3 Table). This significant difference in the wave I latency was confirmed in two more detailed analyses, in which Fukuyama CMD patients were divided into the following two groups according to the genetic abnormality: one with SVA homozygous mutation and the other with compound heterozygous mutation. Significantly delayed latency of wave I was detected in both homozygous patients (2.07 ± 0.24 ms, \( P = 0.0006 \)) and compound heterozygous patients (2.00 ± 0.37 ms, \( P = 0.0010 \)) compared with normal volunteers (homozygous, 1.57 ± 0.15 ms and heterozygous, 1.66 ± 0.15 ms) (Fig 8B and 8C and S4 and S5 Tables). The amplitude of wave I and the interpeak latency between waves I and V were not significantly different in all three analyses (Fig 8A–8C and S3–S5 Tables). These clinical findings suggest that Fukuyama CMD patients have latent/subclinical SNHL.

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<th>Fig 8</th>
<th>Delayed latency of wave I of ABR in Fukuyama CMD patients.</th>
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**Discussion**

The present study indicates that Large<sup>myld/myld</sup> and POMGnTI-KO mice, models of MD-DG, exhibit congenital hearing impairment, and the lesions are localized at the peripheral segment of the cochlear nerve, where myelination is projected by Schwann cells, but not oligodendrocytes [27]. Large and POMGnTI are expressed ubiquitously, including in skeletal muscles; however, very few reports have investigated their spatio-temporal expression patterns. Large is also highly expressed in the nervous system from the embryonic stage, including in the cerebral cortex and trigeminal ganglion [33, 34]; in contrast, expression of POMGnTI in the brain is relatively low [35]. Although POMGnTI and Fukutin expression has been reported in astrocytes, Large, POMGnTI, or Fukutin expression in Schwann cells has not been reported [36]. In peripheral nerves, α-DG is expressed in the outer (axonal) membranes of Schwann cells to bind to basal lamina proteins, such as laminin α2 [30–32]. The mechanism of myelin formation, in which the anchorage of the axonal membranes to the basal lamina provided by the α-DG–laminin complex enables the inner lips of spiraling Schwann cells to progress over the axonal membranes, is well studied [30, 37, 38]. Impairment of peripheral nerve myelination causing various axonal abnormalities, including naked axons and axons with abnormal myelin, was reported in Large<sup>myld/myld</sup> mice [39], Fukutin-deficient chimeric mice [40], a patient with LAMA2 deficiency [41], and a Fukuyama CMD patient [42]. Moreover, the dystroglycan–laminin complex is reportedly more important for
myelination/differentiation of Schwann cells than for their survival/proliferation [43]. The findings in these studies support our results that α-DG and laminin α2 are critical for myelin formation in the peripheral segment of the cochlear nerve.

The mechanisms underlying abnormal DPOAE in Large<sup>mod<sub>mod</sub></sup> mice, but not in POMGnTI-KO mice, are currently unknown because we were unable to detect morphological abnormality of OHCs or connective spaces between OHCs and supporting cells or between OHCs and underlying basal lamina, where α-DG is reportedly expressed [25, 26], in Large<sup>mod<sub>mod</sub></sup> mice (S7 Fig). Since MD-DG is a complex disease, which causes lesions in many organs and tissues, including the peripheral nerves, brainstem, and brain, one simple explanation is that the effector fibers in the cochlear nerve, relaying the feedback from cortico-olivocochlear pathways, which is proposed to modulate otoacoustic emission (OAE) generation [44], could be more severely affected in Large<sup>mod<sub>mod</sub></sup> than in POMGnTI-KO mice. Although abnormal cortico-olivocochlear efferent function is associated with various auditory disorders, such as hyperacusis, tinnitus, and poor speech-in-noise recognition [45, 46], its effects on OAE are controversial; its activation decreases and enhances OAE levels depending on stimuli and conditions [46, 47]. Another possibility is that cochlear in Large<sup>mod<sub>mod</sub></sup> mice may have functional issues, but not morphological ones. Since α-DG is reported to be widely expressed in rodent and human cochlea [25, 26, 48], including in the stria vascularis where endocochlear potential is generated, MD-DG dysfunction may affect cochlear function due to reduced endocochlear potential. Indeed, mild morphological atrophy was reported in the stria vascularis in Charcot-Marie-Tooth (CMT) disease type 1B (CMT1B), which is caused by congenital anomalies in myelination by Schwann cells [49, 50]. Additionally, because SNHL in DM1 shows abnormal OAE [18, 19], the same mechanism may underlie the dysfunction of Large<sup>mod<sub>mod</sub></sup> cochlea.

We found that POMGnTI-KO mice showed milder phenotypes than did Large<sup>mod<sub>mod</sub></sup> mice. There are three possible explanations: 1) POMGnTI-KO mice show the detectable amount of properly glycosylated α-DG [51], whereas properly glycosylated α-DG is completely absent in Large<sup>mod<sub>mod</sub></sup> mice [52]. This difference might be due to different enzyme functions: Large is an enzyme responsible for synthesizing laminin-binding matriligycan, whereas POMGnTI acts as a regulatory enzyme for matriligycan formation [8]; 2) more than 60% of POMGnTI-KO mice usually die within 3 weeks after birth [53]; notably, the present study used mice aged ≥ 4 weeks, which probably had milder phenotypes [53, 54]. In fact, there is broad phenotypic variability in patients with abnormal POMGnTI gene [55–57]; and 3) the genetic backgrounds of Large<sup>mod<sub>mod</sub></sup> and POMGnTI-KO mice are different (C57B/6 and 129SvEv, respectively). Regarding the recovery of wave I latency observed in 10-week-old POMGnTI-KO mice, they showed fewer secondary axonal changes than Large<sup>mod<sub>mod</sub></sup> mice (see S4B and S4D Fig), suggesting that, judged on the basis of morphological phenotypes, surviving POMGnTI-KO mice may have a greater potential for functional recovery than Large<sup>mod<sub>mod</sub></sup> mice. Alternatively, in addition to lesions at the peripheral segment of the cochlear nerve, POMGnTI-KO mice may have other lesions, which affect the early hearing phenotype but improve by 10 weeks of age.

Whether MD-DG pathology can lead to SNHL in humans remains an open question. Recently, mild to moderate HL was found in two patients (siblings) with homozygous truncating mutation in O-mannosyl kinase (POMK, Fig 1B), which is required for α-DG glycosylation, and this disease was classified as MD-DG 12 [58]. Among 9 reported patients in 6 families with mutations in POMK [59–62], SNHL was demonstrated in the above mentioned two patients. Among 23 reported patients [63–65] with mutations in B3GALNT2 (MD-DG 11, Fig 1B), one patient [66] had SNHL. Six patients in four families with LARGE mutations were reported, but hearing function based on intensive evaluation was not described [21, 67–69]. In patients with POMGnTI mutations, symptoms associated with SNHL have not been investigated to date [55–57], and no Fukuyama CMD patient with SNHL has been reported either. The Fukuyama CMD patients showed prolonged ABR wave I latency, but their hearing thresholds are less than 40 dB, which is the minimum sound intensity used in this study. Nevertheless, prolonged wave I latency may cause minor hearing symptoms such as tinnitus, since several studies suggested that prolonged latency and reduced amplitude of wave I are characteristic findings for tinnitus patients with normal hearing [70]. Recently, “hidden HL” has been referred to as hearing dysfunction that cannot detected by standard tests of auditory thresholds, but can be diagnosed by a reduced wave I amplitude in the absence of ABR threshold or latency changes [71]. In addition to cochlear synaptopathy, which is induced by impaired synaptic connection between HCs and the cochlear nerve [72], peripheral neuropathy including demyelination of the cochlear nerve has been reported as a cause of “hidden HL” [71]. Demyelination-related hidden HL reportedly had an additional phenotype of permanently increased wave I latency in Guillain-Barre syndrome [73]. Additionally, demyelination-related hidden HL with abnormal speech recognition ability in noisy backgrounds, but normal ABR amplitude and latency, was reported in CMT type 1A (CMT1A) [74].

CMT1A and CMT1B are neuropathies caused by genetic abnormalities in peripheral myelin protein 22 and myelin protein zero, respectively, which are the main myelination-associated proteins in Schwann cells, and commonly show progressive SNHL starting in adolescence [75]. Although Kovach et al. and Starr et al. reported abnormal ABR accompanied by decreased or absent OAE [49, 76], the principal pathology of CMT1B is myelin damage of the cochlear nerve initiating distally (accompanied by secondary damage of the cochlear nerve axons) without IHC loss [49, 50]. While children with CMT1A showed no prolonged latency of wave I [75], adult patients showed prolonged latency of wave I [77]. Thus, both CMT1 and MD-DG cause primary lesions at the distal portion of the cochlear nerve; however, CMT1 shows a progressive phenotype [75]. This difference is most likely to arise due to from the different etiologies of CMT1 and MD-DG (autosomal dominant vs. autosomal recessive inheritance, respectively), and the degree of cochlear synaptopathy involvement may also be different in the two diseases. Possible dysmyelination at the peripheral cochlear nerve in Fukuyama CMD patients may accompany cochlear synaptopathy, and may cause similar symptoms of “hidden HL”, including tinnitus and hyperacusis. These pathologies that are otherwise missed in routine clinical examination could be detected by more intensive hearing assays, such as speech recognition.
test in noisy backgrounds and estimation of auditory temporal resolution/processing [74, 75, 78], suggesting that other types of MD-DGs also cause previously unrecognized common and specific retrocochlear pathologies with widely varying degrees of hearing impairment.

Fukuyama CMD is characterized by the combination of MD and dysgenesis of the central nerve system (CNS). However, a good mouse model of Fukuyama CMD is not available. Mice with knock-in of the SVA-transposon insertion, the most frequent mutation in Japanese Fukuyama CMD patients, show no phenotype because of the only 50% binding reduction in laminin [51], and conventional Fukutin-KO mice are embryonic lethal [79]. Brain MRI of Fukuyama CMD patients demonstrates that cerebral cortical dysplasia is the most frequently detected anomalies (97/207 patients), and white matter abnormalities are the second detected anomalies (35/207 patients) [10]. To date, it is unclear whether white matter lesions are involved in delayed myelination or dysmyelination. Delayed myelination is supported by the observation that white matter lesions lessen with age (usually until 5-year-old) [80]. In addition, other studies support dysmyelination [81] or/hypomyelination [82] based on the following observations: 1) inconsistent distribution of white matter lesions with the time-course of myelination process, 2) no disappearing of white matter lesions in some patients (elder than 5-year-old) [80, 83, 84], and 3) data using MR spectroscopy [85]. Taken together, white matter lesions are not simply delayed myelination, but dysmyelination or/hypomyelination is more or less involved. Indeed, we detected mildly decreased levels of MBP in the brain and the proximal cochlear nerve of Largemut/mut mice. In addition to the white matter lesions, Fukuyama CMD patients have dysplasia in the brain, which may also affect hearing function. However, we detected the prolonged latency of wave I with normal interpeak latency between waves I and V in ABR in Largemut/mut mice, as well as in Fukuyama CMD patients, indicating that the main lesions for SNHL in MD-DG are at the peripheral segment of the cochlear nerve myelinated by Schwann cells, rather than in the brain.

No HL in Duchenne/Becker MD patients [29, 86], as well as Dmdmut/mut mice [87], may be due to compensations by other types of dystrophins and/or utrophin, a homolog of dystrophin [88]. Indeed, specific dystrophin (Dp116) and utrophin are the major subtypes of dystrophins in Schwann cells [30, 88–90]; furthermore, Duchenne MD patients with Dp116 deficiency have no apparent peripheral neuropathy [86, 91]. In addition, agrin, as well as laminin 2, has been shown as the binding partners of α-DG in Schwann cells [92]. These diversities of dystrophin–dystroglycan complex in Schwann cells may account for the milder hearing phenotypes in Fukuyama CMD patients than in two mouse models.

The present study indicates that both animal models and patients of MD-DG have impaired myelination at the peripheral segment of the cochlear nerve (at the RC and OSL), which potentially causes retrocochlear/retrolabyrinthine hearing impairment. Other types of MDs, including Duchenne/Becker MD, may also have the cryptic lesions at the peripheral segment of the cochlear nerve, leading to various types of hearing impairments/symptoms such as HL, “hidden HL”, hyperacusis, and tinnitus. However, we did not find correlations between genotypes (homozygous vs. heterozygous mutation, which generally predict severity of MD phenotypes) and hearing phenotype (prolonged latency of wave I in ABR) in Fukuyama CMD patients. Studies with larger sample sizes are needed to further investigate HL in diseases with impairments of the dystrophin–dystroglycan complex.

Methods

Ethics statement

All procedures and experiments were reviewed and approved by the Institutional Review Board of Kobe University (clinical research number, 1653; animal research number, 26-03-05 and P15201-R3) and were performed in accordance with the ethical standards stated in the 1964 Declaration of Helsinki. Written informed consent was received from participants prior to inclusion in the study.

Human studies

Nine Fukuyama CMD patients (four males and five females) aged 1–19 years and age-matched normal volunteers participated in the study. The diagnosis of Fukuyama CMD patients was confirmed by genetic analysis in all patients from peripheral blood. All patients with Fukuyama CMD had an ancestral SVA mutation in one allele [93]. Four patients (Case 1–4, Table 1) had homozygous SVA mutations. The compound heterozygous mutations in patients with Fukuyama CMD included a nonsense mutation in exon 3 [94] in four patients (Cases 5–8, Table 1) and a deep intrinsic splicing mutation in intron 5 [95] in one patient (Case 9, Table 1). Generally, phenotypes of Fukuyama CMD are more severe in compound heterozygous patients than in those with homozygous SVA mutations [10, 94].

Animals

Large-deficient (Largemut/mut) mice were obtained from Jackson Laboratories. POMGnTI-KO mice were bred in house as previously described [53]. Dystrophin-deficient mdx (Dmdmut) mice were obtained from CLEA Japan. Offspring were genotyped using PCR with the following primer pairs: Largemut: 5′-GGCGTGTTCCATAGTCCAA-3′ and 5′-GGCATAGCCTTCTGGTAAAC-3′ for wild type (WT) and 5′-ATTCAGCTCCAAAAGGTTGAAG-3′ and 5′-GCCAATGGCAAATTGAGGGAAA-3′ for mutant; POMGnTI: 5′-CAGCACTTTCTCCCTTCAACCC-3′ and 5′-ATTTGGCTCGTCCCTCTTGCT-3′ for WT and 5′-AGCTATCGCTGCTATGCTGGG-3′ and 5′-TACCTCTCTCCCGGGAGCAAGGG-3′ for mutant; Dmdmut: 5′-TCATCAAATATCAAATGCTGTAAGTG-3′ for both WT and mutant, 5′-
GTCACTCAGATAGTTGAAGCCATTAG–3’ for WT and 5’ –GTCACTCAGATAGTTGAAGCCATTAAAG–3’ for mutant. All mice were identified by numbered ear tags. Mice were housed in specific pathogen-free conditions using the individually ventilated cage system (Techniplast, Tokyo, Japan), with food and water ad libitum. The animal facility was maintained on a 14-h light and 10-h dark cycle at 23 ± 2°C and 50 ± 10% humidity. Both males and females were used in the analyses unless otherwise indicated. Age-and sex-matched WT siblings were used as controls (mice younger than 1 week were not differentiated based on sex).

Antibodies and chemicals

The following specific antibodies were used for both immunoblotting (IB) and/or immunohistochemistry (IH) (polyclonal unless indicated): monoclonal MBP (ab40390, Abcam, RRID: AB_141521, 1/750 for IB and 1/250 for IH), CNPase (D83E10) XP (Cell Signaling Technology (CST), RRID: AB_10705455, 1/750 for IB), core α-DG (3D7; 1/100 for IH, [52]), glycosylated α-DG (IHH6C4, Millipore, RRID: AB_309828, 1/100 for IH), β-DG (8D5, Novocastro; 1/100 for IH), laminin (L9393, Sigma-Aldrich, RRID: AB_477163, 1/1000 for IB and 1/100 for IH), and laminin α2 (4H8-2, Enzo, RRID: AB_2051764, 1/50 for IH). HRP-conjugated antibodies against GAPDH (M171-7, 1:20000) and Alexa Fluor 488-conjugated phalloidin (1/1000) were used as described previously [96]. HE solutions were obtained from Muto Pure Chemicals (Japan).

Histocytometry

Dissected tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) and decalciﬁed in 0.12 M EDTA for 1 week at 4°C to examine surface preparations of the cochlea [24]. After permeabilization, fixed tissues were incubated with Alexa Fluor 488-conjugated phalloidin for 2 h at 23°C. Stained tissues were mounted with Prolong anti-fade (Invitrogen) and were observed under an LSM700 confocal microscope (Carl Zeiss, Jena, Germany).

Tissues were isolated from indicated mice and fixed with 4% paraformaldehyde in 0.1 M PB to perform cross-section analyses of cochlea. Decalciﬁed cochlea were embedded into parafﬁn blocks and cut into 6-μm slices on a Leica RM2125 RTS manual rotary microtome (Leica Biosystems, Wetzlar, Germany). Sections were stained (HE staining or immunostaining) after deparaffinization. To analyze cryosections of the brains, adult mice were transcardially perfused with ice-cold 0.9% saline solution and subsequently with 4% paraformaldehyde in 0.1 M PB. For antigen unmasking, the slides were bathed in HistoVT One (Nacalai Tesque, Kyoto, Japan) for 20 min at 80°C. Retrieved tissues were blocked in either 5% fat-free BSA and 3% H2O2 (Nacalai Tesque) or 0.1% phenylhydrazine (Nacalai Tesque) for 20 min at 23°C. The tissues were incubated with primary antibodies for 2 h at 23°C, followed by MACH 2 Universal HRP-Polymer Detection (BIOCARE Medical, Pacheco, CA, USA) for 30 min at 23°C. Sections were visualized after staining with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) and 0.02% H2O2 in Tris-buffered saline (pH 7.6). Slides were washed and were mounted in Entellan New (Merck Millipore, Billerica, MA, USA), coveredslipped, and photographed under a light microscope (Axioplan II; Carl Zeiss) equipped with a DP26 camera (Olympus, Tokyo, Japan).

For the quantitative assessment of DAB intensity, we compared sample pairs (control vs. mutant) prepared (fixed, embedded into parafﬁn, cut into slices, and immunostained) on the same day and as per the same schedule and conditions, such as the duration of developing times. We used ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the color deconvolution plugin for proper separation of the DAB color spectra, as previously described [97]. Briefly, the region of interest (ROI, see Fig 4C) was manually determined with the polygon or freehand tool, and the deconvoluted image was then analyzed pixel-by-pixel. The color threshold for the positive area was deﬁned in the range of 61–125/255, and the ratio of the positive area to the total image area was calculated and presented as a percentage of the control sample ratio.

Immunoblotting

Dissected tissues from P5-7 mice, such as the SG and whole brain, were lysed in homogenizing buffer [98] by sonicating in the presence of a protease inhibitor cocktail (Nacalai Tesque) and 1% Triton X-100. Total cell lysates were centrifuged at 800 × g for 5 min at 4°C, and the supernatants were subjected to SDS-PAGE followed by immunoblotting for 2 h at 23°C using primary antibody. The bound primary antibodies were detected with secondary antibody-HRP conjugates using the ECL detection system.

For quantiﬁcation, the relative expression levels of interested molecules were normalized to that of GAPDH, as previously described [96].

ABR and DPOAE measurements

To assess hearing, ABR and distortion product otoacoustic emission (DPOAE) were measured in mice under anesthetization with a mixture of medetomidine, midazolam, and butorphanol (intraperitoneal, 0.3 mg/kg, 4.0 mg/kg, and 5.0 mg/kg, respectively) and on a heating pad. In mice, ABR and DPOAE are reportedly mature and saturated at 2 weeks and 2–4 weeks after birth, respectively [23]. Blinded data analysis was performed by two otologists.

ABR was measured in Large<sup><small>Wt</small></sup>, PomGonT1-KO, and Dma<sup><small>mdc</small></sup> mice with their littermate control mice at the indicated ages, as described previously [96]. ABR waveforms using sound stimuli of clicks or tone bursts at 8 kHz, 16 kHz, 24 kHz, or 32 kHz were recorded for 12.8 ms at a sampling rate.
of 40,000 Hz using 50–5000 Hz band-pass filter settings, and ABR waveforms from 500 stimuli were averaged. ABR thresholds (dB SPL) were defined by decreasing the sound intensity by 5 dB intervals until the lowest sound intensity level, resulting in a recognizable ABR wave pattern (mainly judged by recognition of wave III), was achieved.

DPOAE was measured in Large\textsuperscript{null}, POMGnT1-KO, Dmd\textsuperscript{null} mice with their littermate controls at the indicated ages, as described previously [96]. DPOAE at frequency of 2f1–f2 were elicited using two primary tone stimuli, f1 and f2, with sound pressure levels of 65 and 55 dB SPL respectively, with f2/f1 = 1.20. DPOAE amplitudes (dB SPL) were measured at f2 frequencies of 4, 6, 8, 10, 12, 16, 18, and 20 kHz and plotted after substitution with noise floor amplitude.

**ABR studies in humans**

The auditory function in Fukuyama CMD patients was evaluated using ABR. In order to avoid risks related to the sedation procedures in Fukuyama CMD patients, which is usually required for conventional ABR testing, we used the Integrity 500 System (Rion Co. Ltd., Tokyo, Japan) that can measure ABR in awake patients using a combination of in situ amplifier-electrodes and Kalman-weighted averaging [99]. Each participant was examined by otoscopy prior to the ABR testing, and participants with abnormal otoscopic findings were excluded from the study. The normal hearing in controls was verified by responses to pure tone audiometry or condition-oriented response audiometry at sound intensity of 40 dB in 1 kHz and 4 kHz. The ABR waveforms using click stimuli presented through insert-earphones (Intelligent Hearing Systems, Miami, FL) were recorded for 25.0 ms at a sampling rate of 27.5 Hz using 30–1500 Hz band-pass filter settings. Equivalent sweeps determined by Kalman weighted algorithm were spontaneously averaged and visualized as ABR waveforms, and the recording was continued until both wave I and V became recognizable and plateaued or the number of the equivalent sweeps became larger than 2000.

Obtained ABR waveforms with sound intensity of 40 dB SPL and 60 dB SPL were evaluated by two otologists. A non-recognizable wave pattern larger than 2000 equivalent sweeps was defined as no response. ABR thresholds were determined as minimal sound intensities at which recognizable wave V was obtained. After the thresholds were determined, the latency and amplitude of each wave were analyzed at 60 dB, since the waveform was more clearly determined at 60 dB than at 40 dB. The amplitudes and the latencies of wave I and V and the interpeak latency between wave I and V in Fukuyama CMD patients were analyzed and compared with those in age- and sex-matched normal controls. An amplitude of a wave was defined as the voltage difference between the peak of the wave and the adjacent negative peak after the wave.

**TEM**

Sample preparation and observation were conducted as reported previously [24]. Briefly, freshly dissected inner ear tissues were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M PB. OC epithelia were dissected in the same buffer and postfixed with 1% OsO\textsubscript{4} in H\textsubscript{2}O for 1 h. For TEM analyses, samples were embedded in Spurr Low-Viscosity Embedding Media after post-fixation (Polysciences, Germany) and polymerized at 70°C for 8 h. Ultra-thin sections (thickness ~70 nm) were cut using an ultramicrotome (EM-UC7; Leica Microsystems, Germany), placed on copper grids, and examined on Hitachi H-7100 electron microscope at 80 kV.

For the quantitative assessment of myelination, we classified abnormal axons into three categories in the transverse section at the OSL (see Fig 6A): “naked axons”, “axons with disrupted myelin”, and “axons with secondary changes (vacuoles and/or aggregates)”. “Axons with abnormal myelination” was defined as the sum of “naked axons” and “axons with disrupted myelin.” An “axon with myelination” (S5B Fig) was defined as a myelinated axon regardless of myelin morphology. We also measured the longest diameter of each myelinated axon (including axons with abnormal myelin, but excluding naked axons) in the transverse section at the OSL; their distribution is shown as a bar graph (x-axis: diameter, y-axis: number of axon) and boxplot.

**Statistical analysis**

All data are presented as mean ± SE. Two groups were compared using the unpaired two-tailed Student’s t-test, Kolmogorov–Smirnov test, or Mann-Whitney’s U-test. For comparisons of more than two groups, one-way ANOVA or two-way ANOVA was performed, followed by Tukey’s or Bonferroni’s post hoc test of pairwise group differences. Statistical analyses were performed using Prism 7.0 software (GraphPad); a P-value of < 0.05 was considered statistically significant.

**Supporting information**
S1 Fig

**DPOAE in Large<sup>myd/myd</sup> and POMGnTI-KO mice.**

A, DPOAE assessed using pure-tone bursts at 4, 6, 8, 10, 12, 16, 18, and 20 kHz in 5-week-old control (Large<sup>wt/wt</sup>, n = 6), Large<sup>myd/myd</sup> (n = 4), and Large<sup>myd/myd</sup> mice (n = 6). DPOAE assessed using pure-tone bursts at 12 and 18 kHz in 2-, 5-, and 9-week-old control mice (n = 6, 6, and 4, respectively) and Large<sup>myd/myd</sup> mice (n = 5, 6, and 4, respectively) was graphed. **p < 0.0001 and ***p = 0.0007** (control vs. Large<sup>myd/myd</sup>) using two-way ANOVA with Tukey’s post-hoc test. B, DPOAE assessed with pure-tone bursts at 4, 6, 8, 10, 12, 16, 18, and 20 kHz in 6-week-old control (wt/wt, n = 4), heterozygous POMGnTI-KO (ko/wt, n = 4), and POMGnTI-KO (ko/ko, n = 4) mice. No significant difference was observed.

(TIF)

Click here for additional data file.(575K, pdf)

S2 Fig

**Decreased CNPase levels in the SG/RC in Large<sup>myd/myd</sup> mice.**

Lysates were obtained from the spiral ganglion (SG)/Rosenthal’s canal (RC) of the P5-7 control and Large<sup>myd/myd</sup> mice. CNPase immunoblotting showed decreased levels of CNPase in the Large<sup>myd/myd</sup> mice. Comparative loading of proteins was confirmed by immunoblotting of GAPDH. Statistical analysis was performed in pairs of the control (n = 9) and Large<sup>myd/myd</sup> mice (n = 7), **p = 0.0015** by Student’s t-test.

(TIF)

Click here for additional data file.(548K, pdf)

S3 Fig

**Normal hearing function and immunoreactivity of α-DG and MBP in Dmb<sup>p<sub>ds</sub>/ind</sub> mice.**

A, ABR with click and pure-tone bursts at 8, 16, 24, and 32 kHz and DPOAE with pure-tone bursts at 8, 12, 16, and 20 kHz were performed in 12-week-old control (Dmb<sup>p<sub>ds</sub>/wt</sub>, n = 6) and Dmb<sup>p<sub>ds</sub>/ind</sub> mice (n = 6). ABR latency of wave I at the click stimulation of 90 dB in control and Dmb<sup>p<sub>ds</sub>/ind</sub> mice (n = 4 and 6, respectively) were graphed. No significant differences were observed in ABR and DPOAE analyses using two-way ANOVA with Bonferroni’s post-hoc test and in ABR latency of wave I analysis using Student’s t-test (P = 0.1230). B and C, Inner ears of 12-week-old control and Dmb<sup>p<sub>ds</sub>/ind</sub> mice were fixed for immunostaining of glycosylated α-DG [α-DG(gly)] and core α-DG [α-DG(core)] proteins (B; n = 4 and 6, respectively) and MBP (C; n = 5). Statistical analysis of immunoreactivity was performed and graphed. No significant difference was observed between control and Dmb<sup>p<sub>ds</sub>/ind</sub> mice using Student’s t-test (P = 0.2039 in α-DG(gly), P = 0.1597 in α-DG(core), and P = 0.4902 in MBP). Scale bars: 50 μm.

(TIF)

Click here for additional data file.(8.4M, pdf)
S4 Fig

Quantification of abnormal myelination and axons at the OSL in Large\abcdef and POMGnTI-KO mice.

Inner ears of control and Large\abcdef mice (A, B) and control and POMGnTI-KO mice (C, D) were fixed for transmission electron microscopy (TEM). TEM images at the osseous spiral lamina (OSL) were obtained. Each percentage was obtained by analyzing 50 axons. Statistical analyses were conducted using the Student’s t-test unless indicated. A, Graph showing the percentages of naked axons (**\*P < 0.0001) and axons with disrupted myelin (**\*P = 0.0016) in 6-week-old control (n = 5) and Large\abcdef (n = 6) mice. B, Graph showing the percentage of axons with secondary changes in 2-week-old control and Large\abcdef mice (n = 3, P = 0.5614), 6-week-old control (n = 5) and Large\abcdef (n = 6) mice (**\*P = 0.0100), and 10-week-old control (n = 5) and Large\abcdef (n = 5) mice (**\*P = 0.0089). *P = 0.0398 (Large\abcdef mice at 2 vs. 10 weeks) using one-way ANOVA with Tukey’s post-hoc test. C–D, Graph showing the percentages of naked axons (**\*P = 0.0024), axons with disrupted myelin (*\*P = 0.0278), and axons with secondary changes (P = 0.6918) in control and POMGnTI-KO mice (n = 3).

(TIF)

Click here for additional data file.(349K, tif)

S5 Fig

Myelination at the OSL and proximal to the glial dome in Large\abcdef mice.

Inner ears of 6- and 10-week-old control and Large\abcdef mice (A) and 5-week-old control and Large\abcdef mice (B) were fixed for transmission electron microscopy (TEM). TEM images at the osseous spiral lamina (OSL, A) and proximal to the glial dome (GD) (B, at the region indicated by the asterisk in Fig 4A) were obtained. Cochlear of 8-week-old control and Large\abcdef mice (C) were fixed for MBP immunostaining. A, The longest diameter of each myelinated axon in the transverse section at the OSL in Large\abcdef mice at 6 weeks (n = 100) and 10 weeks (n = 74) were statistically analyzed (3 cochleae) using the Kolmogorov–Smirnov test. No significant difference was observed (P = 0.3043). B, The percentage of axons with myelination and of axons with abnormal myelinations in control and Large\abcdef mice were calculated per x 5000-field, and statistically analyzed (total 10 fields of each obtained from 3 control and 5 Large\abcdef mice) using the Student’s t-test. Lower panels represent magnified images of the areas indicated by the squares in the upper panels. No significant difference was observed in axons with myelination (P = 0.1657), but a significant difference was observed in axons with abnormal myelination (**\*P < 0.0001). Arrowheads indicate abnormal myelination. Scale bars: 1 \m. C, Immunostaining was performed using an MBP antibody, and statistical analyses were conducted between control and Large\abcdef mice (n = 3). No significant difference was observed by Student’s t-test (P = 0.1984). RC: Rosenthal’s canal. Scale bars: 100 \m.

(TIF)

Click here for additional data file.(6.1M, tif)

S6 Fig

Decreased MBP levels in the brain in Large\abcdef mice.

A, Brain sections of eight-week-old control and Large\abcdef mice at the level of the corpus callosum were obtained for immunostaining for MBP (Scale bars: 200 \m). The panels on the right are magnified images of the corpus callosum indicated by the squares in the panels on the left (Scale bars: 100 \m). Decreased immunoreactivity of MBP was observed in Large\abcdef mice. The results are presented as the mean of at least three experiments. B, Whole-brain lysates of P7 control and Large\abcdef mice (n = 5) were obtained for MBP immunoblotting. Decreased expression levels of MBP were observed in Large\abcdef mice compared with those in control mice. Comparative loading of proteins was confirmed by immunoblotting of GAPDH. *P = 0.0121 by Student’s t-test.

(TIF)

Click here for additional data file.(6.8M, tif)
S7 Fig

No apparent morphological anomaly in the cochlea in \textit{Large\textsuperscript{myd/myd}} mice in TEM images.

Organs of Corti of 10-week-old controls and \textit{Large\textsuperscript{myd/myd}} mice were fixed for transmission electron microscopy (TEM) (A and B). TEM images at the apical connective spaces between outer hair cells (OHCs) and supporting cells (SCs) (A, indicated by the rectangle) and between OHCs and underlying basal lamina (BL) (B, indicated by the rectangle). No apparent difference was observed between control and \textit{Large\textsuperscript{myd/myd}} mice. Representative results from three independent experiments are shown (\(n = 3\)). Scale bars: 500 nm.

(TIF)
Click here for additional data file.(5.4M, tif)

S1 Table

ABR analysis of Fukuyama CMD patients.

Latency of wave I (latency I) and wave V (latency V), interpeak latency between wave I and V (interpeak I-V), and amplitude of wave I (amplitude I) in nine Fukuyama CMD patients analyzed in the present study are shown. Severity is classified based on the physical activity: mild, able to crawl; moderate, able to sit; severe, unable to control head position. CC, cerebellar cyst; ID, intellectual disability.

(DOCX)
Click here for additional data file.(25K, docx)

S2 Table

ABR analysis of healthy volunteers (controls) evaluated in the present study.

Latency of wave I (latency I) and wave V (latency V), interpeak latency between wave I and V (interpeak I-V), and amplitude of wave I (amplitude I) in controls are shown.

(DOCX)
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S3 Table

Comparison between all Fukuyama CMD patients and controls.

Participant number, sex, mean age, and ABR data were compared between all Fukuyama CMD patients (total) and controls.

(DOCX)
Click here for additional data file.(21K, docx)
S4 Table

Comparison between Fukuyama CMD patients with homozygous mutations and controls.

Participant number, sex, mean age, and ABR data were compared between Fukuyama CMD patients with homozygous (homo) mutations and controls.

(DOCX)

Click here for additional data file, (21K, .docx)

S5 Table

Comparison between Fukuyama CMD patients with heterozygous mutations and controls.

Participant number, sex, mean age, and ABR data were compared between Fukuyama CMD patients with heterozygous (hetero) mutations and controls.

(DOCX)

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S1 Data

Raw data of Fig 2A.

(XLSX)

Click here for additional data file, (15K, .xlsx)

S2 Data

Raw data of Fig 2B.

(XLSX)

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S3 Data

Raw data of Fig 3.

(XLSX)

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S4 Data

Raw data of Figs 4–7.

(XLSX)

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S5 Data

Raw data of S1 Fig.

(XLSX)

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S6 Data

Raw data of S2 Fig.

(XLSX)

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S7 Data

Raw data of S3 Fig.

(XLSX)

Click here for additional data file (14K, xlsx)

S8 Data

Raw data of S4–S6 Figs.

(XLSX)

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Data Availability

All relevant data are within the manuscript and its Supporting Information files.

Article information

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Decision Letter 0

Thomas B. Friedman, Guest Editor and Hua Tang, Section Editor: Natural Variation

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31 Dec 2019

Dear Dr. Ueyama,
Thank you very much for submitting your Research Article entitled 'Impaired α-dystroglycan sugar chains at the peripheral cochlear nerve cause congenital hearing loss with dysmyelination' to PLOS Genetics. Your manuscript was fully evaluated at the editorial level and by independent peer reviewers. The reviewers appreciated the attention to an important problem, but raised some major and minor concerns about the current manuscript. Based on the reviews, we will not be able to accept this version of the manuscript, but we would be willing to review again a much-revised version. We cannot, of course, promise publication at that time.

Should you decide to revise the manuscript for further consideration here, your revisions should address the specific points made by each reviewer. Reviewer #1 has raised some significant concerns each of which requires your attention. We will also require a detailed list of your responses to the review comments and a description of the changes you have made in the manuscript.

If you decide to revise the manuscript for further consideration at PLOS Genetics, please aim to resubmit within the next 60 days, unless it will take extra time to address the concerns of the reviewers, in which case we would appreciate an expected resubmission date by email to plosgenetics@plos.org.

If present, accompanying reviewer attachments are included with this email; please notify the journal office if any appear to be missing. They will also be available for download from the link below. You can use this link to log into the system when you are ready to submit a revised version, having first consulted our Submission Checklist.

To enhance the reproducibility of your results, we recommend that you deposit your laboratory protocols in protocols.io, where a protocol can be assigned its own identifier (DOI) such that it can be cited independently in the future. For instructions see our guidelines.

Please be aware that our data availability policy requires that all numerical data underlying graphs or summary statistics are included with the submission, and you will need to provide this upon resubmission if not already present. In addition, we do not permit the inclusion of phrases such as "data not shown" or "unpublished results" in manuscripts. All points should be backed up by data provided with the submission.

While revising your submission, please upload your figure files to the Preflight Analysis and Conversion Engine (PACE) digital diagnostic tool. PACE helps ensure that figures meet PLOS requirements. To use PACE, you must first register as a user. Then, login and navigate to the UPLOAD tab, where you will find detailed instructions on how to use the tool. If you encounter any issues or have any questions when using PACE, please email us at figures@plos.org.

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To resubmit, use the link below and 'Revise Submission' in the 'Submissions Needing Revision' folder.

[LINK]

We are sorry that we cannot be more positive about your manuscript at this stage. Please do not hesitate to contact us if you have any concerns or questions.

Yours sincerely,

Thomas B. Friedman

Guest Editor

PLOS Genetics

Hua Tang

Section Editor: Natural Variation

PLOS Genetics

Reviewer's Responses to Questions

Comments to the Authors:

Please note here if the review is uploaded as an attachment.

Reviewer #1: This is a solid descriptive study of hearing loss in two mouse models of muscular dystrophy. Muscular dystrophy is caused by degeneration of skeletal muscles, often due to changes in the dystrophin-dystroglycan complex, which mediates attachments between cells and the extracellular matrix. Because this complex is present in other tissues, some forms of MD may include additional phenotypes, such as sensorineural
hearing loss. The origin and nature of MD associated hearing loss is not well defined. This study provides new evidence that hearing may be affected in some forms of MD, possibly due to changes in myelination. Specifically, the data show that 1) ABR and DPOAE thresholds are elevated in Large(mdy/mdy) mutant mice, with decreased ABR wave 1 amplitudes and increased wave 1 latencies; 2) ABR but not DPOAE thresholds are modestly elevated in POMGnT1-KO mice; 3) alpha-DG and laminin alpha2 levels are diminished in the myd/myd and POMGnT1-KO mice, as shown by immunostaining and Western blot; 4) myelination is affected, with decreased MBP and CNPase levels and structurally abnormal myelin shown by EM; 5) Wave 1 is also delayed in patients with Fukuyama congenital MD. Thus, the authors conclude that “Impaired alpha-dystroglycan sugar chains at the peripheral cochlear nerve cause congenital hearing loss with dysmyelination.”

Overall, the study presents interesting new data highlighting the possibility of hearing loss in some (but not all) forms of MD. The data convincingly demonstrate that hearing is affected in two mouse models, both of which show associated changes in the levels of glycosylated alphy-a-dystroglycan and laminin in the region of the cochlea where SGN peripheral processes are myelinated. Further, the authors show changes in myelination that are stronger distal to the glial dome than proximal. My major criticism is that the data do not firmly link the observed cellular changes to the auditory phenotype. Specifically, although the data do support the conclusion that both mouse strains exhibit congenital hearing loss with dysmyelination, the data do not prove that the changes in the sugar changes in the peripheral nerve are responsible for all of the reported phenotypes (i.e. elevated thresholds, abnormal DPOAEs). Additionally, the dysmyelination phenotype needs to be quantified better.

Major points:

1. The title is not accurate. The studies convincingly show that alpha-dystroglycan and laminin protein levels are decreased at the peripheral cochlear nerve and that the mice do not hear well, but the experiments do not directly link these two observations. In fact, it is striking that the hearing phenotypes differ in the two mouse models: DPOAEs are essentially absent in the myd/myd mice but normal in the POMGnT1-KO mice. Since there is a strong DPOAE phenotype in the myd/myd mice, it is impossible to interpret the decreased wave 1 amplitude and increased latency, as these changes could be secondary to abnormal cochlear function. The POMGnT1-KO mice show only a transient change in wave 1 latency though thresholds are elevated at all ages. Since the abnormal myelination is shown at 10 weeks in that mutant, a time when latencies are recovered, it seems like the change in myelination cannot be responsible for the change in latency. Also, since both mice show changes in alpha-dystroglycan glycosylation and dysmyelination in the peripheral cochlear nerve yet only one strain shows a DPOAE phenotype, these cellular phenotypes are unlikely to be the full explanation for the observed hearing loss. Other interpretations need to be considered.

2. The authors demonstrate changes in alpha-dystroglycan and laminin using both immunohistochemistry and Western blot. However, it is hard for me to interpret the immunohistochemistry since it is an enzymatic detection using DAB substrate, which is not a quantitatively reliable assay. The authors should note whether the control and mutant tissues were processed on the same slides and developed for the same amount of time. This information was not provided in the Methods section.

3. The dysmyelination phenotype is striking but needs to be better quantified. I do not understand what is meant by “dispersion of the longest diameter in the myelinated axon” (p. 10, 212-210). This made it very hard for me to assess the data shown in Fig. 6. Please explain exactly what was measured and how. Likewise, I do not understand the definition of “normal myelinated axons” or how that was assessed objectively. Since the effect on myelination in both mouse lines is a key finding, these data should be better quantified overall. For instance, Fig 6 illustrates naked axons, disrupted myelin, vacuolated axons, and aggregated axons. The authors should quantify the incidence of each of these pathologies, blind to genotype, in mutants and littermate controls.

4. The authors note that “None of the FCMD patients analyzed had complaint of deafness” (line 236, page 11), but go on to show that wave 1 latencies were increased. The authors should discuss this point in the Discussion, as it has an impact on the clinical value of the information presented. For instance, please discuss how increased latencies might affect hearing and whether there are assays that might reveal a deficit that is otherwise missed by both the audiologists and the patients.

5. Since the primary cellular phenotype seems to be a change in myelination, there should also be some discussion of how the hearing phenotype compares to what is observed in other mouse models with impaired myelination.

6. For the patient analysis, the authors should report auditory thresholds so that the effect on latencies can be properly interpreted. I am also curious whether patients with “mild” motor phenotypes also had “mild” effects on latency.

Minor points:

1. I expect this paper will be interesting to hearing researchers who are unlikely to be familiar with the various forms of muscular dystrophy. The introduction needs to be improved to make it easier for non-experts to follow the differences between the three mouse models presented as well as the human form of MD that is assessed. This could be achieved by reworking the introduction and perhaps adding a figure introducing the various enzymes related to the mouse and human mutants that are ultimately examined.
2. I do not think it is appropriate to use a phrase such as “To prove our hypothesis.” This wording suggests that the authors wanted to be right. It is more appropriate to say “To test our hypothesis.” This may seem minor but I think it is important to demonstrate a value for unbiased and objective approaches in official scientific publications.

3. The paper should be proofread and edited for improved clarity. For instance, I believe “torn bursts” is meant to be “tone bursts” (line 420, p. 20) and “spinal ganglion” should be “spinal ganglion” (line 822, p. 36).

Reviewer #2: In the manuscript by Morioka et al., the authors catalog numerous phenotypes in mainly two mouse models (Large and Pomn1 deletion lines) for muscular dystrophy. Both mouse models show hearing impairment with some aspects being progressive. Importantly, the authors zero in on various Schwann cell phenotypic defects in their models, including defects in myelination, alpha-Dystroglycan expression, laminin expression and others. The data from the mouse models is correlated with auditory defects reported in patients with muscular dystrophy. Thus, with regard to muscular dystrophy, the authors draw a reasonable link between their mouse models and a patient population. In their analyses, the authors performed a battery of quantitative comparisons (between mutants and WT) and the sample power and statistical analyses throughout the manuscript are excellent. The experiments are reported in logical sequence and appropriate control experiments are provided.

One complaint is that the title of the manuscript suggests the authors have discovered a causal link between the loss of alpha-dystroglycan and hearing loss. This is not at all accomplished and so a more conservative title is strongly recommended.

The Discussion should provide more information on important future directions, like an investigation of the temporal and spatial expression patterns of Large and Pomn1. There should also be a more thorough discussion of possible defects in efferent fibers and OHC function, which could underlie the DPOAE defects reported. Also, have the Schwann cells started to die off at some point? Are they poorly differentiated? These questions should also be addressed in the Discussion.

A few minor issues:

The authors use excessive acronyms and these add a lot of visual clutter to the narrative. I suggest choosing only two or three terms to reduce to acronyms, and to otherwise spell out everything else.

The statement in the last two sentences of the 2nd paragraph (Discussion section, line 278-281) needs references to back up the hypothesis.

Figure 4D and S2B: Can authors show quantitative data for this experiment?

Line 399: Is this reference to figure 4C accurate?

Figure S6: Is this figure missing quantitative data? The Figure legend says that the results are presented as mean of at least 3 representative experiments.

Figure S6: The cartoon has some thick lines extending from HC. What are these?

********

Have all data underlying the figures and results presented in the manuscript been provided?

Large-scale datasets should be made available via a public repository as described in the PLOS Genetics data availability policy, and numerical data that underlies graphs or summary statistics should be provided in spreadsheet form as supporting information.

Reviewer #1: Yes

Reviewer #2: No: It does not appear that all numerical data that goes into graphs and statistics has been provided.

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Do you want your identity to be public for this peer review? For information about this choice, including consent withdrawal, please see our Privacy Policy.

Reviewer #1: No

Reviewer #2: No

2020 May; 16(5): e1008826.
Author response to Decision Letter 0

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27 Feb 2020

Attachment

Submitted filename: Step-by-step response_PLOS Genetics_after editing_200227.doc

Click here for additional data file.(141K, doc)

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Published online 2020 May 26. doi: 10.1371/journal.pgen.1008826.r003

Decision Letter 1

Thomas B. Friedman, Guest Editor and Hua Tang, Section Editor: Natural Variation

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28 Apr 2020

* Please note while forming your response, if your article is accepted, you may have the opportunity to make the peer review history publicly available. The record will include editor decision letters (with reviews) and your responses to reviewer comments. If eligible, we will contact you to opt in or out. *

Dear Dr Ueyama,

Thank you very much for submitting your Research Article entitled 'Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy' to PLOS Genetics. Your manuscript was fully evaluated at the editorial level and by independent peer reviewers. The reviewers appreciated the attention to an important topic but identified some aspects of the manuscript that should be improved.

We therefore ask you to modify the manuscript according to the review recommendations before we can consider your manuscript for acceptance. Your revisions should address the specific points made by each reviewer.

In addition we ask that you:

1) Provide a detailed list of your responses to the review comments and a description of the changes you have made in the manuscript.

2) Upload a Striking Image with a corresponding caption to accompany your manuscript if one is available (either a new image or an existing one from within your manuscript). If this image is judged to be suitable, it may be featured on our website. Images should ideally be high resolution, eye-catching, single panel square images. For examples, please browse our archive. If your image is from someone other than yourself, please ensure that the artist has read and agreed to the terms and conditions of the Creative Commons Attribution License. Note: we cannot publish copyrighted images.

We hope to receive your revised manuscript within the next 30 days. If you anticipate any delay in its return, we would ask you to let us know the expected resubmission date by email to plosgenetics@plos.org.

If present, accompanying reviewer attachments should be included with this email; please notify the journal office if any appear to be missing. They will also be available for download from the link below. You can use this link to log into the system when you are ready to submit a revised version, having first consulted our Submission Checklist.

While revising your submission, please upload your figure files to the Preflight Analysis and Conversion Engine (PACE) digital diagnostic tool. PACE helps ensure that figures meet PLOS requirements. To use PACE, you must first register as a user. Then, login and navigate to the UPLOAD tab,
Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy

where you will find detailed instructions on how to use the tool. If you encounter any issues or have any questions when using PACE, please email us at figures@plos.org.

Please be aware that our data availability policy requires that all numerical data underlying graphs or summary statistics are included with the submission, and you will need to provide this upon resubmission if not already present. In addition, we do not permit the inclusion of phrases such as "data not shown" or "unpublished results" in manuscripts. All points should be backed up by data provided with the submission.

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To resubmit, you will need to go to the link below and 'Revise Submission' in the 'Submissions Needing Revision' folder.

[LINK]

Please let us know if you have any questions while making these revisions.

Yours sincerely,

Thomas B. Friedman

Guest Editor

PLOS Genetics

Hua Tang

Section Editor: Natural Variation

PLOS Genetics

Reviewer's Responses to Questions

Comments to the Authors:

Please note here if the review is uploaded as an attachment.

Reviewer #2: The authors put forth great effort to address the concerns that came from the first submission. The manuscript is well-suited for publication. One note -- during the revisions, the authors apparently had some survival issues in their POMGnT1-KO mice, which left them with limited sample numbers for quantifying Western blots. They included what they had in Supplementary Figures 2A and 2C. However, the small sample size precluded statistical significance and so statistical data are not shown. Given the small sample size, my opinion is that these data are preliminary and not suitable for publication. I suggest the authors remove Supplementary Figures 2A and C and note the trends and limited sample numbers in the Results section.

***********

Have all data underlying the figures and results presented in the manuscript been provided?

Large-scale datasets should be made available via a public repository as described in the PLOS Genetics data availability policy, and numerical data that underlies graphs or summary statistics should be provided in spreadsheet form as supporting information.

Reviewer #2: None

***********

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Reviewer #2: No

Author response to Decision Letter 1

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2020 May;18(5): e1008826.
Published online 2020 May 26. doi: 10.1371/journal.pgen.1008826.r005

Decision Letter 2

Thomas B. Friedman, Guest Editor and Hua Tang, Section Editor: Natural Variation

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4 May 2020

Dear Dr Ueyama,

We are pleased to inform you that your manuscript entitled "Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy" has been editorially accepted for publication in PLOS Genetics. Congratulations!

Before your submission can be formally accepted and sent to production you will need to complete our formatting changes, which you will receive in a follow up email. Please be aware that it may take several days for you to receive this email; during this time no action is required by you. Please note: the accept date on your published article will reflect the date of this provisional accept, but your manuscript will not be scheduled for publication until the required changes have been made.

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Thank you again for supporting open-access publishing; we are looking forward to publishing your work in PLOS Genetics!

Yours sincerely,

Thomas B. Friedman

Guest Editor
PLOS Genetics

Hua Tang

Section Editor: Natural Variation

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Acceptance letter

Thomas B. Friedman, Guest Editor and Hua Tang, Section Editor: Natural Variation

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20 May 2020

PGENETICS-D-19-01933R2

Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy

Dear Dr Ueyama,
We are pleased to inform you that your manuscript entitled "Congenital hearing impairment associated with peripheral cochlear nerve dysmyelination in glycosylation-deficient muscular dystrophy" has been formally accepted for publication in PLOS Genetics! Your manuscript is now with our production department and you will be notified of the publication date in due course.

The corresponding author will soon be receiving a typeset proof for review, to ensure errors have not been introduced during production. Please review the PDF proof of your manuscript carefully, as this is the last chance to correct any errors. Please note that major changes, or those which affect the scientific understanding of the work, will likely cause delays to the publication date of your manuscript.

Soon after your final files are uploaded, unless you have opted out or your manuscript is a front-matter piece, the early version of your manuscript will be published online. The date of the early version will be your article's publication date. The final article will be published to the same URL, and all versions of the paper will be accessible to readers.

Thank you again for supporting PLOS Genetics and open-access publishing. We are looking forward to publishing your work!

With kind regards,

Jason Norris

PLOS Genetics

On behalf of:

The PLOS Genetics Team

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