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Genetic mutations in adipose triglyceride lipase and myocardial up-regulation of peroxisome proliferated activated receptor-γ in patients with triglyceride deposit cardiomyovasculopathy

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ABSTRACT

Adipose triglyceride lipase (ATGL, also known as PNPLA2) is an essential molecule for hydrolysis of intracellular triglyceride (TG). Genetic *ATGL* deficiency is a rare multi-systemic neutral lipid storage disease. Information regarding its clinical profile and pathophysiology, particularly for cardiac involvement, is still very limited. A previous middle-aged *ATGL*-deficient patient in our institute (Case 1) with severe heart failure required cardiac transplantation (CTx) and exhibited a novel phenotype, "Triglyceride deposit cardiomyovasculopathy (TGCV)". Here, we tried to elucidate molecular mechanism underlying TGCV. The subjects were two cases with TGCV, including our second case who was a 33-year-old male patient (Case 2) with congestive heart failure requiring CTx. Case 2 was homozygous for a point mutation in the 5′ splice donor site of intron 5 in the *ATGL*, which results in at least two types of mRNAs due to splicing defects. The myocardium of both patients (Cases 1 and 2) showed up-regulation of peroxisome proliferated activated receptors (PPARs), key transcription factors for metabolism of long chain fatty acids (LCFAs), which was in contrast to these molecules' lower expression in *ATGL*-targeted mice. We investigated the intracellular metabolism of LCFAs under human *ATGL*-deficient conditions using patients' passaged skin fibroblasts as a model. *ATGL*-deficient cells showed higher uptake and abnormal intracellular transport of LCFA, resulting in massive TG accumulation. We used these findings from cardiac specimens and cell-biological experiments to construct a hypothetical model to clarify the pathophysiology of the human disorder. In patients with TGCV, even when hydrolysis of intracellular TG is defective, the marked

Abbreviations: ATGL, adipose triglyceride lipase; BSA, bovine serum albumin; CTx, cardiac transplantation; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; LCFA, long chain fatty acids; LSM, laser scanning microscopy; LVAS, left ventricular assist system; MEM, modified Eagle's medium; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PPAR, peroxisome proliferated activated receptors; TGCV, triglyceride deposit cardiomyovasculopathy.

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up-regulation of PPARγ and related genes may lead to increased uptake of LCFAs, the substrates for TG synthesis. This potentially vicious cycle of LCFAs could explain the massive accumulation of TG and severe clinical course for this rare disease.

1. Introduction

Adipose triglyceride lipase (ATGL), a protein expressed mainly in muscles and adipose tissues, is an essential molecule for hydrolysis of intracellular TG [1,2]. Since the first report of human *ATGL* mutants in patients with mild myopathy [3], only up to 40 cases with *ATGL* mutations have been reported [4–8]. Although *ATGL* knockout mice exhibited premature cardiac death resulting from massive accumulation of TG in the heart [9], cardiac involvement in human *ATGL* deficiency is still unclarified. Our previous report concerned a cardiac transplant recipient with a genetic *ATGL* mutation [10]. His myocardium and coronary arteries showed massive accumulation of TG, which constituted a novel clinical entity, and we named this abnormality as "Triglyceride deposit cardiomyovasculopathy (TGCV)" [10,11]. More recently, we demonstrated a novel type of atherosclerosis in their coronary arteries with TG-deposit smooth muscle cells [12].

The current report concerns possible molecular mechanism underlying TGCV. The subjects were two patients with TGCV, including our second case with a novel *ATGL* mutation. We found that peroxisome proliferated activated receptors (PPARs) were up-regulated in the myocardium of both patients. We used the patient's passaged skin fibroblasts to investigate the intracellular metabolism of long chain fatty acids (LCFAs) and to construct a pathophysiological model which could possibly explain human *ATGL* deficiency.

2. Materials and methods

2.1. Subjects

Case 1 was a 41-year-old male reported previously [10]. Case 2 was a 33-year-old male first experienced modest exertional dyspnea 12 years ago. He was diagnosed with a specific cardiomyopathy featuring lipid droplets in the cardiomyocytes, peripheral leucocytes (Jordan's anomaly), and type I fibers of skeletal muscle [13]. In spite of appropriate medical treatment, congestive heart failure developed rapidly and a left ventricular assist system (LVAS) was implanted and the patient underwent CTx.

2.2. DNA sequencing

All exons and exon/intron boundaries in the *ATGL* and comparative gene identification-58 (CGI-58) genes were sequenced using genomic DNA from Case 2, his relatives, and controls. Ninety-five Japanese healthy normal subjects were screened to confirm that the substitutions were actual pathological mutations. cDNA structures were analyzed in Case 2 fibroblasts.

2.3. Pathological, lipid, and molecular analyses of cardiac specimens

When the LVAS was implanted, a sample specimen of apical myocardium with 8 mm in size was obtained and frozen immediately and stored at −80 °C. These specimens were divided into three parts for analyses of pathology, lipid measurement, and real time PCR. GAPDH was used for the internal standard for real time PCR. Primers used were listed in the supplementary table. Two hearts from autopsied cases without apparent cardiovascular disease were used as controls (Controls 1 and 2).

2.4. Animal study

ATGL knockout mice were kindly provided by Professor Rudolph Zechner (University of Graz, Austria) [9] and were maintained with normal lab chow. Because these mice would become lethal when they were around 11 weeks old, the experiments were performed during 7–9 weeks after birth, at which we confirmed that massive TG had accumulated in the hearts. C57BL/6J mice were used as controls.

2.5. Skin fibroblasts

Skin fibroblasts obtained from Case 2 were used to establish cell lines. Normal fibroblasts ($n = 3$) were kindly provided by Dr. Yasuko Miyake (National Cerebral and Cardiovascular Research Institute, Suita, Japan). We confirmed that there were no substitutions in the *ATGL* of the normal fibroblasts.

2.6. Preparation of LCFAs

For the experiments in cell biology, fatty acids were dissolved in 95% ethanol at 60 °C and then mixed with pre-warmed BSA (10%) to yield a stock concentration of 7.5 mM [14]. Cells were treated with 50 and 250 μM of the fatty acids for 48 h.

2.7. Fluorescent labeling experiments

For studies of the uptake and intracellular trafficking of LCFAs, 4, 4-difluro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY FL C16, Invitrogen Life Technologies Inc., Carlsbad, CA, USA) was used as a tracer, which binds with intracellular fatty acids binding proteins, as well as Mitotracker Red CMXRos (Invitrogen Life Technologies Inc., Tokyo, Japan), which is an established tracer that is transferred to mitochondria. Cells were plated on chamber slides and incubated in MEM/10% FCS. Before the start of labeling experiments, cells were washed with PBS and incubated for 10 min with media without FCS. Both BODIPY FL C16 and Mitotracker Red CMXRos (final quantity: 5 μM for both) were added to the media and the cells were examined with a confocal laser scanning microscopy (LSM, Carl Zeiss, Eching, Germany). For the relevant experiments, cells were fixed with 4% formaldehyde, and then examined by LSM.

2.8. Gene silencing experiments with siRNA

siRNA was transferred using the Nucleofector device and its reagent (Amaxa, Cologne, Germany). *ATGL* siRNA was found to reduce *ATGL* mRNA, measured by real time PCR, by 90% in normal fibroblasts. This effect lasted for at least 3 days after nucleofection. Samples were obtained 2 days after nucleofection. Data were presented as mean ± SD from at least three different experiments.

2.9. Statistical analysis

All data are expressed as the mean ± SD. Mean values were compared by means of unpaired Student's *t*-test. Probabilities of less than 0.05 were considered to be significant.

The entire study was approved by the Ethics and Animal Experiments Committees of Osaka University and written informed consents were obtained from patients and their family members.

3. Results and discussion

3.1. Molecular Basis of patients with TGCV (Fig. 1)

Fig. 1-A shows the location of the *ATGL* mutations in Cases 1 and 2. Case 1 was homozygous for a missense mutation in the exon 7 (c.865C>T). The predicted protein lacks the lipid biding domain, as reported previously [3,10]. Case 2 was homozygous for a point mutation at the 5′ splice donor site of intron 5 (c.696+1G>C) (Fig. 1-B, upper panel). Genetic analyses of family members showed that his parents and a brother were heterozygous for the mutation (Fig. 1-B, lower panel). This mutation was not detected in any of the 95 Japanese control subjects. To confirm that the point mutation at the intron 5 splice donor site results in a splicing defect, we cloned and sequenced two distinct cDNAs from the patient's cultured fibroblasts. Fig. 1-C shows the cloned cDNA structures. In one cDNA, read-through into 93 bp of intron 5 resulted in a new reading frame shift at position 92 in a stop at position 162 (p.Val233LeufsX162) (Fig. 1-C, upper panel). The predicted protein reportedly lacks the hydrophobic carboxy terminus responsible for mediating lipid droplet binding [2]. In the other cDNA, exon 5 skipping resulted in the predicted ATGL protein lacking 70 amino acids (p.Arg163_Leu232del) (Fig. 1-C, lower panel), including Asp166, which is important for the catalytic dyad [2]. We therefore speculate that both predicted proteins may lack activities associated with ATGL.

3.2. Myocardial up-regulation of PPARs in human ATGL deficiency

Fig. 2 shows the myocardial TG contents and their related molecular expression in the TGCV patients (Cases 1 and 2). Measurements of TG content in the specimens obtained from the apical portion of the heart at LVAS implantation showed massive TG accumulation in the patients' myocardium (Fig. 2-A). Because it was reported that PPARs were down-regulated in ATGL-targeted mice [15], we examined expression of PPARγ, and -α in human mutants. We found that PPARγ mRNA expression was more than 4 times higher in the TGCV patients' myocardium than in that of controls and confirmed that the PPARγ immunoreactive mass

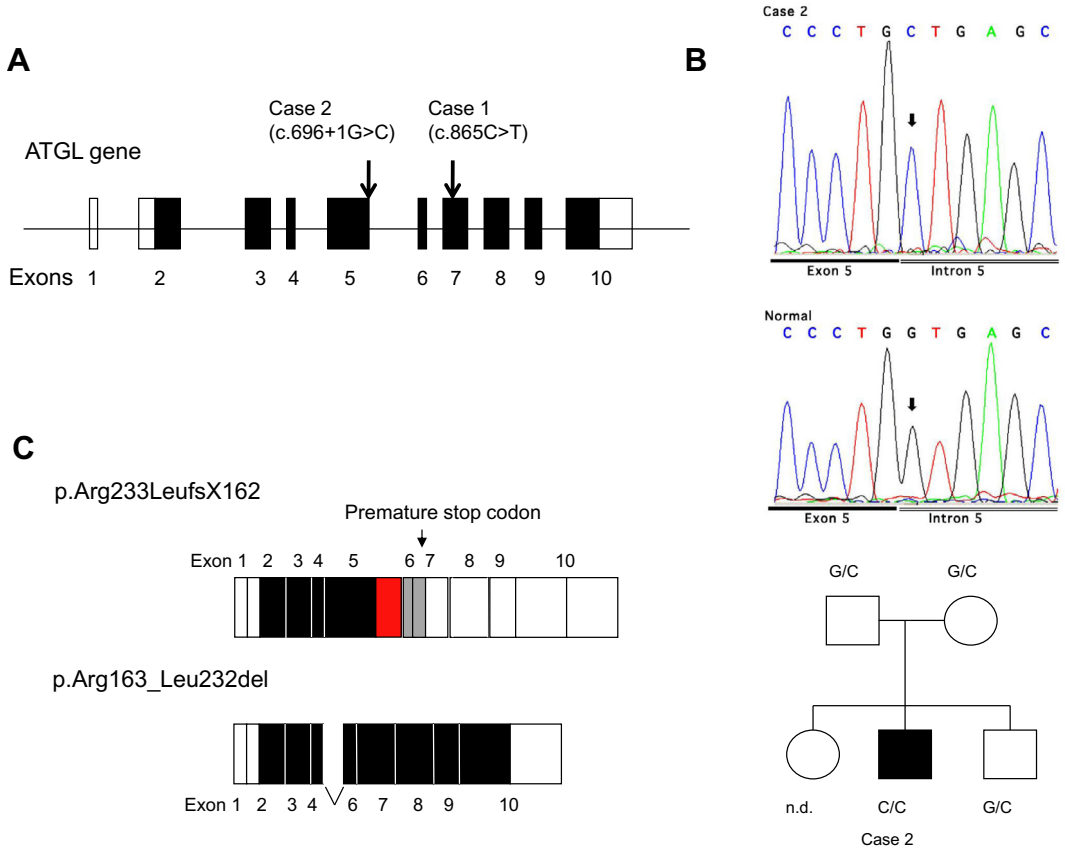

Fig. 1. Molecular Basis of two patients with TGCV (A) Structure of ATGL gene and locations of mutations in Cases 1 and 2. (B) Mutational analyses of ATGL. Electropherograms of the mutant and normal alleles of ATGL showing sequences around the site of the mutation (arrow; upper panel). Family pedigree of the case with genotype of the exon 5/intron 5 boundary (lower panel). Circles and squares represent females and males, respectively. Open symbols indicate unaffected family members, and closed symbols represent an affected individual with cardiomyopathy. C/C represents the sequence of the proband's intron 5 allele with C indicating the mutant-type alleles. G/C represents the sequence of unaffected family members' intron 5 allele with G indicating the wild type allele. (C) cDNA structures cloned from patient's fibroblasts. The two different cDNAs were cloned, showing that the mutation results in an intron 5 splicing defect. Open bars represent the non-coding exonic sequences and filled bars the coding exonic sequences. Red bars represent 92 bp intron 5 sequences read through as a result of intron 5 splicing defect. Grey bars represent sequences of exons 6 and 7 encoding 23 amino acids with frameshift changes. A: adenine; C: cytosine; G: guanine; T: thymine; n.d.: not done.

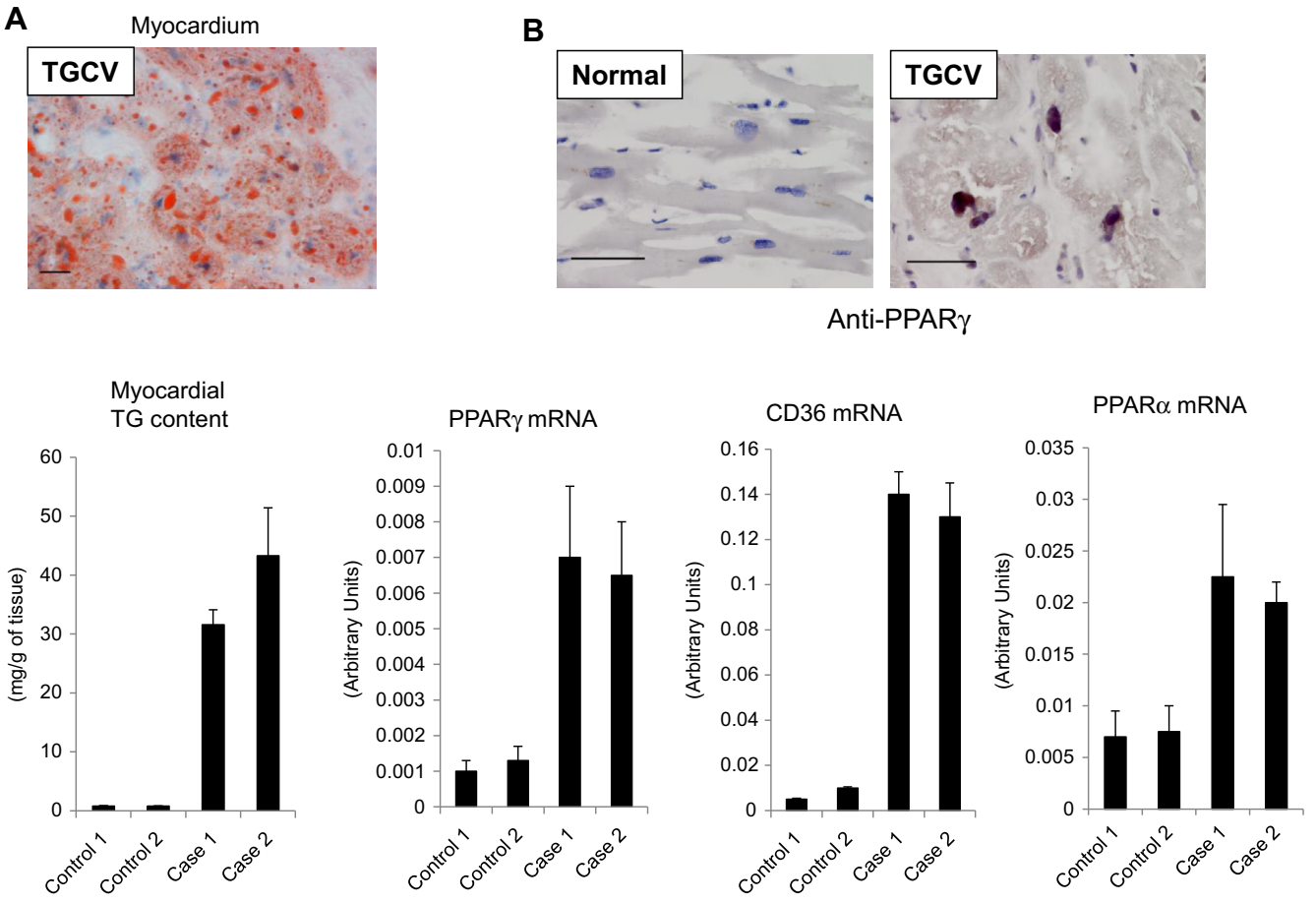

Fig. 2. Myocardial TG contents (A) and expression of PPARs and related genes (B). (A) Oil red O staining of endomyocardial biopsy specimens from Case 1 (upper panel) and myocardial TG contents of specimens at surgery (lower panel). The scale bar represents 40 μm. (B) Expression of PPARγ and related genes. Immunohistochemical analysis of PPARγ (upper panel) and mRNA expression detected by quantitative real time PCRs (lower panel).

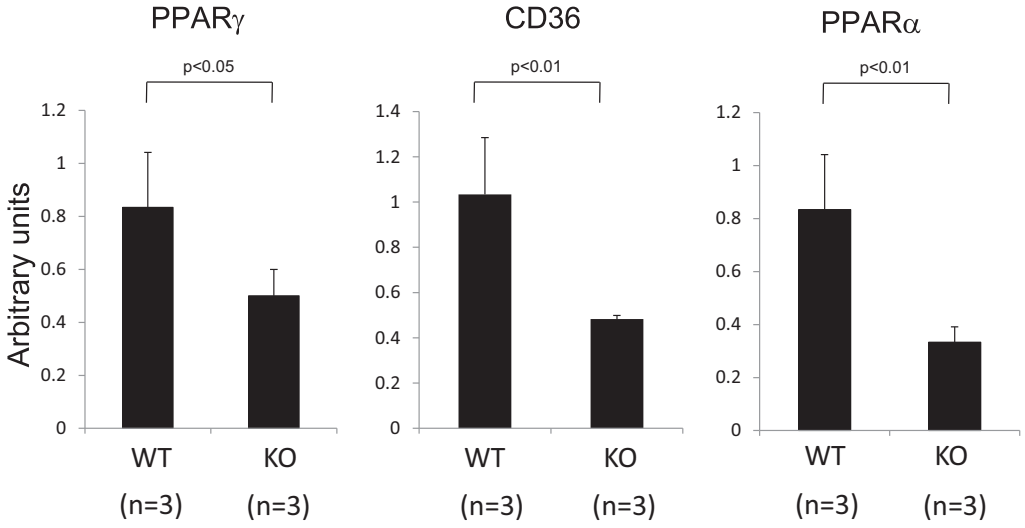

Fig. 3. Expression of PPARs and related genes in hearts from ATGL knockout mice ATGL KO and WT (C57BL6/J) mice were fed with normal chow and sacrificed when 7 weeks old ($n = 3$ each). mRNA was extracted from heart and subjected to quantitative real time PCR.

had increased in the patients' cardiomyocytes (Fig. 2-B). CD36, one of the downstream genes of PPARγ, had markedly increased (Fig. 2-B, lower panels). It should be noted that CD36 is a receptor or transporter responsible for LCFAs in human myocardium, as indicated in our previous study of genetic *CD36* deficiency [16]. Fatty acid binding protein-4 mRNA, which is another downstream gene, was also markedly up-regulated (data not shown). In addition, PPARα expression was also increased in human *ATGL* deficiency

(Fig. 2-B, lower panel). These findings are apparently the opposite of those for ATGL-targeted mice which showed down-regulation of these genes (Fig. 3). The observed down-regulation in the KO mice was consistent with the previous report [15]. A comparison of human with mice mutants showed that both the primary gene defect and its resultant myocardial phenotype with massive TG accumulation were identical. However, the molecular consequences secondary to *ATGL* mutation for human and mice mutants appear to be different. The exact molecular mechanism involved here remains unknown, but it is important to know the possible species differences when KO mice are used as a model to find therapeutic targets for human deficiencies.

3.3. Cellular LCFA metabolism in patients' skin fibroblasts

After confirming that *ATGL* deficiency results in the up-regulation of PPARγ in passaged skin fibroblasts (Fig. 4-A), we used a fluorescent LCFA probe (BODIPY FL C_{16}) as a tracer to analyze cellular metabolism of LCFA in these cells. Fig. 4-B shows the cellular uptake of BODIPY FL C_{16}. Fibroblasts with mutant *ATGL* had higher uptake of fluorescent LCFAs than did normal cells. Normal cells transfected with ATGL siRNA also showed similar phenotypes. Fig. 4-C shows the result of a time lapse study using confocal laser scanning microscopy. Five minutes after the addition of BODIPY FL C16 and Mitotracker, normal cells showed overlapping images of the two tracers. These findings indicate that a majority of the LCFAs were transported to mitochondria. In contrast, part of the BODIPY FL C16 was carried separately from Mitotracker in fibroblasts with *ATGL* mutations and normal fibroblasts treated with ATGL siRNA (Fig. 4-C), indicating that some LCFAs may be transported to lipid droplets in *ATGL*-deficient cells. Fig. 4-D shows that the addition of palmitate (C16:0) for 48 h induced massive accumulation of TG in mutant cells and cells transfected with ATGL siRNA. Addition of other LCFAs such as C18:1, C18:2, C20:4 and C20:5 produced similar phenotypic changes (data not shown), indicating that LCFA may be important for the development of neutral lipid deposition in *AGTL* deficiency.

3.4. Construction of a pathophysiological model for human ATGL deficiency

On the basis of all these data obtained from cardiac specimens and fibroblasts *in vitro*, we tried to construct a pathophysiological model as shown in the Graphic Abstract. Under normal conditions, cells take up LCFAs and transfer most of them to mitochondria for β-oxidation. The remaining LCFAs are then utilized as a source of TG and rapidly hydrolyzed by intracellular lipases such as ATGL. In the *ATGL* mutants, on the other hand, LCFAs are taken up and utilized as a source of TG which cannot be hydrolyzed, thus resulting in massive TG deposition. Especially in human deficiency, PPARγ and related genes are up-regulated, which increases the

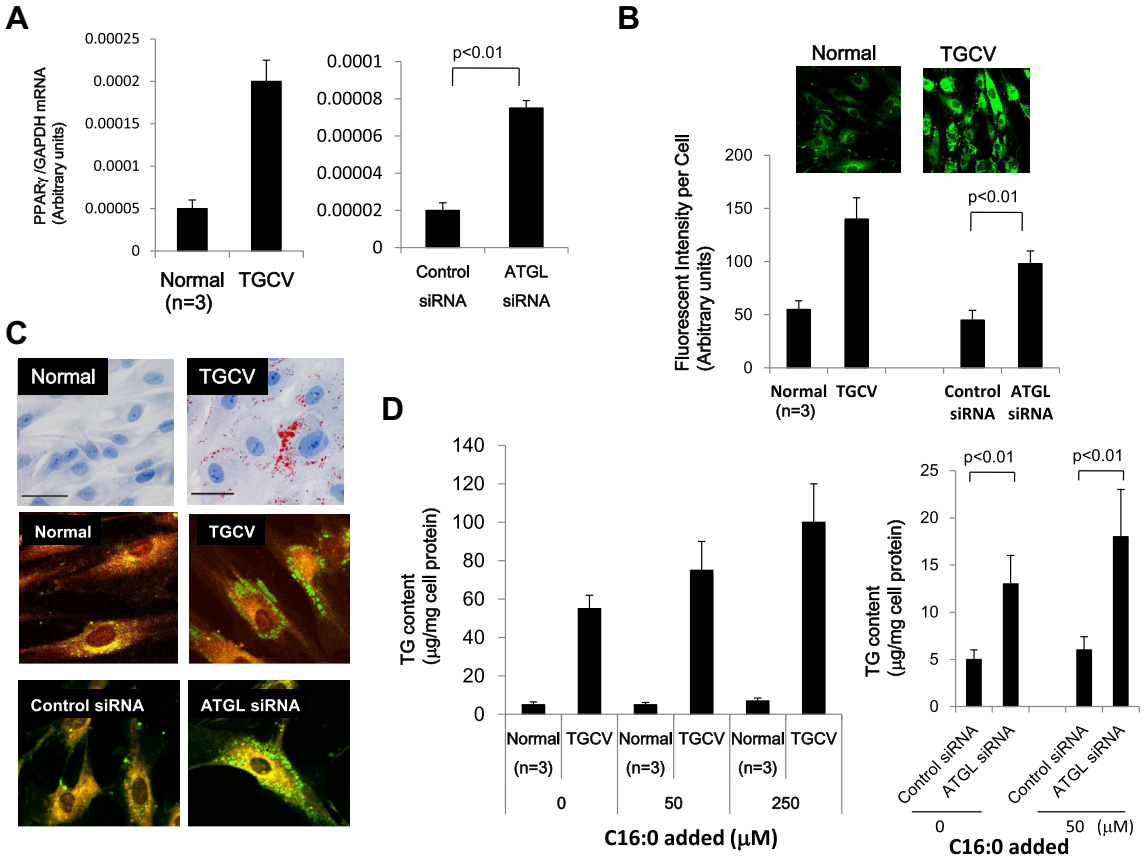

Fig. 4. Cellular phenotype and metabolism of long chain fatty acids (LCFAs) of fibroblasts with ATGL deficiency. (A) Expression of PPARγ in TGCV- (left) and ATGL- (right) targeted fibroblasts. (B) Uptake of fluorescent LCFAs in TGCV- and ATGL-targeted fibroblasts. Cells were incubated with BODIPY FL C16 for 5 min and fixed with formalin. Images were obtained with a confocal laser microscopy. Uptake of LCFAs is expressed as mean fluorescent intensity per cell. (C) Lipid droplets (upper panels) and detection of intracellular transport of LCFAs (middle and lower panels) in fibroblasts. Upper panels show oil red O and HE staining in normal and TGCV fibroblasts. Cells shown in the middle and lower panels were plated on chamberslides and incubated in MEM/10% FCS. Before the start of labeling experiments, cells were washed with PBS and incubated for 10 min without FCS. Both BODIPY FL C16 and MitoTracker Red CMXRos (both, final 5 μM) were added to media and cells and were examined with a confocal laser scanning microscopy (LSM510, Carl Zeiss). Images were obtained 5 min after incubation with fluorescent probes. (D) Effects of 48-h-incubation with 50 and 250 μM palmitate (C16:0) added to the media on intracellular TG contents in TGCV- (left) and ATGL- (right) targeted fibroblasts.

uptake of LCFA and induces abnormal transport of LCFAs to lipid droplets.

There are some reports concerning the association between up-regulation/overexpression of PPARs and cardiac dysfunction in humans and mice. In patients with arrhythmogenic right ventricular cardiomyopathy, which is characterized by lipid accumulation in right ventricles, myocardial up-regulation of PPARγ and related signals may contribute to severe myosin dysfunction [17]. Experimental overexpression of these molecules induced myocardial accumulation of TG and cardiac dysfunction in mice [18,19]. The potentially vicious cycle of LCFAs related to the up-regulation of PPARγ observed in our study may explain the massive accumulation of TG and subsequent severe clinical course in patients with TGCV.

Conflict of interest disclosures

No conflict to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.003.

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