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Clinical, muscle pathological, and genetic features of Japanese facioscapulohumeral muscular dystrophy 2 (FSHD2) patients with *SMCHD1* mutations.

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Abstract

Facioscapulohumeral muscular dystrophy 2 (FSHD2) is a genetic muscular disorder characterized by DNA hypomethylation on the 4q-subtelomeric macrosatellite repeat array, D4Z4. FSHD2 is caused by heterozygous mutations in the gene encoding structural maintenance of chromosomes flexible hinge domain containing 1 (*SMCHD1*). Because there has been no study on FSHD2 in Asian populations, it is not known whether this disease mechanism is widely seen. To identify FSHD2 patients with *SMCHD1* mutations in the Japanese population, bisulfite pyrosequencing was used to measure DNA methylation on the D4Z4 repeat array, and in patients with DNA hypomethylation, the *SMCHD1* gene was sequenced by the Sanger method. Twenty patients with D4Z4 hypomethylation were identified. Of these, 13 patients from 11 unrelated families had ten novel and one reported *SMCHD1* mutations: four splice-site, two nonsense, two in-frame deletion, two out-of-frame deletion, and one missense mutations. One of the splice-site mutations was homozygous in the single patient identified with this. In summary, we identified novel *SMCHD1* mutations in a Japanese cohort of FSHD2 patients, confirming the presence of this disease in a wider population than

previously known.

Keywords

Facioscapulohumeral muscular dystrophy 2; Structural maintenance of chromosomes flexible hinge domain containing 1; D4Z4; DNA methylation; Pyrosequence

1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most prevalent genetic muscle diseases [1]. It is clinically characterized by asymmetric involvement of facial, shoulder-girdle, and upper arm muscles. In 95% of patients, the D4Z4 repeat, which is a subtelomeric macrosatellite tandem repeat on chromosome 4q35 is contracted, and this type of FSHD is called FSHD1. FSHD1 patients worldwide have 1 to 10 D4Z4 units, whereas the vast majority from any general population have more than 10 units. In Japan, we previously proposed that a definite diagnosis of FSHD should only be made when the repeat size is 6 or fewer, because most Japanese FSHD1 patients have 1 to 6 D4Z4 repeats [2]. This contraction leads to epigenetic changes on the D4Z4 repeat, namely, a decrease in DNA methylation and histone H3 lysine 9 tri-methylation [3,4]. These epigenetic aberrations result in ectopic upregulation of flanking genes, including double-homeobox gene 4 (*DUX4*) [5-7]. DUX4 is toxic to myogenic cells both *in vitro* and *in vivo* [8,9]. *DUX4* can be stably expressed only on the 4qA haplotype, because only this haplotype includes the polyadenylation signal of *DUX4* [7]. Therefore, all FSHD patients have the 4qA haplotype [10].

On the other hand, less than 5% of FSHD patients do not have D4Z4 repeat contraction, though they show DNA hypomethylation in a wider genomic region, including the D4Z4 repeats on both chromosomes 4 and the highly homologous D4Z4 repeat array on chromosome 10, whereas DNA hypomethylation is observed only on the pathogenic chromosome 4 allele in FSHD1 [11-13]. This type of FSHD is called FSHD2.

Recently, heterozygous loss-of-function mutations in the structural maintenance of chromosome hinge domain containing 1 (*SMCHD1*) gene were identified in FSHD2 [12]. *SMCHD1* is an epigenetic modifier of the inactivated X chromosome, but is also involved in epigenetic modification of the D4Z4 repeat [14-16]. Heterozygous *SMCHD1* mutations are associated with DNA hypomethylation at the D4Z4 repeat, resulting in FSHD2 [12]. In addition, it was also shown that *SMCHD1* mutations can act as a modifier in FSHD1 patients with mildly contracted D4Z4 repeat arrays of 8 to 10 units [17]. This idea is supported by the fact that although 8 to 10 D4Z4 units are observed in healthy subjects, FSHD patients with 8 to 10 D4Z4 units exhibit a wide range of severity [18].

Up to now, no Japanese FSHD2 patient has been reported. We therefore

quantified D4Z4 CpG methylation in clinically suspected FSHD patients in Japan with more than 6 D4Z4 units, using pyrosequence analysis after bisulfite conversion. We first identified patients with DNA hypomethylation on D4Z4 repeat array, and then searched for *SMCHD1* gene mutations in these patients.

2. Patients and Methods

2.1. Patients

All clinical information and materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethics Committee of the National Center of Neurology and Psychiatry. We have analyzed 926 patients from 838 families who were clinically suspected of FSHD by their clinicians and, whose DNA were sent to our institute for D4Z4 repeat analysis from 2002 to 2014. Genetic analysis showed that 602 from 527 families carried an FSHD-sized D4Z4 array (6 or fewer units), while 324 from 311 families carried a normal-sized D4Z4 array (7 or greater untis). Among the 324, we analyzed 308 by bisulfite pyrosequence. Severity of the disease was evaluated for each patient by their local neurologist according to a modified version of clinical severity scale (CSS) described by Ricci et al. [19] (Table S1).

2.2. D4Z4 repeat size analysis

D4Z4 repeat size was analyzed as previously described [20]. Briefly, genomic DNA was digested with *Eco*RI (Takara) or *Eco*RI/*Bln*I (Takara). The digested DNA was

electrophoresed in Gel Electrophoresis Apparatus GNA-200 (Amersham), transferred to Hybond-XL (GE Healthcare), and hybridized with ^{32}P-labeled p13E-11 probe. The membrane was washed twice in 2×SSC, 0.1% SDS for 20min. Digested bands in *Eco*RI with 3kb shorter band in *Eco*RI/*Bln*I was regarded as chromosome 4-type D4Z4 fragment. D4Z4 units was calculated as follows: D4Z4 unit = (D4Z4 length in *Eco*RI digestion (kb) - 6.6) ÷ 3.3. To determine haplotype of D4Z4, genomic DNA was digested with *Hin*dⅢ (Takara), hybridized with 4qA probe, and washed twice in 1×SSC, 0.1% SDS for 15min, followed by autoradiography.

2.3. Quantification of D4Z4 methylation by bisulfite pyrosequencing

DNA (500 ng) extracted from the patients' blood was subjected to bisulfite treatment using EpiTect DNA bisulfite kit (QIAGEN) according to the manufacturer's protocol. The level of D4Z4 methylation was quantified using the pyrosequencing technique. Briefly, the polymerase chain reaction (PCR) was performed by PyroMark PCR Kit (QIAGEN), and ten μl of the biotinylated PCR product was subjected to affinity purification using Streptavidin Sepharose High

Performance (GE Healthcare Life Science) and PyroMark Q24 Advanced CpG Reagents (QIAGEN). PCR primers and sequencing primers were designed using PyroMark Assay Design 2.0 Software (QIAGEN). We designed two sets of PCR primers targeting a different sequence within the DR1 region that is reported to be highly hypomethylated in FSHD2 patients [21]. The primer sequences used are as follows: forward primer 1, GAAGGCAGGGAGGAAAAG; biotinylated reverse primer 1, GCTCAGCCTGGGGATGTGCGGTCT; sequencing primer 1, GGTAGGAGGGGTATTATTT; forward primer 2, TAGGGAGGAAAGGAGGGAAAGATAG; biotinylated reverse primer 2, ACTATAAACCCAACCCTCAAC; sequencing primer 2, GGTTTTAGGGAGTAG. Pyrosequencing was performed using PyroMark Q24 Advanced System (QIAGEN). The seven CpG methylation sites were quantified by PyroMark Q24 Advanced Software.

2.4. Quantification of D4Z4 methylation by Southern blot analysis

Analysis of DNA methylation on D4Z4 was performed using a methylation-sensitive restriction enzyme and Southern blot analysis [12]. Briefly, genomic DNA

was digested with *Eco*RI and *Bgl*II. The DNA was purified with QIAquick PCR purification kit (QIAGEN). Additionally, the purified DNA was digested with *Fse*I. The DNA was electrophoresed in a 0.8% Agarose S gel (Wako). The electrophoresed DNA was capillary-transferred to Hybond-N+ membranes (Amersham). The membrane was prehybridized and hybridized with a p13E-11 probe in DIG EASY Hyb (Roche) according to the manufacturer's instructions. The probe was generated by PCR using the p13E-11 primers (forward: TGGCTCAGTAAAGGGGGATA, reverse: CCCAAAAGCAATGCCAAA). Briefly, the product of the genomic PCR was cloned into pBluescript. The resulting vector was amplified with PCR DIG Probe Synthesis Kit (Roche) using the p13E-11 primers and the amplicon as a probe. After hybridization of the probe to a Hybond-N+ membrane (Amersham), the membrane was incubated with an anti-DIG alkaline phosphatase antibody (Roche), and detected using CDP-*Star* (Roche) according to the manufacturer's instructions. Intensities of the 4,061 bp and 3,387 bp bands were quantified using ImageJ [22]. DNA methylation status was presented as the percentage of the 4,061 bp band intensity to the total intensity of the 4,061 bp band and 3,387 bp band.

2.5. Sanger sequence

We performed Sanger sequencing of all exons and flanking regions of *SMCHD1* in each of the 20 patients with reduced D4Z4 DNA methylation levels measured by bisulfite sequencing. Genomic DNA was extracted from peripheral blood lymphocytes using a standard technique. For mutation screening of the *SMCHD1* gene (NM_015295.2), all exons and their flanking intronic regions were amplified by PCR and directly sequenced using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems). Sequence data were analyzed and visualized using SeqScape software (PE Applied Biosystems). Primer sequences are available on request. The allele frequency of the variants were compared in the following public databases: Exome Variant Server (ESP6500), dbSNP138, and Human Genetic Variation Browser (HGVD). HGVD is the exome database of 1208 individuals in the Japanese population. The pathogenicity of missense mutations were predicted using PolyPhen2 and SIFT. Identified *SMCHD1* mutations were submitted to Leiden Open Variation Database (http://www.LOVD.nl/SMCHD1)

2.6. Analysis of aberrant splicing

Total RNA was extracted from the patients' biopsied muscles and reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) according to the manufacturer's instructions. To detect aberrant splicing, exons 1 to 5, 3 to 8, and 42 to 45 were amplified from the cDNAs of patients 10, 11, and 13, respectively. The amplicons were cloned with TOPO® TA Cloning® Kit with PCR®2.1 TOPO® (Life Technologies) and One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies) according to the manufacturer's instructions. The cloned insert was amplified using KOD Dash (Toyobo) according to the manufacturer's instructions and sequenced as described above.

2.7. Quantitative PCR

cDNA was amplified with the previously described primers for *SMCHD1* and QuantiTect SYBR® Green PCR Kit (QIAGEN) according to the manufacturer's instructions. cDNA was also amplified with primers for *POLR2A* (TaqMan® Gene Expression Assays, Mm00839493_m1). The reactions were quantified with StepOnePlus™ Real-Time PCR System (Applied Biosystems) and analyzed using

StepOne Software v2.3. The amount of the *SMCHD1* transcript was normalized to that of the POLR2A transcript. As control samples, cDNA from two skeletal muscles with normal pathology and two with dystrophic pathology were used.

2.8. Protein analysis

Whole cell extracts from fibroblasts were separated on NuPAGE® Novex® 3%-8% Tris-Acetate Protein Gels (Life Technologies) with NuPAGE® Tris-Acetate SDS running buffer (Life Technologies) using XCell SureLock® Mini (Life Technologies) according to the manufacturer's instructions. Proteins were wet-transferred onto an Immobilon-P transfer membrane (Millipore), using an XCell II™ Blot Module (Life Technologies). Primary antibodies used were a mouse monoclonal anti-α-tubulin antibody (clone B-5-1-2; Sigma-Aldrich) and a mouse monoclonal anti-SMCHD1 antibody kindly provided by Prof. Obuse [14]. Secondary antibodies used were goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) and HRP activity was detected by Can Get Signal Immunoreaction Enhancer Solution (Toyobo). Chemilluminescence was detected with ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

2.9. Histochemical analysis of biopsied muscle

Biopsied muscle was frozen and sectioned at a thickness of ten µm according to standard procedures and stained with a battery of routine histochemical stains, including hematoxylin and eosin (H&E) and nicotinamide adenine dinucleotide hydrogenase-tetrazolium reductase (NADH-TR), as previously described [20].

3. Results

We first measured DNA methylation on D4Z4 by bisulfite pyrosequencing in 19 control samples (average ± standard deviation (SD): 46(%) ± 10) (Figure 1A) and defined a cut off level of DNA hypomethylation on D4Z4 as <26(%), which is the average value minus 2SD (Figure 1A). Among the 308 patients that were clinically suspected of having FSHD by their clinicians and subjected to D4Z4 repeat analysis in our institution, which revealed that their D4Z4 repeat was 7 or greater units, we found 20 patients with DNA hypomethylation on D4Z4 based on bisulfite pyrosequencing (Figure 1B). Among them, 13 patients from 11 unrelated families had mutations in *SMCHD1* detected by Sanger sequence (Table 1). DNA hypomethylation on D4Z4 in the 13 patients was also confirmed by Southern blot with *Fse*I digestion [12] (Figure 1C). Ratio of the number of the patients with *SMCHD1* mutations to FSHD1 (6 or fewer D4Z4 units) was 2% (11/527).

The identified 11 different *SMCHD1* mutations were the following: four splice-site, two nonsense, two in-frame deletion, two out-of-frame deletion, and one missense mutations. These mutations were distributed throughout the *SMCHD1* coding region (Figure 2A). All of the identified mutations were novel, except for

one aberrant splice mutation (NC_000018.9:g.2732488_2732491del) [23]. None of the mutations were present in the public databases ESP6500, dbSNP138, or HGVD. Patients 1 and 2, and patients 6 and 7 were related and shared the same mutation. We obtained cDNA from biopsied muscles of patients 7 and 9 and confirmed the presence of both the wild-type transcript and aberrantly spliced transcripts that cause frameshifts (Figure 2B). Patient 10 had a homozygous splice-site mutation. No consanguinity among the parents was reported. cDNA analysis showed no presence of the wild-type transcript, but showed three aberrantly spliced transcripts that cause 13-, 18-, and 131-bp deletions (Figure 2B). The proportions of the three aberrantly spliced transcripts were 15%, 46%, and 35% in 26 colonies of cDNA clones, consistent with the electrophoresis result (Figure 2B). The total amount of *SMCHD1* transcript was 29% of the wild-type transcript in controls (Figure 2C). Therefore, the in-frame transcript with 18-bp deletion was 13% of the wild-type transcript in controls. Patient 11 had a heterozygous missense mutation that localizes in the putative ATPase domain (Figure 2A). This amino acid change was predicted to be damaging by SIFT and PolyPhen2 analyses. The mutated amino acid was a conserved amino acid among

species (Figure 2D). Patients 12 and 13 had heterozygous mutations that predicted to cause one and five amino acid deletions, respectively. These deleted amino acids were also conserved among species (Figure 2D). The amount of SMCHD1 protein in the fibroblasts from patient 12 was not clearly different compared with that of the controls (Figure 2E).

Clinical features of the patients were evaluated by their local neurologists (Table 2). All 13 patients showed a clinical course consistent with FSHD1. Additionally, 3 of the 13 patients in the early stages of the disease had dysphagia, although this usually occurs in the late stages of this disease.

Muscle pathology data were available for seven of the patients (Figure 3A-I, Table 3). Muscle pathology was variable, from normal to dystrophic changes.

We observed that the median of 4qA D4Z4 unit in 10 unrelated families with heterozygous *SMCHD1* mutation was 13 (Table 1). We did not include patient 5 and 10 in this calculation because we could not assess D4Z4 haplotype in patient 5 due to the lack of sufficient DNA sample and patient 10 was homozygous for *SMCHD1* mutation which might have distorted any relationship observed with D4Z4 length for heterozygous cases. Indeed patient 10 was the only patient

observed with *SMCHD1* mutation and D4Z4 length >14 units.

4. Discussion

In this study, we identified *SMCHD1* gene mutations in 13 Japanese FSHD2 patients from 11 families. Lemmers et al. reported that there are two types of mutation patterns regarding the pathomechanism; putative dominant negative *SMCHD1* mutations (preserving the open reading frame: P-ORF) and haploinsufficiency mutations (disrupting the open reading frame: D-ORF). In our FSHD2 patients with *SMCHD1* mutation, three splice-site mutations in patient 7, 9, and 10 caused aberrant splicing leading to out-of-frame disruption of the reading frame, suggesting that these mutations cause *SMCHD1* haploinsufficiecy (Figure 2B and C). On the other hand, one splice-site mutation in patient 8 was reported to cause aberrant splicing but which leaves the reading frame intact, possibly causing a dominant negative effect [24]. In addition, we identified only one missense mutation and two small in-frame deletion mutations in our cohort. From these results, we demonstrated that the underlying pathogenic mechanism of seven *SMCHD1* mutations (patient 1-7, 9, and 10) was *SMCHD1* haploinsufficiency, while that of the other four *SMCHD1* mutations (patient 8 and 11-13) was dominant negative or unknown (Figure 2E). Thus, we observed a

higher ratio of D-ORF mutation to P-ORF mutations (ratio 64%: 36%) compared to that reported in European and USA population (ratio 31%: 69%) [24]. This may be due to smaller sample size than in the previous report [24], or due to a different genetic background with the different ethnicity. We speculate that there might be a difference in the pool of *SMCHD1* mutations in each ethnicity due to the absence of a hot spot for mutations [24] and due to the possible low penetrance of *SMCHD1* mutations which requires the additional rare genetic factors of D4Z4 contraction with 4qA haplotype to cause FSHD2 [24]. Lemmers *et al.* also reported that P-ORF mutations cause more marked DNA hypomethylation and more deleterious effect than D-ORF mutations. Consistent with their observation, P-ORF mutations in patient 8 and 11 tended to show more marked DNA hypomethylation value than D-ORF mutations in our study (Table 1), although these tendencies are not conclusive yet due to small sample size. Considering D-ORF mutation is more common in Japan than Europe, there may be a protective factor(s) such as repeat length in the Japanese population, although this interesting possibility needs further future investigations.

The median of D4Z4 size in our FSHD2 patients with *SMCHD1* mutation is

clearly smaller than that in previously reported healthy individuals, albeit of different ethnicity [25], suggesting that mildly contracted D4Z4 in addition to the *SMCHD1* mutation may be essential for the development of FSHD2 [11,24]. The median of D4Z4 size in our FSHD2 patients with heterozygous *SMCHD1* mutation (Table 1) is comparable to that (13 units) in European and USA patients [24].

Interestingly, we identified a putative homozygous *SMCHD1* mutation in patient 10. Although we could not obtain the parents' DNA, we suspected that this patient has bi-allelic mutation because we detected no wild-type *SMCHD1* transcript in biopsied muscle. Furthermore, we found comparable amplicon depth in *SMCHD1* to controls (Supplemental data) suggesting that the patient does not have a large deletion in *SMCHD1*. These data may suggest that this patient has homozygous *SMCHD1* mutation. Patient 10 had milder clinical symptoms than other FSHD2 patients. Additionally, the parents of patient 10, who are possibly heterozygous for the mutation, were healthy. This may be explained by two alternative hypotheses. One is that the mutant allele might only be minimally hypomorphic, because the mutant allele expresses an in-frame transcript that might be translated into a functional SMCHD1 protein. The other is that D4Z4 repeat arrays in patient 10

and the parents might be relatively long, which may prevent deleterious effects of the mutant allele, and hence only homozygosity of this allele can cause the disease. In support of this notion, the length of D4Z4 repeat arrays in this patient was longer than that in all patients with heterozygous *SMCHD1* mutation (Table 1), consistent with a previous report of a patient with compound heterozygosity for damaging *SMCHD1* mutations [26]. Patient 10 did not show more marked DNA hypomethylation on D4Z4 repeat array than that of other patients (Table 1), though bi-allelic *SMCHD1* mutation was reported to have more significant effect on DNA methylation status than mono-allelic mutations. Because DNA methylation status is affected by overall length of D4Z4 arrays on chromosome 4 and 10, the significant effect of bi-allelic *SMCHD1* mutation on DNA methylation might be masked in patient 10. To know the precise effect of the homozygous mutation, a delta 1 score should be obtained. This measures the difference between expected D4Z4 CpG methylation based on the total number of D4Z4 units and experimentally observed DNA methylation level [24]. Unfortunately we could not obtain a delta 1 score due to the lack of high-quality DNA.

We found ratio of FSHD2 with *SMCHD1* mutations to FSHD1 (<7 units) was 2%

in Japan. We could not compare the ratio between Japan, and Europe and USA because the precise ratio in Europe and USA was not reported. The ratio is dependent on the frequency of *SMCHD1* mutations and mildly contracted D4Z4. Considering the different ratio of D-ORF and P-ORF mutation as mentioned above, the frequency of *SMCHD1* mutations might be different in each ethnicity. Moreover, D4Z4 size distribution is different in each ethnicity [27]. Thus, the ratio of FSHD2 with *SMCHD1* mutations to FSHD1 might be different in each ethnicity.

Out of our 18 unrelated patients with potential FSHD2 and DNA hypomethylation, we found 7 (39%) patients showed no mutation in *SMCHD1*. Lemmers et al. identified 9 families without *SMCHD1* mutations among 60 potential FSHD2 families with hypomethylation (15%) [24,28]. If deletion of *SMCHD1* can be excluded, this suggests that there may be novel genes that play a role in the epigenetic regulation of D4Z4, and which may cause FSHD.

In addition to the D4Z4 repeat array, SMCHD1 can also affect other genomic regions, such as the X chromosome [14]. Therefore, FSHD2 may have additional phenotypes to those seen in FSHD1. Mild dysphagia, which is uncommon in the early stages of mild FSHD1, was seen in 3 of the 13 patients with *SMCHD1*

mutations in our study; however, we did not detect any other additional clinicopathological features (Table 2 and 3, and Figure 3). Dysphagia is known to be observed particularly in some infantile-onset severe FSHD1 patients [29]. As the three FSHD patients with dysphagia in this study had a later onset, our results raise the possibility that dysphagia may be a distinctive feature of FSHD2, although additional data on more patients are required to draw any conclusions. Among the 13 patients, 7 showed from normal to severely affected muscle pathology. The severity of muscle pathology was not correlated with clinical severity scale (data not shown). This discrepancy is possibly due to selectivity of affected muscle in FSHD and different biopsy site among the 7 patients. Nevertheless, we speculate that phenotypic effects of the *SMCHD1* mutation may be restricted to the consequences of its influence on the D4Z4 repeat, as overall clinicopathological features are essentially identical between FSHD1 and FSHD2.

5. Conclusion

In summary, we report for the first time the presence of *SMCHD1* mutations in Japanese patients, providing evidence of the presence of FSHD2 in a wider population than previously known. We would like to emphasize that standard FSHD1-sized repeat array in Japan ranges between 1 and 6 units and recommend searching further for D4Z4 hypomethylation and *SMCHD1* mutations in FSHD patients carrying a medium short (7-14 units) D4Z4 array.

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Disclosures

All authors approved the final article and declared no conflict of interest.

Contributors

KH: collection of clinical, pathological, and molecular data, data analysis and interpretation, literature review, and drafting the manuscript. YKH: conceptualization and design of the study, and manuscript revision for intellectual content. AM and KG: collection of molecular data. KN and CO: conceptualization of the study. SN: supervision of conceptualization and design of the study content data interpretation. SM and IN: supervision of all aspects, including study design, data analysis and interpretation, and manuscript preparation.

Figure legends

Figure 1. DNA methylation analysis in Japanese cohort

(A) DNA methylation on D4Z4 of 19 controls analyzed by pyrosequencing. The bars indicate the average and standard deviation. (B) DNA methylation on D4Z4 of 308 Japanese patients with FSHD-like features carrying larger than 6 units D4Z4 array cohort analyzed by pyrosequencing. The line indicates 26% DNA methylation level. (C) DNA methylation on D4Z4 of 13 patients with an *SMCHD1* mutation, analyzed by Southern blot with *Fse*I

Figure 2. *SMCHD1* mutation analysis

(A) A diagram of the *SMCHD1* gene showing the mutations identified in FSHD patients. Putative domains are depicted. (B) Aberrant splicing of the *SMCHD1* transcript in FSHD patients. Detection of the aberrantly spliced cDNA is shown for patients 7 (top), 9 (middle), and 10 (bottom). Patient 7 had the c.186_187insTAGA mutation, patient 9 had the c.5367_5416del mutation, and patient 10 had the c.626_638del, c.621_638del, and c.508_638del mutations. cDNA was amplified by primers indicated by the arrows. Wt and mt indicates wile-type transcript and mutated transcript, respectively. (C) Quantitative PCR analysis of

the *SMCHD1* transcript level of patient 10. The amount of *SMCHD1* transcript was normalized to that of the POLR2A transcript, and demonstrated as the ratio to the average of four controls. (D) Patients 11, 12, and 13: Conservation of the mutated or deleted amino acids among species. All the mutated amino acids were well conserved among species. The red letters indicate the amino acids affected. The numbers above the amino acids indicate the amino acid position number. (E) Western blot analysis of SMCHD1 in fibroblasts from patient 12. α-Tubulin is shown as an internal control.

Figure 3. Muscle pathology of patients with *SMCHD1* mutations

Representative images of muscle pathology. (A) No pathological change is seen in patient 13. (B) No pathological change is seen in patient 7. (C) A small angular fiber (arrow) in patient 2. (D) A small angular fiber (arrow) in patient 12. (E and F) Marked variation in fiber size, endomysial fibrosis, endomysial adipose tissue infiltration, and lobulated fibers were detected in patient 9. (G and H) Marked variation in fiber size, endomysial lymphocyte infiltration, endomysial fibrosis, endomysial adipose tissue infiltration, and lobulated fibers were detected in

patient 10. (I) Very small fibers, endomysial fibrosis, endomsyial lobulated fibers, and perivascular lymphocyte infiltration were detected in patient 3. (F) and (H) are NADH-TR staining, whereas the other images are H&E staining. The bar indicates 100 μm in (A), (B), (E), (G), and (I), and 50 μm in (C), (D), (F), and (H).

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