A Novel Titin Truncation Variant Linked to Familial Dilated Cardiomyopathy Found in a Japanese Family and Its Functional Analysis in Genome-Edited Model Cells

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Summary

Dilated cardiomyopathy (DCM) is a common cause of heart failure. TTN, which encodes titin protein, is a representative causative gene of DCM, and is presented mainly as a truncation variant. However, TTN truncation variants are also found in healthy individuals, and it is therefore important to evaluate the pathogenicity of each variant. In this study, we analyzed 67 cardiomyopathy-associated genes in a male Japanese patient who was hospitalized for recurrent severe heart failure and identified a novel truncation variant, TTN Ser17456Arg fs*14. This TTN truncation variant was located in the A-band region. Moreover, the patient’s mother with heart failure harbored the same variant, whereas the father and brother without heart failure did not harbor the variant. To examine the functional changes associated with the truncation variant, H9c2 cells were subjected to genome editing to generate cells with a homologous truncation variant. The cells were differentiated using all-trans-retinoic acid, and the mRNA expression of skeletal actin and cardiac actin were found to be increased and decreased, respectively, consistent with known changes in patients with DCM or heart failure. In contrast, another cell with the titin truncation variant used as a control showed no changes in heart failure-related genes. In summary, we found a novel TTN truncation variant in familial DCM patients and confirmed its functional changes using a relatively simple cell model. The novel truncation variant was identified as a pathogenic and disease-causing mutation.

Key words: Genome editing, Heart failure

Heart failure is a life-threatening disease often caused by cardiomyopathy. Primary cardiomyopathy can be classified as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, or left ventricular noncompaction (LVNC).1,2 DCM is characterized by ventricular enlargement and progressive heart failure with a prevalence of 1:250-2500 adults.1,2 Additionally, 20-50% of DCMs have a family history of DCM (FDCM), predominantly showing autosomal dominant traits.3,4

More than 40 genes have been reported as causative genes for DCM. Among these genes, TTN, encoding the titin protein, is frequently reported in patients with DCM; in particular, heterozygous truncation variants of TTN are noted in 14-25% of all DCM cases.4,6 Titin is the largest cytoskeletal protein and plays important structural, developmental, and mechanical regulatory roles in striated muscle, i.e., heart and skeletal muscles, particularly with regard to passive stiffness.3,6 Titin truncation variants are expected to result in loss of functions, leading to passive tension and to the impairment of various titin-mediated signaling pathways, and ultimately to DCM.4,5,6 However, 1-2% of the general population without DCM carry TTN truncation variants, and therefore the pathological significance of each TTN truncation variant should be investigated.5,12,13

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with the truncation variant in the same domain were constructed using a relatively simple method in order to examine whether changes in gene expression in the cells were similar to those observed in general DCM.

Methods

Cardiac imaging and histological examinations: To evaluate the patient’s cardiac function, echocardiography (EPIQ 7G; Phillips Healthcare, MA, USA) was performed. In addition, cardiac catheterization and myocardial biopsy at the right ventricle myocardium were performed when the proband patient was 46 years old. The specimen was fixed with 10% formaldehyde, embedded in conventional-dehydrated paraffin after dehydration, sectioned, stained with hematoxylin and eosin (HE) and Masson trichrome, and observed under an optical microscope.

Genetic analysis and evaluation of genetic variants: Genomic DNA was extracted from the peripheral blood of the patient using a Wizard DNA purification kit (Promega Corporation, WI, USA). Genetic analysis of the genomic DNA was performed using an Ion Torrent PGM system (Thermo Fisher Scientific, CA, USA) for 67 genes reported as inherited primary and secondary cardiomyopathy-associated genes: ABCC9, ACTC1, ACTN2, ANKRD1, BAG3, CALR3, CAV3, CRYAB, CSRP3, DES, DOLK, DSGH2, JUP, LAMA4, LAM2, LDB3, LMNA, MTO1, MURC, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYL2, DSP, DTRA, EMD, EYA4, FHL1, FHL2, FHOD3, FRTN, GAA, GATA1, GJA1, GLA, ILK, ISL1, JPK2, MYOZ2, MYPN, NEBL, NEXN, OBSCN, PK2, PLN, PRDM16, PRKAG2, PSEN1, PSEN2, RBP2, SCN5A, SDHA, SGCD, TAZ, TCA1, TGBF3, TIEG1, TMEM43, TMPO, TNCC1, TNIN2, TNNT2, TPM1, TTN, TTR, and VCL. Sequenced lead data were mapped to the reference genome, hg19, using Torrent Suite 4.4 (Thermo Fisher Scientific) to extract the variants. Next, variants with a minor allele frequency (MAF) of less than 0.2% were selected from the following databases: the 1000 Genome Project, the Genome Aggregation Database (gnomAD), and the Human Genetic Variations Database (HGVD), which is a general Japanese variant database. In addition, nonsynonymous variants were selected using CLC Genomic Workbench (Qiagen, Hilden, Germany). To confirm the identified TTN variant, Sanger sequencing was performed using the following primers: TTN 17456F, 5'-GACATGGATGAGCTAATGG-3' (forward), TTN 17456R, 5'-CATTGCTGACATTGGAAC-3' (reverse). The PCR products were purified using ExoSAP-IT (Affymetrix, CA, USA) and sequenced using a BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI 3130 (Thermo Fisher Scientific). In addition, the PCR product of the TTN variant was cloned into the pMD 20-T vector using a Mighty TA-cloning Kit (Takara Bio, Shiga, Japan), and each allele of the gene mutation was confirmed by sequencing. Written informed consent was obtained from patients who agreed to undergo genetics analysis. Segregation analyses were performed for the proband patient’s family members. This study was approved by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University (TMDU) and Tokyo Medical University School of Medicine.

Functional analysis using genome-edited cell line: H9c2 cell line (ATCC, VA, USA) derived from rat embryonic cardiomyocytes was used as a model system. H9c2 cells with TTN frameshift variants were generated using the Guide-it CRISPR/Cas9 system (Clontech, CA, USA). Target genomic sequences in single guide RNAs (sgRNAs) were customized by designing pairs of oligos (5’-TACCA CACACTCGAATTTTC-3’ and 5’-GAAATTCAGAAGTGT GGTGA-3’) for generating variants in TTN 16334fs, and 5’-CAGATTCGTCTCAGGC-3’ and 5’-GCGTCA GAGACAGAATGTG-3’ for TTN V17602fs). After hybridization of the target oligo, it was cloned into pGuide-it-ZsGreen1 vector with the cas9 sequence. H9c2 cells were transfected with pGuide-it-ZsGreen1 vector having the generated target gRNA sequence using Lipofectamine TM 3000 Reagent (Thermo Fisher Scientific). After 48 hours, H9c2 cells expressing ZsGreen were selected from cells transfected with the pGuide-it-ZsGreen1 vector using MoFlo XDP (Beckman Coulter, CA, USA) and cultured as single cells. Each single cell clone was cultured and subjected to Sanger sequencing for validation of gene editing. The mutant and wild-type H9c2 cells were seeded in 24-well plates at a density of 3 × 10^4 cells/cm^2 and cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After reaching 70-80% confluence, the cells were cultured for 1 week in medium supplemented with 1% FBS with 100 nM all-trans-retinoic acid (ATRA; Sigma-Aldrich, MO, USA) for differentiation. Cells cultured in 10% FBS without ATRA were used as controls. Total RNA was extracted from cultured cells using a Nu- cleoSpin RNA kit (Takara Bio). The extracted RNA was converted into cDNA using a PrimeScript II first strand cDNA synthesis kit (Takara Bio) according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed on the obtained cDNA using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). The relative expression of the target gene was evaluated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. The target genes and primers are listed in Table 1. For relative gene expression analysis, calibration curves for the target gene and reference gene were prepared using a dilution series of standard samples, and the relative expression levels of the target and reference genes were calculated based on the calibration curves. Statistical analysis was performed using the unpaired or Welch’s t-tests; statistical significance was indicated by P values lower than 0.05. For the TTN gene sequence, the following accession numbers in GeneBank were used: human TTN, NM_001256850; rat TTN, XM_017592328.

Results

Clinical course of a proband patient with DCM: The proband male patient (Figure 1, IV-1) was admitted to Tokyo Medical University Hospital because of acute heart failure associated with left ventricular systolic dysfunction (NYHA class IV) at 46 years of age. Echocardiography
findings showed marked dilation of the left ventricle and decreased ejection fraction; coronary angiography showed no stenosis of the coronary artery. Myocardial biopsy showed abnormal arrangement and vacuolation of cardiomyocytes and interstitial fibrosis, suggesting DCM (Figure 2A, B). Two years later, at the age of 48, despite appropriate medical treatment and a heart failure management program, his heart failure suddenly worsened, and the patient was readmitted to hospital. An echocardiogram showed left ventricular diffuse hypokinesis and 4-chamber enlargement (Figure 3A, B). The left ventricular end-diastolic/end-systolic dimension was 69/65 mm, and the left ventricular ejection fraction was 17%. The thicknesses of the interventricular septum and posterior wall were 7 and 8 mm, respectively, and there was no cardiac hypertrophy. In addition, marked trabeculae formation was observed around the apex of the left ventricle, and LVNC was also suspected but did not match the diagnostic criteria (Figure 3C).¹⁸ The patient received an infusion of a phosphodiesterase inhibitor, the dose of beta-blocker was gradually increased, and he continued to receive ACE inhibitors and aldosterone antagonists. Fortunately, he was able to leave the hospital without a heart transplantation. About 3 years have passed since the patient was discharged from the hospital; the echocardiographic findings did not show any improvement in the cardiac contractility
or ventricular diameter, and no reverse remodeling was obtained.

**Genetic analysis of the proband and family members:** Mutation analysis of 67 reported cardiomyopathy-associated genes in the patient’s genome identified a frameshift variant, **TTN** c. 52368_52370 delinsGCAAA, p. Ser17456Arg fs* 14. This variant consists of a 3-base deletion, a 5-base insertion, and a frameshift, resulting in a truncation (Figure 4), in the 7-domains super-repeat segment (D-zone) of the A-band region of titin (Figure 5). This region is evolutionarily conserved, and the variant was novel because it was not found in multiple genome variation databases including gnomAD, which has accumulated more than 140,000 individuals from multiple ethnic groups, and HGVD, which contains data from the general Japanese population.15,16) The proband’s maternal family members had also experienced heart disease, and his mother (III-7) developed heart failure in her 40s (Figure 1). The truncation variant was observed in his mother, but both the proband’s father (III-4) and brother (IV-2), who did not have heart disease, were confirmed not to carry the variant (Figure 1). Thus, this **TTN** truncation variant was co-segregated with the heart failure in his family.

**Functional analysis using a genome-edited cell line:** To investigate the functional alterations associated with the **TTN** truncation variant, a cell model was constructed using genome editing. H9c2 rat cardiomyoblasts exhibited low titin expression; however, when differentiated using a low concentration of bovine serum plus ATRA, the expression levels of titin (**TTN**) and cardiac actin (**ACTC1**) increased, whereas those of skeletal muscle actin (**ACTA1**) remained low, resulting in an enhanced cardiac phenotype (Figure 6A). Cardiac troponin T in H9c2 cells were expressed before differentiation (data not shown).

H9c2 cells with the rat **TTN** V17602fs truncation variant were generated by genome editing. Codon 17602 in rat titin is a homologous sequence site on the same exon as codon 17456 of human titin, and rat **TTN** V17602fs is considered a homologous truncation variant to human **TTN** S17456Rfs found in the proband case (Figure 5). In addition, as the external control, H9c2 cells with the rat **TTN** I6334fs (homolog of human **TTN** V6382fs) were generated. This variation, located in the titin I-band region, has been reported to be on an exon with a relatively low transcription level owing to alternative splicing and is considered to be a benign genetic variant11) (Figure 5). H9c2 cells with the respective truncation variants, **TTN** V17602fs and **TTN** I6334fs, were selected as heterozygotes, as was observed in humans, and differentiated...
Figure 3. Two-dimensional echocardiography from the proband patient at the age of 48 years old, showing the parasternal long-axis view (A) and apical 4-chamber view (B, C). All 4 chambers of the heart were enlarged. Marked trabeculae carneae were observed in the left ventricular apex (circled area).

Figure 4. Nucleotide sequence of TTN from the proband patient. The upper panel shows the results of Sanger sequencing, and a frameshift was identified (arrow). The middle and lower panels show the sequences of the cloned wild-type and mutant alleles. A 3-base (TGT) deletion followed by a 5-base (GCAAA) insertion resulted in a heterozygous frameshift variant, yielding the truncation variant TTN Ser17456Arg fs*14, as shown in the box.
TTN mRNA expression was elevated in both cell lines (Figure 6B). The mRNA expression of ACTA1, which is usually increased in human heart failure and DCM, was not changed in H9c2 cells carrying TTN I6334fs compared with wild-type, but was increased in H9c2 cells with TTN V17602fs found in this FDCM (Figure 6B). In addition, the mRNA expression of ACTA1 was not changed in the cells with the TTN I6334fs from the wild-type, but was decreased in the cells with TTN V17602fs (Figure 6B).

Discussion

In this study, we identified a TTN truncation variant, TTN Ser17456Arg fs*14 in FDCM, by genetic analysis of cardiomyopathy-associated genes. The male proband with DCM presented with severe heart failure, and echocardiographic findings similar to LVNC were suggestive of a severe form of DCM. The same variant was found in his brother, who did not have heart failure, indicating a genetic co-segregated finding. This truncation variant was not found in multiple gene variant databases of the general population and was considered to be novel.

Titin, also called connectin, is a large cytoskeletal protein that extends from the Z-disc to the M-line and contributes to the maintenance of sarcomere and myofibril elasticity and to various signaling pathways.\(^8\,19\,20\) TTN mutations in patients with DCM were found for the first time in the Z-disc and A-band regions; thereafter further genetic analysis of TTN was carried out with the development of next-generation sequencing (NGS) technology, indicating that TTN truncation variants were the most frequent cause of DCM.\(^6,13\,12\) Although the mechanism for the development of DCM from titin truncation variants is still unclear, the major mechanism is thought to involve nonsense-mediated mRNA decay.\(^11,13,22\) However, TTN truncation variants are also found in approximately 1-2% of the healthy population, and the pathogenicity of each truncation variant should be investigated separately.\(^5,12\,13\) Currently identified TTN truncation variants in DCM may explain the differences in terms of alternative splicing. Indeed, DCM tends to occur in patients with TTN truncation variants in regions with a high percent spliced in (PSI) value, i.e., the fraction of mRNAs that contain an exon, and truncation variants found in healthy individuals tend to occur in regions with low PSI values.\(^5,12,25\) However, TTN truncation variants in regions of high PSI have also been reported in healthy individuals, and therefore DCM cannot be explained by PSI alone; thus, the pathogenicity of individual truncating mutants should be assessed.\(^13\)

In this study, genome editing for truncation variants was performed in rat H9c2 cells, which are inexpensive to maintain and relatively easy to handle, in order to assess whether the TTN truncation variant identified in this case could indeed be associated with the phenotype of DCM or heart failure. H9c2 cells with the rat TTN V17602fs, equivalent to the TTN S17456fs found in the FDCM family, showed increased expression of skeletal actin and decreased expression of cardiac actin. The altered gene expression pattern, which increases fetal gene expression, is common in patients with heart failure.\(^26\,27\) In contrast, H9c2 cells harboring rat TTN I6334fs, homologous to human TTN V6382fs, were used as control. This variant has been reported to be present in exons at a relatively low PSI in the adult myocardium.\(^11,13\) In these control cells, expression of TTN was increased, but no significant changes in skeletal or cardiac actin expression were observed.

Considering clinical applications, there is a need for a simple assessment of the pathogenicity for each mutation; for example, in secondary cardiomyopathies with therapeutics, such as cardiac Fabry disease, the individual
**Figure 6.** A: The mRNA expressions levels of titin (TTN), cardiac actin (ACTC1), and skeletal actin (ACTA1) were measured using qPCR. The expression levels were normalized to those of GAPDH expression. Gene expression levels in undifferentiated H9c2 cells cultured in normal medium (10% FBS) were used as a control, and differentiated H9c2 cells cultured in medium with reduced FBS concentrations plus all-trans-retinoic acid (ATRA; 1% FBS + ATRA) are shown. Expression levels of each mRNA are presented as the relative quantification (RQ) values relative to the control levels (mean ± SEMs (error bars); n = 3). Statistical differences were examined using the unpaired and Welch’s t-tests (*P < 0.05, **P < 0.01). B: mRNA expression of TTN, ACTC1, and ACTA1 in H9c2 cells with wild-type, or with the heterozygous TTN truncation variants, I6334fs and V17602fs, after differentiation. The expression levels were normalized to GAPDH expression and are presented relative to that in the wild-type cell (WT). Data are presented as the mean ± SEM (error range); n = 3, except for the expression of ACTA1 in TTN V17602fs (n = 2). Statistical differences were tested using the unpaired and Welch’s t-tests (*P < 0.05, **P < 0.01).

gene mutations identified were introduced into cells, and the potential for therapeutic effects was assessed at the cellular level.30) In terms of cell types, it is ideal to induce the differentiation from human-induced pluripotent stem cells or other stem cells; however, the cost and time for differentiation in culture are problematic. In rats, TTN truncation mutation models had the phenotype of heart failure and a cell line from rat origin, such as H9c2, may be used for evaluation of human TTN truncation variants.25) This approach could be used as a convenient
screening method if cells with mutations equivalent to those of humans would be generated to evaluate their pathogenicity with a limited number of heart failure-associated markers.

Further studies are needed to develop more precise and simpler methods for evaluating the pathological significance of individual mutations. In particular, changes in cellular function due to TTN truncating variants in the exons of high PSI in healthy individuals need to be evaluated to determine whether they are at risk of developing heart failure. In this regard, it will be necessary to build simpler cellular models with developing concise methods for genome editing.

Conclusions

In this study, we identified a novel TTN truncation variant, TTN Ser17456Arg fs*14, in a Japanese proband patient with severe DCM and his mother, who had heart failure. We examined the effects of the TTN truncation variant in a cell model with a homologous truncation variant, which was generated relatively easily by genome editing of H9c2 cells. In these cells, a gene expression status similar to that observed in heart failure was recognized in the truncation variation found in our patient with DCM, suggesting that TTN Ser17456Arg fs*14 is a disease-causing mutation.

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Disclosure

Conflicts of interest: The authors declare that they have no conflicts of interest.

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