Tubular aggregate myopathy caused by a novel mutation in the cytoplasmic domain of \textit{STIM1}

\textbf{ABSTRACT}

\textbf{Objective}: To identify the gene mutation of tubular aggregate myopathy (TAM) and gain mechanistic insight into the pathogenesis of the disorder.

\textbf{Methods}: We described a family affected by autosomal dominant TAM and performed exome and Sanger sequencing to identify mutations. We further analyzed the functional significance of the identified mutation by expression studies and intracellular Ca\textsuperscript{2+} measurements.

\textbf{Results}: A 42-year-old man presented with slowly progressive muscle weakness and atrophy in all 4 limbs and the trunk. Muscle biopsy and microscopic examination revealed tubular aggregates in his skeletal muscle. Genetic analysis of this family identified a novel heterozygous mutation, c.1450_1451insGA (p.Ile484ArgfsX21), in stromal interaction molecule 1 (\textit{STIM1}), a Ca\textsuperscript{2+} sensor in sarcoplasmic reticulum. We transfected cultured cells with \textit{STIM1} and demonstrated that the mutant \textit{STIM1} exhibited aggregation-like appearance in shrunk cytoplasm. Furthermore, we revealed that the intracellular Ca\textsuperscript{2+} influx is decreased by the mutant \textit{STIM1}.

\textbf{Conclusions}: The novel mutation p.Ile484ArgfsX21 is located in the cytoplasmic C-terminal inhibitory domain (CTID) of \textit{STIM1}. However, all mutations reported so far in TAM reside in the luminal N-terminal EF hand region. The aggregation-like appearance of \textit{STIM1} and the decreased intracellular Ca\textsuperscript{2+} influx in cells transfected with CTID mutant are in sharp contrast to these previous reports. Taken together, these findings indicate that mutations of \textit{STIM1} cause TAM through the dysregulation of Ca\textsuperscript{2+} homeostasis.

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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/ng for full disclosure forms. The Article Processing Charge was paid by the authors.

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RESULTS Clinical data. A 42-year-old Japanese man presented with difficulty walking. He first noticed slight difficulty walking when he was 37 years old. At the age of 40, running and climbing stairs became difficult. His mother also had difficulty walking and was in a wheelchair when she was 40 years old. His parents were not consanguineous. On neurologic examination, pupils and ocular movement were normal. The temporals and masseter muscles on both sides were atrophic. Muscle atrophy and weakness was evident in all 4 limbs, and the bilateral triceps surae muscles were most prominently affected (figure 1A). The paraspinal muscles were also atrophic and he had winged scapulae (figure 1B). His gait was lordotic. Ankle joint contracture was observed bilaterally. He did not exhibit myalgia, cramps, scoliosis, rigid spine, or symptoms suggesting Stormorken syndrome, including miosis, thrombocytopenia, aspension, or ichthyosis. He had no history of repetitive infections indicative of immunodeficiency. Blood cell counts and routine blood biochemistry were normal, except serum creatine kinase was elevated to 798 IU/L (normal range <150). Needle EMG showed chronic myopathic changes. Muscle MRI revealed disseminated high-intensity areas on T1-weighted images in the quadriceps femoris, triceps surae, and paraspinal muscles, indicating fatty replacement in these muscles (figure 1C).

Pathologic findings. H&E staining demonstrated increased variability of muscle fiber diameter and occasionally centrally nucleated myofibers. In addition, many fibers showed irregularly shaped dark red staining (figure 1D). These dark red accumulations were stained intense blue by NADH-TR (figure 1E) and bright purple with Gomori trichrome staining (figure 1F), indicating that these are tubular aggregates. A serial section stained with ATPase (pH 4.6) further demonstrated that the tubular aggregates were in type 2 fibers (figure 1G). Electron microscopic analysis revealed that some areas in the myofiber were replaced by numerous tubular structures (figure 1H) and that each tubule was arranged in a honeycomb-like structure that showed double-walled membranes at higher magnification (figure 1H, inset).

Genetic analysis and localization of STIM1 in skeletal muscle. The clinical and microscopic findings characteristic of TAM prompted us to perform genetic analysis. Exome sequence analysis and subsequent Sanger sequencing of the proband revealed a novel heterozygous insertion mutation, c.1450_1451insGA (p.Ile484ArgfsX21), in exon 7 of STIM1 (figure 2A). The same mutation was identified in his mother but not in healthy family members (figure 2B). The amino acid 484 resides in the CTID located in the cytoplasmic region of STIM1 (figure 2C). To assess the localization of STIM1 in the skeletal muscle of the patient, we
performed immunofluorescent microscopic analysis. The immunolabelling of STIM1 and ORAI1, a calcium channel in plasma membrane, colocalized to the tubular aggregates visualized by Gomori trichrome staining (figure 2D), which is consistent with previous reports.4,7 Because premature termination codons often result in nonsense-mediated decay of mRNA, we analyzed mRNA transcript in the muscle biopsy specimen. Sequencing of the RT-PCR product demonstrated that both wild-type and the c.1450_1451insGA mutant transcripts are present (figure 2E).

Expression of mutant STIM1 in C2C12 myoblasts. To investigate the functional significance of the c.1450_1451insGA mutation, we transfected C2C12 myoblasts with wild-type and mutant STIM1. Typically, wild-type
STIM1 displayed diffuse distribution in C2C12 cells (figure 3A). In sharp contrast, mutant STIM1 concentrated intensely around nuclei and exhibited an aggregation-like appearance in shrunk cytoplasm (figure 3A). Counting cells with the diffuse vs aggregation-like localization of STIM1 confirmed that the majority of cells expressing wild-type STIM1 showed diffuse localization whereas most cells expressing mutant STIM1 exhibited an aggregation-like pattern (figure 3B).
(A) C2C12 myoblasts were transfected with wild-type (WT) and the C-terminal inhibitory domain mutant STIM1 and labeled with anti-STIM1 antibody, phalloidin, and 4',6-diamidino-2-phenylindole (DAPI). WT STIM1 displays diffuse distribution in C2C12 cells, whereas the signal of the mutant STIM1 concentrates intensely around nuclei and exhibits an aggregation-like appearance in the shrunk cytoplasm. Bar = 50 μm. (B) Seven stitching images (5 × 5) of the C2C12 myoblasts were captured using a fluorescence microscope, and the number of cells with diffuse vs aggregation-like localization of STIM1 was counted. The majority of cells expressing WT STIM1 show diffuse localization, whereas most cells expressing the mutant STIM1 exhibit the aggregation-like pattern. (C) Intracellular Ca²⁺ concentration was measured in HEK293 cells expressing WT STIM1 (gray square; n = 40 cells), the mutant STIM1 (black triangle; n = 42 cells), or control (open circle; n = 47 cells). The perfusion solution was changed from HEPES-buffered saline (HBS) containing 2 mM Ca²⁺ to Ca²⁺-free solution containing 0.5 mM ethylene glycol tetraacetic acid (EGTA). To evaluate Ca²⁺ influx across the plasma membrane, 2 mM Ca²⁺ was applied to the cells. The duration of exposure to Ca²⁺-containing HBS and Ca²⁺-free HBS is indicated above the graph. (D) Maximal intracellular Ca²⁺ rises after readdition of 2 mM Ca²⁺ to the cells. Data points are mean ± SEM. p = 0.0028, 0.0104, and 0.5299 for WT vs mutant, control vs WT, and control vs mutant, respectively.


Effects of STIM1 mutation on store-operated Ca\(^{2+}\) entry–mediated Ca\(^{2+}\) influx. Finally, we performed Ca\(^{2+}\) measurements to examine whether the STIM1 mutation affects store-operated Ca\(^{2+}\) entry (SOCE). The wild-type or the mutant STIM1 was expressed in HEK293 cells, and changes in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) were evaluated. As shown in figure 3C, overexpression of wild-type STIM1 caused an increased [Ca\(^{2+}\)]\(i\), in the perfusion solution containing 2 mM Ca\(^{2+}\), suggesting that wild-type STIM1 enhanced Ca\(^{2+}\) influx through ORAI1 endogenously expressed in HEK293 cells. This notion was further confirmed by the fact that removal of the external Ca\(^{2+}\) by ethylene glycol tetraacetate acid led to decreased [Ca\(^{2+}\)]\(i\), and that readdition of Ca\(^{2+}\) to the perfusion solution resumed the Ca\(^{2+}\) response. However, the mutant STIM1–expressed cells did not show significant Ca\(^{2+}\) responses by the changes in the external Ca\(^{2+}\) concentration (figure 3, C and D). These results collectively suggest that the STIM1 mutation we found in this study causes dysregulated Ca\(^{2+}\) homeostasis.

**DISCUSSION** In the present study, we described a family with dominant TAM with a novel frameshift mutation in the CTID of STIM1. Of note, the triceps surae muscles were most severely involved in our patient. This is in contrast to the previous reports of TAM, in which the proximal muscles were predominantly affected.\(^{4,5}\) In skeletal muscle, Ca\(^{2+}\) homeostasis is regulated by SOCE. In SOCE, STIM1 forms an oligomer upon the depletion of Ca\(^{2+}\) in SR and activates ORAI1 channels at sarcolemma, triggering extracellular Ca\(^{2+}\) entry.\(^{10}\) All STIM1 mutations in TAM reported so far are missense mutations residing in the EF hand (EF1 and EF2 in figure 2B), and this region is considered a hotspot.\(^{4,6}\) These mutations are presumed to induce the constitutive activation of ORAI1 and cause excessive Ca\(^{2+}\) influx into muscle cells.

In contrast, a frameshift mutation, which creates a premature stop codon in the CTID located in the C-terminal cytoplasmic region of STIM1, was identified in our family (figure 2B). The CTID is known to interact with SOCE-associated regulatory factor (SARAF), a regulator of slow Ca\(^{2+}\)-dependent inactivation of ORAI1.\(^{11}\) Following the transfection of C2C12 myoblasts with the CTID mutant STIM1, aggregation-like signals of STIM1 were observed around the nuclei. The aggregation-like appearance is distinct from that in C2C12 myoblasts transfected with STIM1 harboring EF hand mutations, which exhibit numerous puncta-like small clusters in cytoplasm.\(^{4,6}\)

Furthermore, intracellular Ca\(^{2+}\) measurements revealed that the Ca\(^{2+}\) influx is significantly decreased in the cells transfected with the CTID mutant compared to the wild-type STIM1-transfected cells. This result is in marked contrast to the previous reports, in which increased Ca\(^{2+}\) influx caused by constitutive activation of SOCE was observed.\(^{4,7}\) The transcript of CTID mutant was present in the affected skeletal muscle of our patient, suggesting that nonsense-mediated mRNA decay is not induced. We speculate that the CTID mutant may inhibit the oligomer formation of STIM1 necessary to activate the ORAI1 channels, leading to the decreased intracellular Ca\(^{2+}\) influx. Alternatively, the STIM1 oligomer including the CTID mutant may not translocate properly to the subsarcolemma where the oligomer interacts with ORAI1, because the CTID mutant lacks the polybasic motif (K in figure 2C) necessary for the STIM1 targeting to plasma membrane.\(^{12}\) It is of particular interest to note that STIM2, an alternatively spliced form of STIM2, converts STIM isoforms from an activator to an inhibitor of ORAI1.\(^{13}\) Our findings provide further evidence that the dysregulation of Ca\(^{2+}\) homeostasis underlies the pathomechanism of TAM.

**ACKNOWLEDGMENT** The authors thank the patients and their families for participation in this study and Dr. Toshihiro Maasaki and Dr. Hiroki Hagowara (Teikyo University of Science) for useful discussion.

**STUDY FUNDING** No targeted funding reported.

**DISCLOSURE** Dr. Okuma reports no disclosures. Dr. Saito has received travel funding/speaker honoraria from Eisai Co. Ltd., Takeda Pharmaceutical Co. Ltd., Daiichi Sankyo Co. Ltd., and Ono Pharmaceutical Co. Ltd.; has received research support from Eisai Co. Ltd., Takeda Pharmaceutical Co. Ltd., and Daiichi Sankyo Co. Ltd.; and was supported by an Intramural Research Grant (26-8) for Neurological and Psychiatric Disorders of NCNP from the Ministry of Health, Labour and Welfare of Japan and a Grant-in-Aid for Scientific Research (C) 26461281 from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Dr. Mitsui has received research support from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research (C) 15K09334). Dr. Hara contributed to the study concept and design, data acquisition, interpretation, and analysis. Dr. Matsumura contributed to the study concept, design, and data analysis. Dr. J. Tsuji contributed to the study concept and design. Dr. Sonoo contributed to the study concept and design, data acquisition, interpretation, and analysis.

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Science Foundation of Japan, the Kowa Life Science Foundation, and the Salt Science Research Foundation. Dr. Hatanaka and Ms. Ikeda report no disclosures. Dr. T. Shimizu was supported by a Grant-in-Aid for Scientific Research C (15K09328) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Dr. Matsumura was supported by a Grant-in-Aid for Scientific Research C (25430101) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Dr. J. Shimizu was supported by a Health and Labour Sciences Research Grant on Intractable Diseases (Evidence-based Early Diagnosis and Treatment Strategies for Neuroimmunological Diseases) from the Ministry of Health, Labour and Welfare of Japan, a Grant-in-Aid for Scientific Research (C) (22590002) of the Japan Agency for Medical Research and Development, a Grant-in-Aid for Scientific Research on Innovative Areas (22129001 and 22129002), the Japan Agency for Medical Research and Development (Grant-in-Aid [H26-Nanchiseshikkan Jisuyoka-Ippan-080, 26310101, and Ninchisho Kenkyu 26340101]), and a Grant-in-Aid (H23-Jisuyoka [Nanbyo-Ippan-004]) of the Research on Measures for Intractable Diseases from the Ministry of Health, Welfare and Labour, Japan. Dr. Sonoo has served on the editorial boards of Muscle and Nerve, Clinical Neurology, and the Japanese Journal of Clinical Neurophysiology; and was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (23591285), and a Health and Labour Sciences Research Grant on Rare and Intractable Diseases (Evidence-based Early Diagnosis and Treatment Strategies for Neuroimmunological Diseases) from the Ministry of Health, Labour and Welfare of Japan. Go to Neurology.org/ng for full disclosure forms.

Received March 17, 2015. Accepted in final form December 7, 2015.

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Tubular aggregate myopathy caused by a novel mutation in the cytoplasmic domain of STIM1
Hidehiko Okuma, Fumiaki Saito, Jun Mitsui, et al.

Neurol Genet 2016;2;
DOI 10.1212/NXG.0000000000000050

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