

# DAG1 mutations associated with asymptomatic hyperCKemia and hypoglycosylation of $\alpha$ -dystroglycan

Mingrui Dong, MD  
Satoru Noguchi, PhD  
Yukari Endo, MD  
Yukiko K. Hayashi, MD,  
PhD  
Shinobu Yoshida, MD,  
PhD  
Ikuya Nonaka, MD, PhD  
Ichizo Nishino, MD,  
PhD

Correspondence to  
Dr. Noguchi:  
noguchi@ncnp.go.jp

## ABSTRACT

**Objectives:** To identify gene mutations in patients with dystroglycanopathy and prove pathogenicity of those mutations using an in vitro cell assay.

**Methods:** We performed whole-exome sequencing on 20 patients, who were previously diagnosed with dystroglycanopathy by immunohistochemistry and/or Western blot analysis. We also evaluated pathogenicity of identified mutations for phenotypic recovery in a DAG1-knockout haploid human cell line transfected with mutated DAG1 complementary DNA.

**Results:** Using exome sequencing, we identified compound heterozygous missense mutations in DAG1 in a patient with asymptomatic hyperCKemia and pathologically mild muscular dystrophy. Both mutations were in the N-terminal region of  $\alpha$ -dystroglycan and affected its glycosylation. Mutated DAG1 complementary DNAs failed to rescue the phenotype in DAG1-knockout cells, suggesting that these are pathogenic mutations.

**Conclusion:** Novel mutations in DAG1 are associated with asymptomatic hyperCKemia with hypoglycosylation of  $\alpha$ -dystroglycan. The combination of exome sequencing and a phenotype-rescue experiment on a gene-knockout haploid cell line represents a powerful tool for evaluation of these pathogenic mutations. *Neurology*® 2015;84:273–279

## GLOSSARY

cDNA 5 complementary DNA; DAG1 5 dystroglycan 1 (dystrophin-associated glycoprotein 1); KO 5 knockout; WES 5 whole-exome sequencing.

Dystroglycan is a central component of the dystrophin–glycoprotein complex, which links the cytoskeleton and extracellular matrix through sarcolemma.<sup>1,2</sup> Dystroglycan has important roles in the development and maintenance of skeletal muscle, the CNS,<sup>3</sup> and other organs.<sup>4–6</sup> It is encoded by the DAG1 gene. The synthesized polypeptide is posttranslationally cleaved into 2 subunits, namely,  $\alpha$ - and  $\beta$ -dystroglycan; then the former is highly glycosylated.<sup>7,8</sup>  $\alpha$ -Dystroglycan is composed of 3 distinct domains: the N-terminal region, the mucin-like domain, and the C-terminal domain, at which the mucin-like domain is highly glycosylated by O-linked mannosyl-oligosaccharides and binds to ligands such as laminin and agrin by its sugar chains.<sup>9,10</sup> Reports show that the N-terminal region is required for functional glycosylation of the mucin-like domain by LARGE, an intracellular enzyme-substrate recognition motif necessary for initiation of specific glycosylation.<sup>8,11</sup>

Defects in glycosylation of  $\alpha$ -dystroglycan lead to a subgroup of muscular dystrophies and brain and eye malformations, termed dystroglycanopathies.<sup>12</sup> There is a broad spectrum of severity in these diseases, ranging from Walker-Warburg syndrome, muscle-eye-brain disease, and Fukuyama congenital muscular dystrophy to the milder form of limb-girdle muscular dystrophy, such as LGMD2I.<sup>13,14</sup> Recent advances in DNA sequencing techniques facilitated identification of new causative genes in dystroglycanopathies<sup>15–18</sup>; to date, 18 causative genes have been identified. Among them, DAG1 mutations cause primary dystroglycanopathy in limb-girdle muscular dystrophy<sup>19</sup> and muscle-eye-brain disease.<sup>20</sup>

Supplemental data  
at [Neurology.org](http://Neurology.org)

From the Department of Neuromuscular Research, National Institute of Neuroscience (M.D., S.N., Y.E., Y.K.H., I. Nonaka, I. Nishino) and Department of Clinical Development, Translational Medical Center (S.N., Y.E., Y.K.H., I. Nishino), NCNP, Tokyo, Japan; Department of Neurology (M.D.), China-Japan Friendship Hospital, Beijing, China; Department of Neurophysiology (Y.K.H.), Tokyo Medical University; and Department of Pediatrics (S.Y.), Omihachiman Community Medical Center, Shiga, Japan.

Go to [Neurology.org](http://Neurology.org) for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

Herein, we report the case of a patient in whom dystroglycanopathy was caused by novel compound heterozygous missense mutations in *DAG1* identified by whole-exome sequencing (WES) and we prove the pathogenicity of the mutations.

**METHODS** Standard protocol approvals, registrations, and patient consents. The ethics committee of the National Center of Neurology and Psychiatry approved this study. All patients gave written informed consent before study participation.

**Subjects.** To identify the cause of  $\alpha$ -dystroglycanopathy, we selected a cohort of 20 unrelated individuals who were diagnosed with  $\alpha$ -dystroglycanopathy by negative reactivity with an antibody for glycoepitope of  $\alpha$ -dystroglycan (VIA4-1; Millipore, Billerica, MA) on a muscle biopsy and/or decreased VIA4-1 immunoreactivity and laminin binding ability as shown by Western blotting.<sup>21</sup> We immunostained muscle with antibodies for  $\beta$ -dystroglycan (43DAG1; Leica, Wetzlar, Germany), dystrophin (NCL-DYS1, Leica), merosin (4H8-2; Alexis, Lausen, Switzerland), and  $\beta$ -sarcoglycan (5B1, Leica), and conducted Western blotting using the core antibody for  $\alpha$ -dystroglycan peptide, GT20ADG. We confirmed that all study patients did not have 3-kb retrotransposal insertion at *FKTN*.

**Whole-exome sequencing.** WES was performed as reported previously.<sup>22</sup> Briefly, after genomic DNA isolation from muscle specimens or peripheral blood lymphocytes using standard techniques, we performed exon capture according to the manufacturer's instructions (SureSelect Human All Exon kit V4, 50 Mb; Agilent, Santa Clara, CA), followed by paired-end 100-base massively parallel sequencing on an Illumina HiSeq1000 (Illumina, Inc., San Diego, CA). Then, we mapped and aligned to the human genome chromosomal sequence using the Burrows-Wheeler Aligner. We removed duplicate reads using Picard for downstream analysis and conducted local realignments around indels and regions for low base quality scores using the Genome Analysis Toolkit for recalibration. We identified single-nucleotide variants and small indels using the Genome Analysis Toolkit Unified Genotyper (version 1.6) and filtered according to the Broad Institute's best-practice guidelines. We used ANNOVAR to annotate genetic variations. Data filtering included the following conditions: (1) mutation effect—splicing, start lost, exon deletion, frame shift, stop gained or lost, nonsynonymous codon change, codon insertion or deletion; (2) variation frequency less than 0.01 in HapMap and in 1000 Genomes Project database; and (3) inheritance mode—homozygous mutations, hemizygous mutation, or more than 2 mutations in the same genes. We used Sanger sequencing to confirm mutations.

**Validation for the pathogenicity of identified mutations.** To examine the pathogenicity of identified mutations, we analyzed functional recovery of dystroglycans in *DAG1*-knockout (KO) haploid human cell line (HAP1) cells using transfection of lentivirus vectors, pLV5IN-IRES-ZsGreen (Clontech, Mountain View, CA), harboring wild-type or mutated human *DAG1* complementary DNA (cDNA). Jae et al.<sup>23</sup> established the *DAG1*-KO HAP1 cell as reported previously. For the glycosylation in  $\alpha$ -dystroglycan assay, we cultured HAP1 cells on laminin-coated glass-bottom dishes. Five days after lentivirus infection, we incubated live cells with IIH6-C4 antibody against glycoepitope of  $\alpha$ -dystroglycan (Millipore) in medium and then

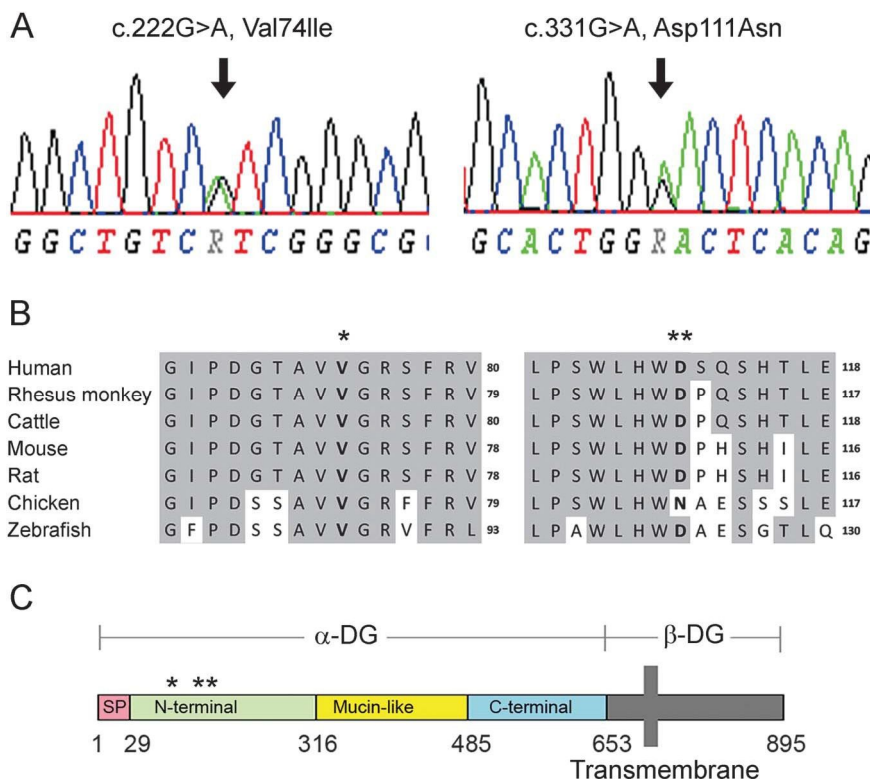
visualized the cells with Alexa Fluor 568-labeled anti-mouse immunoglobulin M secondary antibody. We observed the cultured cells using a fluorescent microscope (BZ-9000; Keyence, Itasca, IL) with Z-axis scanning throughout whole cells to acquire green fluorescent protein and  $\alpha$ -dystroglycan images together (7 images with 1-mm intervals) for full-focus images. After staining with 43DAG1 antibody and GM130 antibody (Cell Signaling Technology, Beverly, MA), we observed localization of  $\beta$ -dystroglycan in formalin-fixed HAP1 cells.

**Biotinylation of cell-surface proteins on HAP1 cells.** We labeled living HAP1 cells with the membrane-impermeable biotin reagent, Sulfo-NHS-LC-Biotin, according to manufacturer's instructions (Thermo Scientific, Waltham, MA) and then subjected streptavidin-purified proteins to Western blotting using standard techniques. We detected  $\beta$ -dystroglycans with 43DAG1 antibody.

**RESULTS** Identification of *DAG1* mutation by WES. After analysis of a cohort of 20 unrelated patients with  $\alpha$ -dystroglycanopathy, we identified one patient who harbored mutations in *DAG1* genes. WES analysis summary is presented in table e-1 on the *Neurology*<sup>®</sup> Web site at [Neurology.org](http://Neurology.org). We identified 7 genes with homozygous mutations, 18 genes with compound heterozygous mutations, and 8 genes with hemizygous mutations in this patient (data not shown). Among them, we identified compound heterozygous mutations, c.220G.A (rs189360006) and c.331G.A (rs117209107) in *DAG1*, which are predicted to lead to missense mutations, p.Val74Ile and p.Asp111Asn, respectively. We did not find any other genes involved in the glycosylation pathway in the patient. We confirmed the 2 mutations in *DAG1* by Sanger sequencing (figure 1A) and the compound heterozygosity by transcript analysis (data not shown). Residues at both mutated sites are located in the N-terminal region of  $\alpha$ -dystroglycan and are highly conserved during evolution (figure 1, B and C). In silico analyses of mutation function demonstrated that p.Val74Ile and p.Asp111Asn, respectively, were predicted as damaging and tolerated by SIFT and probably damaging and benign in PolyPhen-2, and both mutations were predicted as disease-causing in MutationTaster. Other than *DAG1* mutations, the compound heterozygous missense alterations were found in *TTN* and *AHNAK* genes among muscle-related genes.

**Clinical phenotype and histologic features of muscle biopsy.** This is a 7-year-old boy coming from a nonconsanguineous marriage who has compound heterozygous mutations in *DAG1*. He was born normally (length at birth, 51.5 cm; birth weight, 3,672 g) and demonstrated normal development milestones. At the age of 4 years and 7 months, he was 98 cm tall, weighed 17 kg, and had a head circumference of 50.8 cm. When he was 4 years

Figure 1 Compound heterozygous mutations in the DAG1 gene in the described patient



(A) Electropherograms around the mutation sites in DAG1 genes from Sanger sequencing. (B) Amino acid conservation in mutation sites among species. (C) Localization of mutation sites (\* and \*\*) in domain structures in DAG1 protein. DG 5 dystroglycan.

and 5 months, he became dehydrated in the wake of acute tonsillitis, and was diagnosed with hyperCKemia by chance. After recovery from dehydration, hyperCKemia continued (range, 1,855–6,512 IU/L; normal range, 45–287 IU/L). Physical examination showed no symptomatic muscle weakness but we observed calf pseudohypertrophy. Muscle CT imaging showed low intensity in the rectus femoris, semimembranosus, and gastrocnemius muscles. Brain CT images showed no morphologic abnormality.

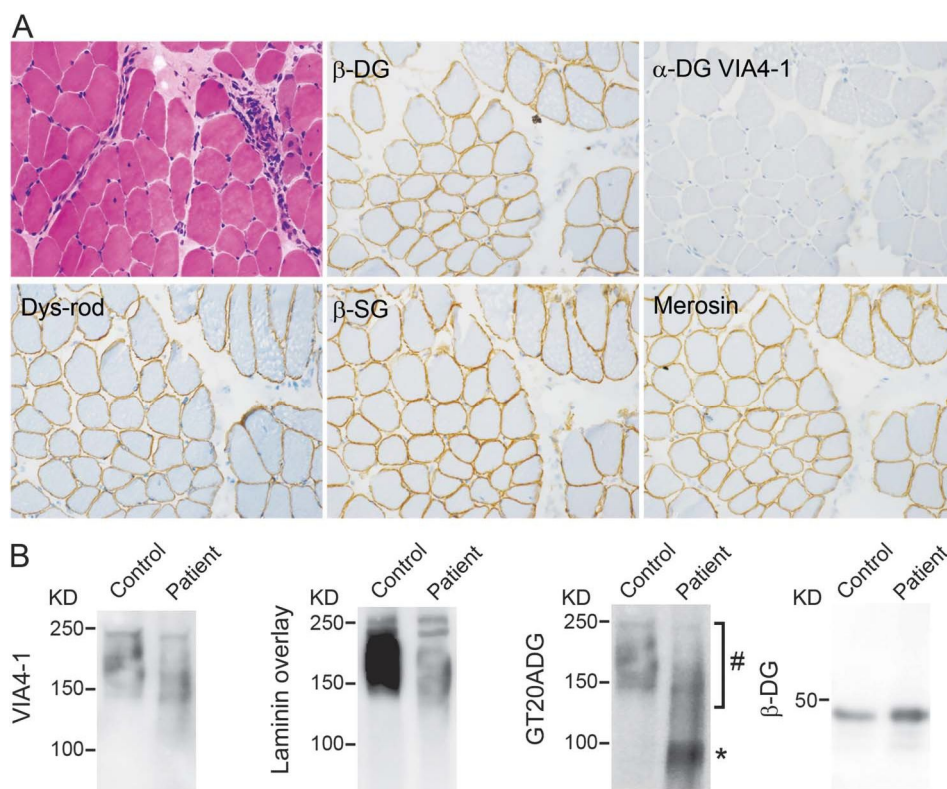
Muscle histologic analysis showed muscular dystrophy-like appearance including a few regenerating fibers, internal nuclei, and mild endomysial fibrosis. Immunohistochemical analysis was positive for dystrophin, merosin, sarcoglycans, and b-dystroglycan, but negative for glycoepitope of a-dystroglycan (figure 2). Results of Western blotting and the laminin overlay assay of muscle proteins corroborated the reduction in glycosylation of a-dystroglycan (figure 2); in contrast, we detected strong immunoreactivity to GT20ADG at lower molecular mass. b-Dystroglycan was normal.

Pathogenesis is proven by rescue of DAG1-KO HAP1 cells by the wild-type and mutant DAG1 gene. To prove the pathogenicity of the 2 missense mutations harbored by this patient, we transfected lentivirus vectors

with wild-type or mutated DAG1 cDNAs (p.Val74Ile and p.Asp111Asn) into DAG1-KO HAP1 cells, which showed defects in reactivity for the anti-a-dystroglycan antibody, IIH6 (figure 3A). DAG1-KO HAP1 cells were rescued by introduction of wild-type cDNA showing recovery of strong IIH6 immunoreactivity similar to that of wild-type HAP1 cells (figure 3A). On the contrary, cDNAs with p.Val74Ile and p.Asp111Asn mutations failed to rescue (figure 3A).

We also analyzed mutated b-dystroglycan transport to the cell surface in HAP1 cells. DAG1-KO cells were negative for b-dystroglycan staining (figure 3B). Introduction of wild-type and mutated DAG1 cDNAs into DAG1-KO cells resulted in recovery of b-dystroglycan staining at the cell surface (in red) but not in the Golgi apparatus (GM130, blue), suggesting that processing and transport of dystroglycan was not affected by the mutations. Cell-surface biotinylation experiments in DAG1-KO cells transfected with wild-type and mutated DAG1 cDNAs also showed recovery of b-dystroglycan in the biotinylated protein fraction (figure 3C). These results demonstrate that these 2 mutations are pathogenic and impair glycosylation of a-dystroglycan, but not dystroglycan expression.

Figure 2 Hypoglycosylation of  $\alpha$ -dystroglycan in the described patient



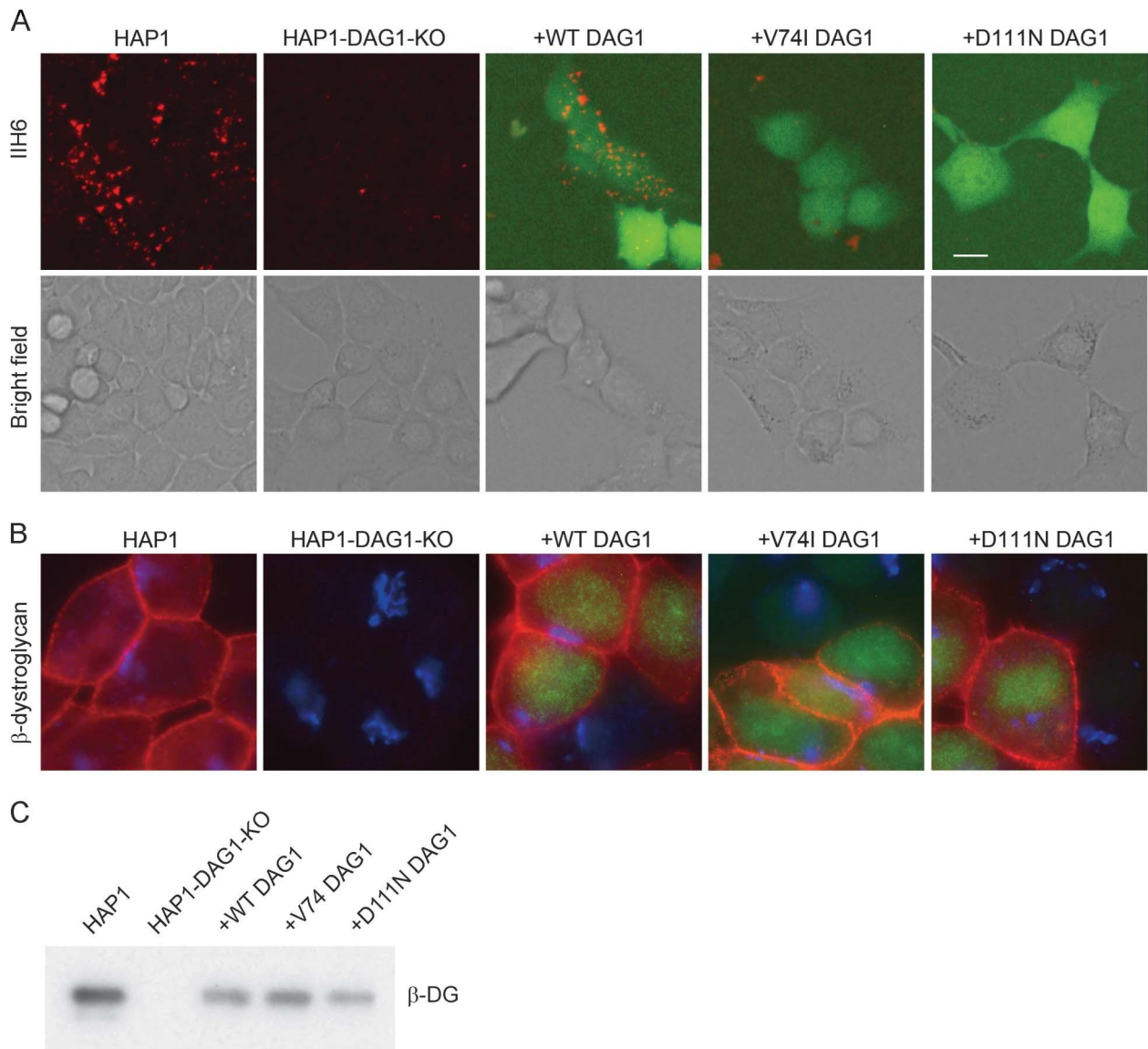
(A, top) Histology and immunostaining of skeletal muscle from the patient. Muscle histology showed muscular dystrophy-like appearance including a few regenerating fibers, internal nuclei, and mild endomysial fibrosis. (A, bottom) Muscle stained positive for antibodies to dystrophin (Dys-rod), merosin,  $\beta$ -sarcoglycan ( $\beta$ -SG), and  $\beta$ -dystroglycan ( $\beta$ -DG), but negative for glycoepitope antibody to  $\alpha$ -dystroglycan ( $\alpha$ -DG VIA4-1). (B) Western blotting with VIA4-1 antibody and the laminin overlay assay of muscle proteins showing reduced glycosylation of  $\alpha$ -dystroglycan; in contrast, strong immunoreactivity to GT20ADG for core peptide was detected at lower molecular mass (\*). After Western blotting with VIA4-1 antibody, the same membrane was used for GT20ADG. The bands labeled with # were the VIA4-1 antibody-reactive bands.  $\beta$ -Dystroglycan staining was normal.

**DISCUSSION** Herein, we report on a patient with dystroglycanopathy, who has compound heterozygous mutations in *DAG1*. This patient had asymptomatic hyperCKemia with mild muscular dystrophy and deficiency in laminin-binding glycosylation in  $\alpha$ -dystroglycan. Although the patient could be presymptomatic for muscle weakness or intellectual disability, the clinical phenotype is much milder compared with a previous report of a patient who had limb-girdle-type muscular dystrophy accompanied by mild cognitive impairment.<sup>19</sup> Our finding expands the clinical and pathologic spectrum of dystroglycanopathy associated with *DAG1* mutation from a muscle-eye-brain disease-like phenotype and mild limb-girdle muscular dystrophy<sup>19,20</sup> to asymptomatic hyperCKemia. Myopathic asymptomatic hyperCKemia has been reported in secondary dystroglycanopathies, with mutations in *FKRP* and *FKTN* genes.<sup>24–26</sup> By WES, we also identified 2 missense alterations in each of the *TTN* and *AHNAK* genes, which have been known to be expressed in skeletal muscles. Both alterations in *TTN* were

predicted as probably damaging in PolyPhen-2 or disease-causing in MutationTaster in silico functional analyses. These alterations in *TTN* were not localized in the exons in which the mutations have been identified in other muscle diseases, such as hereditary myopathy with early respiratory failure, cardiomyopathy, or tibial muscular dystrophy. *AHNAK* missense alterations were predicted as probably damaging and benign in PolyPhen-2 or polymorphism in MutationTaster. Functional experiments for the mutated proteins would be required for final conclusion of their pathogenicities.

Although one could argue whether the c.220G.A and c.331G.A variants previously annotated in the dbSNP131, 1000 Genomes, and HapMap databases can be the candidate pathogenic mutations, we still presume they are pathogenic because we did not find any other strong candidate gene for dystroglycanopathy in this patient. Because it is known that the 3-kb retrotransposal insertion in *FKTN* with a frequency of 1/88 allele is associated with a high prevalence of Fukuyama congenital muscular dystrophy in Japan,<sup>27</sup>

**Figure 3** Mutant DAG1 observed in patients does not rescue hypoglycosylation of  $\alpha$ -dystroglycan in DAG1-KO cells



(A) IIH6-4C2 staining of wild-type HAP1 cells (in red), DAG1-knockout cells (HAP1-DAG1-KO), and DAG1-KO cells transfected with wild-type (1WT-DAG1), Val74Ile-mutated (1V74I DAG1), and Asp111Asn-mutated DAG1 (1D111N DAG1). Transfected cells are positive for ZsGreen expression (in green). (B, C) Recovery of  $\beta$ -dystroglycan on cell surface in DAG1-KO cells by transfection with wild-type (1WT-DAG1), p.Val74Ile-mutated (1V74I DAG1), and p.Asp111Asn-mutated DAG1 (1D111N DAG1). HAP1, wild-type haploid cells; HAP1-DAG1-KO, DAG1-KO HAP1 cells. (B) Immunostaining of  $\beta$ -dystroglycan (red) and Golgi protein, GM130 (blue). (C) Western blot analysis of cell-surface biotin-labeled fraction. Scale bar denotes 20  $\mu$ m.  $\beta$ -DG 5  $\mu$ g  $\beta$ -dystroglycan.

it is logical to suspect a mutation with a variation frequency of more than 0.01. Because the c.331G.A mutation has a variation frequency of 0.005 in all populations in 1000 Genomes and a higher frequency (0.028) in the Japanese population in the Human Genetic Variation Database, there is a possibility that a higher incidence of potential dystroglycanopathy caused by p.Asp111Asn substitution exists in the Japanese population. However, in other populations, the frequency has not been known.

As reported, hypoglycosylation levels of  $\alpha$ -dystroglycan do not consistently correlate with clinical severity.<sup>28</sup>

Our patient should be classified as having a primary dystroglycanopathy with mutations in DAG1; he had typical hypoglycosylation of  $\alpha$ -dystroglycan in terms of low molecular mass of the protein, positive reactivity to anti-core peptide antibody, and decreased binding to laminin, but he showed a milder phenotype. The level of hypoglycosylation of  $\alpha$ -dystroglycan is not necessarily predictive of phenotypic severity in dystroglycanopathy.

Our results suggest that the missense mutation of p.Val74Ile or p.Asp111Asn in the N-terminal region of  $\alpha$ -dystroglycan does not influence expression of the dystroglycan, but it does cause a defect in

posttranslational modification. Similarly, Hara et al.<sup>19</sup> reported a missense mutation (p.The192Met) in the N-terminal region, which is also associated with hypoglycosylation of  $\alpha$ -dystroglycan but with normal  $\beta$ -dystroglycan localization. LARGE catalyzes the extension of specific disaccharide structures [23GlcAa124Xylb12] on a phosphorylated O-mannosyl glycan in the mucin-like domain, which is required for laminin binding, within the Golgi apparatus.<sup>29</sup> The N-terminal region in  $\alpha$ -dystroglycan serves as a recognition site for LARGE<sup>8</sup>; of note, Hara et al. demonstrated that the p.The192Met mutation in DAG1 impairs interaction between  $\alpha$ -dystroglycan and LARGE. This N-terminal region is predicted to have L-shaped modular architecture and comprises 2 autonomous domains; domain 1 contains residues 28–168 in murine  $\alpha$ -dystroglycan and belongs to the I-set domain of the immunoglobulin superfamily, and domain 2 contains residues 180–303 with a ribosomal RNA-binding protein fold.<sup>30</sup> Both mutated residues, Val74 and Asp111, are present in domain 1 and are neighbors of Gly75 and Gln113 (corresponding to Gly73 and His111 in murine dystroglycan); each of these is predicted to be aligned on the interaction between domain 1 and 2, and the trimer interface of domain 1, respectively, in the crystal structure of the N-terminal region of murine  $\alpha$ -dystroglycan. Both mutations may affect higher-order structural formation of the N-terminal region of  $\alpha$ -dystroglycan. Another possibility is that the mutations may impair direct interaction of the N-terminal globular region of  $\alpha$ -dystroglycan with extracellular matrix molecules, as suggested by Hall et al.<sup>31</sup> Remarkable secondary structure and hydrophobic character changes of the mutated fragment are reported to lead to weaker interaction of this domain with laminin.<sup>32</sup>

Previously, Willer et al.<sup>15</sup> have demonstrated the rescuing experiments using patients' fibroblasts in dystroglycanopathy for evaluation of the pathogenicity of gene mutations. In this study, we used gene-modified HAP1 cells because the patient's cells were not available. The phenotypic rescue experiments described here, using DAG1-KO HAP1 cells with lentivirus-mediated expression of mutated cDNA, enabled rapid and easy evaluation of the pathogenicity of the mutations. This is a simple method based on the recovery of the function of  $\alpha$ -dystroglycan. Theoretically, this method can be applied to evaluate any of the mutations in all known causative genes as well as mutations in novel candidate genes for dystroglycanopathies without requiring enzymatic activity measurement, as long as the specific gene-KO HAP1 cells are available. This method would be applicable by any researcher for confirming the data from WES for each causative

mutation in any disease, if the phenotypes of cells were characterized.

## AUTHOR CONTRIBUTIONS

M.D. conducted acquisition, analysis and interpretation of data, and drafted and edited the manuscript. S.N. supervised all aspects of this study including study design, data interpretation, and drafted and edited the manuscript. Y.E. made WES pipeline and analyzed the data. Y.K.H. selected patients and performed WES. S.Y. collected clinical information of the patient. I. Nonaka and I. Nishino supervised manuscript preparation and edited the manuscript.

## ACKNOWLEDGMENT

The authors thank Nozomi Matsuyama, Megumu Ogawa, Kanako Goto, and Asako Kaminaga for technical support, Thijn R. Brummelkamp for supplying HAP1 cells, and Kevin P. Campbell for supplying GT20ADG antibody.

## STUDY FUNDING

This study was partially supported by Intramural Research Grant (25-5, 26-8) for Neurological and Psychiatric Disorders of NCNP (to S.N., I. Nishino); Research on Applying Health Technology from the Ministry of Health, Labour and Welfare (to I. Nishino).

## DISCLOSURE

M. Dong reports no disclosures relevant to the manuscript. S. Noguchi serves as an editor of *Acta Neuropathologica Communications* and received research support from the Ministry of Health, Labour and Welfare. Y. Endo reports no disclosures relevant to the manuscript. Y. Hayashi received research support from the Ministry of Health, Labour and Welfare. S. Yoshida and I. Nonaka report no disclosures relevant to the manuscript. I. Nishino serves as an associate editor of *Neuromuscular Disorders and Neurology and Clinical Neuroscience*, serves on the speakers bureau of Genzyme and Kitano Hospital, serves as a consultant of Novartis Pharma, and received research support from Genzyme and the Ministry of Health, Labour and Welfare. Go to [Neurology.org](http://Neurology.org) for full disclosures.

Received May 31, 2014. Accepted in final form September 29, 2014.

## REFERENCES

1. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 1992;355: 696–702.
2. Michele DE, Barresi R, Kanagawa M, et al. Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* 2002;418:417–422.
3. Moore SA, Saito F, Chen J, et al. Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* 2002;418:422–425.
4. Saito F, Moore SA, Barresi R, et al. Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron* 2003;38: 747–758.
5. Durbeek M, Talts JF, Henry MD, Yurchenco PD, Campbell KP, Ekblom P. Dystroglycan binding to laminin alpha1LG4 module influences epithelial morphogenesis of salivary gland and lung in vitro. *Differentiation* 2001;69:121–134.
6. Matsumura K, Chiba A, Yamada H, et al. A role of dystroglycan in schwannoma cell adhesion to laminin. *J Biol Chem* 1997;272:13904–13910.

7. Noguchi S, Wakabayashi E, Imamura M, Yoshida M, Ozawa E. Formation of sarcoglycan complex with differentiation in cultured myocyte. *Eur J Biochem* 2000;267: 640–648.
8. Kanagawa M, Saito F, Kunz S, et al. Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* 2004;117:953–964.
9. Brancaccio A, Schulthess T, Gesemann M, Engel J. Electron microscopic evidence for a mucin-like region in chick muscle  $\alpha$ -dystroglycan. *FEBS Lett* 1995;368: 139–142.
10. Chiba A, Matsumura K, Yamada H, et al. Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve  $\alpha$ -dystroglycan: the role of a novel O-mannosyl-type oligosaccharide in the binding of  $\alpha$ -dystroglycan with laminin. *J Biol Chem* 1997; 272:2156–2162.
11. Hara Y, Kanagawa M, Kunz S, et al. Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. *Proc Natl Acad Sci USA* 2011;108:17426–17431.
12. Muntoni F, Brockington M, Brown SC. Glycosylation eases muscular dystrophy. *Nat Med* 2004;10:676–677.
13. Brown SC, Torelli S, Brockington M. Abnormalities in  $\alpha$ -dystroglycan expression in MDC1C and LGMD2I muscular dystrophies. *Am J Pathol* 2004;164:727–737.
14. Mendell JR, Boué DR, Martin PT. The congenital muscular dystrophies: recent advances and molecular insights. *Pediatr Dev Pathol* 2006;9:427–443.
15. Willer T, Lee H, Lommel M, et al. ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome. *Nat Genet* 2012;44: 575–580.
16. Roscioli T, Kamsteeg EJ, Buysse K, et al. Mutations in ISPD cause Walker-Warburg syndrome and defective glycosylation of  $\alpha$ -dystroglycan. *Nat Genet* 2012;44: 581–585.
17. Manzini MC, Tambunan DE, Hill RS, et al. Exome sequencing and functional validation in zebrafish identify GTDC2 mutations as a cause of Walker-Warburg syndrome. *Am J Hum Genet* 2012;91:541–547.
18. Carss KJ, Stevens E, Foley AR, et al. Mutations in GDP-mannose pyrophosphorylase B cause congenital and limb-girdle muscular dystrophies associated with hypoglycosylation of  $\alpha$ -dystroglycan. *Am J Hum Genet* 2013; 93:29–41.
19. Hara Y, Balci-Hayta B, Yoshida-Moriguchi T, et al. A dystroglycan mutation associated with limb-girdle muscular dystrophy. *N Engl J Med* 2011;364:939–946.
20. Geis T, Marquard K, Rödl T. Homozygous dystroglycan mutation associated with a novel muscle-eye-brain disease-like phenotype with multicystic leucodystrophy. *Neurogenetics* 2013;14:205–213.
21. Hayashi YK, Ogawa M, Tagawa K, et al. Selective deficiency of  $\alpha$ -dystroglycan in Fukuyama-type congenital muscular dystrophy. *Neurology* 2001;57:115–121.
22. Saito H, Nishimura T, Muramatsu K, et al. De novo mutations in the autophagy gene WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood. *Nat Genet* 2013;45:445–449.
23. Jae LT, Raaben M, Riemersma M, et al. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. *Science* 2013;340:479–483.
24. de Paula F, Vieira N, Starling A, et al. Asymptomatic carriers for homozygous novel mutations in the FKRP gene: the other end of the spectrum. *Eur J Hum Genet* 2003;11:923–930.
25. Fernandez C, de Paula AM, Figarella-Branger D, et al. Diagnostic evaluation of clinically normal subjects with chronic hyperCKemia. *Neurology* 2006;66:1585–1587.
26. Fiorillo C, Moro F, Astrea G, et al. Novel mutations in the fukutin gene in a boy with asymptomatic hyperCKemia. *Neuromuscul Disord* 2013;23:1010–1015.
27. Colombo R, Bignamini AA, Carobene A, et al. Age and origin of the FCMD 3'-untranslated-region retrotransposal insertion mutation causing Fukuyama-type congenital muscular dystrophy in the Japanese population. *Hum Genet* 2000;107:559–567.
28. Jimenez-Mallebrera C, Torelli S, Feng L, et al. A comparative study of  $\alpha$ -dystroglycan glycosylation in dystroglycanopathies suggests that the hypoglycosylation of  $\alpha$ -dystroglycan does not consistently correlate with clinical severity. *Brain Pathol* 2009;19:596–611.
29. Yoshida-Moriguchi T, Yu L, Stalnaker SH, et al. O-mannosyl phosphorylation of  $\alpha$ -dystroglycan is required for laminin binding. *Science* 2010;327:88–92.
30. Bozic D, Sciandra F, Lamba D, Brancaccio A. The structure of the N-terminal region of murine skeletal muscle  $\alpha$ -dystroglycan discloses a modular architecture. *J Biol Chem* 2004;279:44812–44816.
31. Hall H, Bozic D, Michel K, Hubbell JA. N-terminal  $\alpha$ -dystroglycan binds to different extracellular matrix molecules expressed in regenerating peripheral nerves in a protein-mediated manner and promotes neurite extension of PC12 cells. *Mol Cell Neurosci* 2003;24:1062–1073.
32. Bhattacharya S, Das A, Ghosh S, Dasgupta R, Bagchi A. Hypoglycosylation of dystroglycan due to T192M mutation: a molecular insight behind the fact. *Gene* 2014;537: 108–114.

