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Case Report

A novel homozygous missense mutation in *PNPLA2* in a patient manifesting primary triglyceride deposit cardiomyovasculopathy

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ABSTRACT

Primary triglyceride deposit cardiomyovasculopathy (P-TGCV), caused by a rare genetic mutation in *PNPLA2* encoding adipose triglyceride lipase (ATGL), exhibits severe cardiomyocyte steatosis and heart failure. Here, we report the case of a 51-year-old man with P-TGCV homozygous for a novel *PNPLA2* mutation (c.446C > G, P149R) in the catalytic domain of ATGL. Analyses of endomyocardial biopsy specimens and *in vitro* expression experiments showed mutant protein expression with conserved lipid binding, but reduced lipolytic activity, indicating mutation pathogenicity.

1. Introduction

Primary triglyceride deposit cardiomyovasculopathy (P-TGCV) is an ultra-rare genetic deficiency of adipose triglyceride lipase (ATGL) [1,2] encoded in *PNPLA2*, inherited in an autosomal recessive fashion (ORPHA code:565612), that we first reported in Japanese patients who required cardiac transplantation (CTx) in 2008 [3–5]. Defective intracellular lipolysis of TG results in cardiomyocyte steatosis and severe heart failure [6]. To date, only ten patients have been reported globally [3–5,7,8]. Clinically, the patients were diagnosed in middle-age. Some patients showed dilated, whereas others showed hypertrophic, cardiomyopathy-like phenotypes [9]. Among the ten patients, six died of heart failure or suddenly [5], and three received CTx [4,8]. Therefore, P-TGCV is a rare but important cause of adult-onset heart failure with poor prognosis. (See Fig. 1.)

Here, we report a case of P-TGCV with a novel homozygous missense *PNPLA2* mutation in the ATGL catalytic domain.

2. Material and methods

2.1. Pathological analyses of endomyocardial biopsy specimens

An endomyocardial biopsy of the right ventricle was performed for the differential diagnosis of heart failure. Paraffin-embedded sections were stained with hematoxylin-eosin (HE) and immune-stained with an anti-perilipin-2 (PLIN2) antibody (No. 690102; 1:100. PROGEN, Germany) [10], and anti-ATGL antibody (No. 2138; 1:100. Cell Signaling, Danvers, MA, USA) [11].

Abbreviations: ATGL, adipose triglyceride lipase; BMIPP, ^{123}I-β-idophenyl-p-pentadecanoic acid; CTx, cardiac transplantation; HE, hematoxylin-eosin; NLSD, neutral lipid storage disease; NLSD-I, NLSD with ichthyosis; NLSD-M, NLSD with myopathy; PCR, polymerase chain reaction; PLIN2, perilipin-2; TGCV, triglyceride deposit cardiomyovasculopathy; WR, washout rate.

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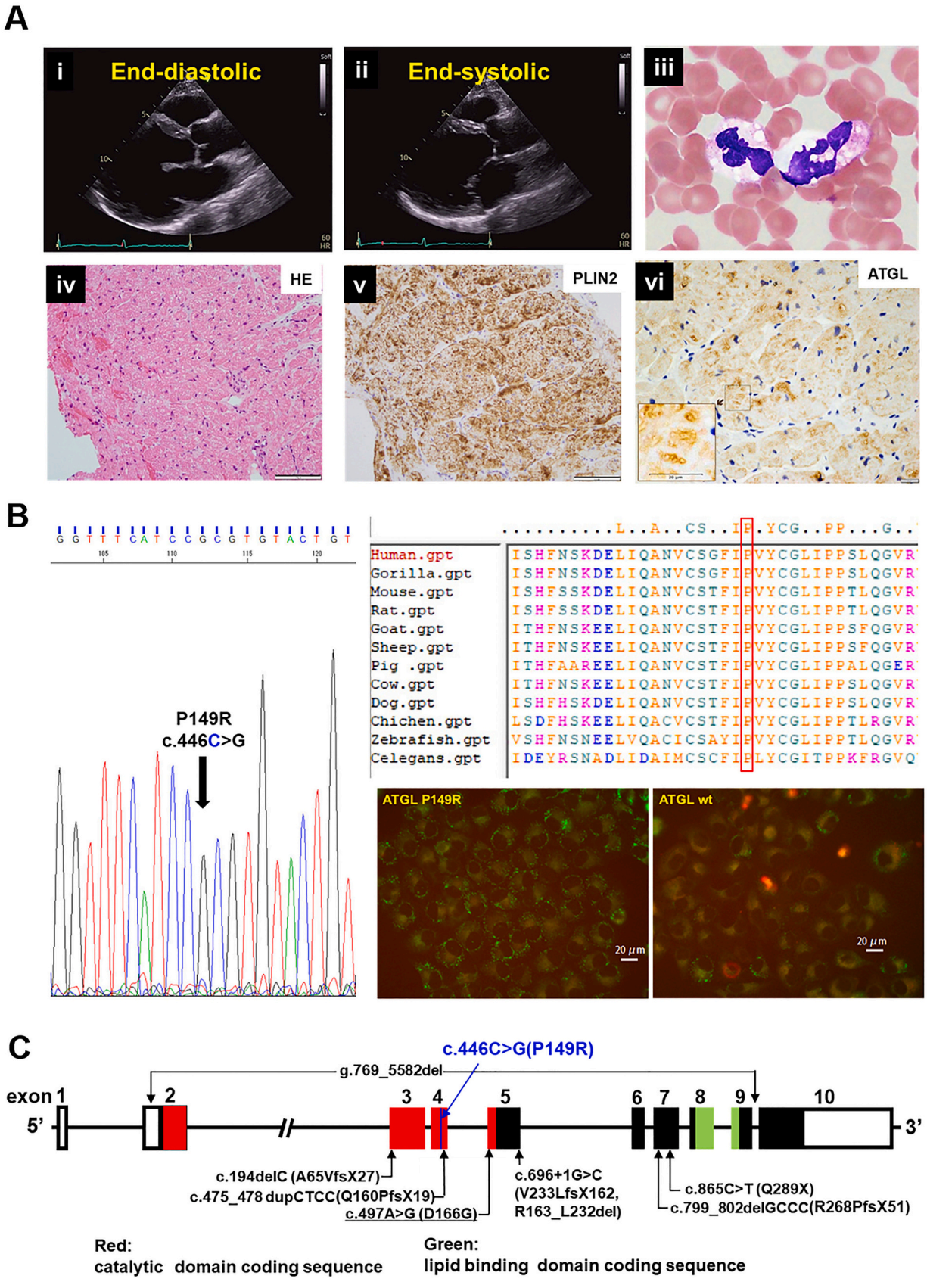

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Fig. 1. A. i, ii) Parasternal long axis views of echocardiography at end-diastolic and end-systolic phases, respectively. iii) Jordans' anomaly in peripheral leukocyte shown in May-Giemsa staining. iv) Hematoxylin-eosin (HE) staining, v) immunostaining for perilipin-2 (PLIN2) and vi) for adipose triglyceride lipase (ATGL) of endomyocardial biopsy specimens. Scale bars are 100, 100, and 20 μm. Magnified image is shown bottom left in vi) to show ring appearance of ATGL immunoreactivities, indicating the association with lipid droplets.
B. Left: sequence electropherogram of *PNPLA2* of the patient. The arrow indicates the homozygous c > g mutation (c.446C > G, P149R).
Upper right: alignments of ATGL sequence around the mutation in different species. The red box indicates amino acid residue P149, which is conserved between species.
Lower right: imaging of oleic acid-treated HeLa cells transfected with DsRed-fused P149R (left) or wild-type ATGL (right). The panels show merged images of lipid droplets (green) and DsRed (red). Scale bar = 20 μm.
C. Schematic representation of *PNPLA2* mutations in the patients with primary triglyceride deposit cardiomyovasculopathy (P-TGCV).
Protein-coding sequences are shown as solid blocks. Blue letters indicate the present case. The blue arrow indicates the position of the c.446C > G mutation. Underlined letters indicate a patient originating from South Asia.

2.2. Sequence of PNPLA2 gene from the patient

All protein-coding regions (exon 2–10) of the *PNPLA2* gene, including exon/intron boundaries, were amplified from the patient's genomic DNA by polymerase chain reaction (PCR). The PCR products were subjected to conventional Sanger sequencing.

2.3. Construction of the C-terminal DsRed-fused human ATGL

The wild-type ATGL cDNA was amplified by reverse transcription-PCR from total RNA of the human heart. The ATGL c.446C > G mutation was introduced using primers harboring this site. The DsRed coding sequence was amplified using the pDsRed-Monomer-C1 vector (Clontech, Mountain View, CA, USA). Both ATGL and DsRed PCR products were inserted into the pEB Multi-Hyg vector (FUJIFILM Wako Pure Chemical, Osaka, Japan) to generate a fusion protein consisting of an ATGL protein fused to DsRed at the C terminus. The products were confirmed by DNA sequencing.

2.4. Cell culture

HeLa cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2.

2.5. Transfection and generation of stable transformants

HeLa cells were plated in standard 35-mm dishes and cultured to 80% confluence. The cells were then transfected with a pEB Multi-Hyg plasmid harboring either ATGL-DsRed or ATGL P149R-DsRed using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was replaced with fresh medium containing 200 μg/ml hygromycin B (Hyg) (FUJIFILM Wako Pure Chemical). Hyg-resistant stable cell pools were maintained in 200 μg/ml Hyg medium.

2.6. Treatment of cells with oleic acid

After treatment with fresh DMEM (+FBS) containing 300 μM oleic acid (Sigma-Aldrich, St. Louis, MO, USA) for 48 h, lipid droplets were stained with 2 μM Lipidye (Funakoshi, Tokyo, Japan). Fluorescence images were acquired using a BZ-X800 microscope (Keyence, Osaka, Japan). Filters (ex/em, 545/605 nm) and (ex/em, 470/525 nm) were used to detect DsRed and Lipidye, respectively.

3. Case report

A 51-year-old man (body mass index = 17.8) was admitted to hospital for first-time heart failure with dilated cardiomyopathy-like morphology. His parents, who have been free from heart diseases, were born in the same small village in the Kyushu island, Japan; consanguinity was denied. The patient complained of orthopnea. Echocardiography (Panel A i,ii) and cardiac magnetic resonance imaging (data not shown) revealed diffuse hypokinesis of the left ventricle. The left ventricular ejection fraction was 20%. In scintigraphy, the myocardial washout (WR) of ^{123}I-β-iodophenyl-p-pentadecanoic acid (BMIPP), a radioactive long-chain fatty acid analogue, was −7.4%, which was comparable to that of other patients with P-TGCV [5] (cut-off value of BMIPP-WR for the diagnosis for TGCV is <10%, according to the latest diagnostic criteria established by the Japan TGCV study group [12]). A peripheral blood smear showed Jordans' anomaly in the polymorphonuclear leukocytes (Panel A iii).

Detailed analyses of paraffin-embedded endomyocardial biopsy specimens showed multiple vacuoles in cardiomyocytes (Panel A iv) positive for PLIN2 (Panel A v), which is a marker for lipid droplets as well as for P-TGCV, as reported in proteomic analyses using TGCV patient-derived fibroblasts in culture [13]. Immunostaining for ATGL (Panel A vi) was positive on the rim and surrounding area of lipid droplets, indicating that ATGL in this patient was expressed and associated with lipid droplets. The patient had mild hearing loss as an extracardiac symptom. Neurological examination revealed normal manual muscle test results. No skin lesions or fatty liver were observed. Therefore, neutral lipid storage disease with myopathy (NLSD-M) [14] and NLSD-ichthyosis [15,16] were clinically excluded.

Genetic analyses revealed that this patient was homozygous for a novel point mutation in exon 4 (c.446C > G, P149R) of *PNPLA2* (LC705680) (Panel B). The amino acid residue P149, located in the catalytic domain of ATGL, is conserved between species (upper right, Panel B). HeLa cells stably expressing this mutant ATGL showed more lipid droplets when treated with oleic acid, compared with those expressing wild-type ATGL, suggesting reduced lipolytic activity (lower right, Panel B).

Combining the clinical phenotype, pathological analysis, and *in vitro* experiments, we conclude that this mutation is pathogenic for the expression of P-TGCV.

4. Discussion

As summarized in Panel C, 11 patients with P-TGCV, including the present case (blue character, Panel C), have been reported. Ten patients were Japanese, and the remaining patient originated from South Asia (underlined character, Panel C) [8,17]. The genetic bases of the 11 patients with P-TGCV were as follows: eight mutations in eight families, one large deletion, one premature stop codon, three frame shifts, one splicing defect, and two missense mutations. As described in the Introduction, the prognosis of P-TGCV is very poor. The mutations found were diverse, but all affected either the catalytic or lipid-binding domain or both. Our data also suggest that the amino acid residue P149, which is close to the known important residue D166 [8], is crucial for the catalytic activity of ATGL.

A significant future study will include *in vitro* biochemical measurement of the ATGL P149R activity to understand the roles of the amino acid residue P149 in the structure and function of ATGL.

Genetic *PNPLA2* mutations are known to cause NLSD-M and P-TGCV. Italian and Chinese registries for NLSD-M [18,19] have reported that

patients with NLSD-M suffer from slowly progressive, disabling skeletal myopathy, and that life prognoses were not affected. However, some patients showed severe cardiac involvement, as described in the first report of NLSD-M by Fischer et al. [14]. Further studies are required to elucidate the mechanisms underlying phenotypic differences between P-TGCV and NLSD-M.

Finally, we recently reported that in ATGL-knockout mice, which exhibit heart failure and death [2] similar to those observed in patients with P-TGCV, tricaprin ameliorated myocardial TG deposition and improved cardiac function [20,21]. Therefore, further awareness and data accumulation of genetic ATGL deficiency under global collaboration (https://clinicaltrials.gov/ct2/show/NCT02918032) is required to overcome this ultra-rare disease. In clinical settings, medical professionals in different specialties such as neurology, cardiology, and clinical laboratories should share information about this ultra-rare disease.

Ethics approval and consent to participate

Not applicable.

Consent for publication

We obtained the written informed consent of the patient to publish the case report.

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Authors' contributions

YH, YI, and KH wrote the manuscript and contributed to the data analyses and discussion. YI and HK performed pathological analyses of the patient. YH performed genetic analysis and cell-biological experiments. SS and HS took care of the patient. MI performed a literature search. KK, HN, and other authors contributed to the discussion. All authors have read and revised the manuscript and approved the final revision.

Declaration of Competing Interest

YH and KH have held the positions of the Joint Research Chair in collaboration with Toa Eiyo Ltd. (Tokyo, Japan) since February 2021. KH has been serving as a medical advisor for Toa Eiyo Ltd. since December 2021. The other authors have no competing interests to declare.

Data availability

The datasets generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

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