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Novel missense mutations in *PNPLA2* causing late onset and clinical heterogeneity of neutral lipid storage disease with myopathy in three siblings

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ABSTRACT

Neutral lipid storage disease with myopathy (NLSD-M) is a rare autosomal recessive disorder characterised by an abnormal accumulation of triacylglycerol into cytoplasmic lipid droplets (LDs). NLSD-M patients are mainly affected by progressive myopathy, cardiomyopathy and hepatomegaly. Mutations in the *PNPLA2* gene cause variable phenotypes of NLSD-M. *PNPLA2* codes for adipose triglyceride lipase (ATGL), an enzyme that hydrolyses fatty acids from triacylglycerol. This report outlines the clinical and genetic findings in a NLSD-M Italian family with three affected members. In our patients, we identified two novel *PNPLA2* missense mutations (p.L56R and p.I193F). Functional data analysis demonstrated that these mutations caused the production of ATGL proteins able to bind to LDs, but with decreased lipase activity. The oldest brother, at the age of 38, had weakness and atrophy of the right upper arm and kyphosis. Now he is 61 years old and is unable to raise arms in the horizontal position. The second brother, from the age of 44, had exercise intolerance, cramps and pain in lower limbs. He is currently 50 years old and has an asymmetric distal amyotrophy. One of the two sisters, 58 years old, presents the same *PNPLA2* mutations, but she is still oligo-symptomatic on neuromuscular examination with slight triceps muscle involvement. She suffered from diabetes and liver steatosis. This NLSD-M family shows a wide range of intra-familial phenotypic variability in subjects carrying the same mutations, both in terms of target-organs and in terms of rate of disease progression.

1. Introduction

NLSD-M (MIM 610717) and NLSD-I (neutral lipid storage disease with ichthyosis, MIM 275630) are due to mutations in adipose triglyceride lipase (ATGL) or in α/β-hydrolase domain-containing protein 5 (ABHD5) and are characterised by systemic triacylglycerol deposition in cytoplasmic lipid droplets (LDs) in most tissues, including skeletal and cardiac muscles, liver and peripheral blood [1]. LDs are virtually present in all cell types, representing a reservoir of bioactive lipids and lipid-derived hormones in adipose and non-adipose tissues [2]. The white blood cell diagnostic feature of NLSD is characterised by lipid-containing vacuoles in leukocytes, which was originally described by Jordans [3,4]. Clinically, the main difference between NLSD-I and NLSD-M is that a lack of ABHD5 results in early ichthyosis and often liver involvement whereas a lack of ATGL essentially causes the predominant clinical phenotype with skeletal and cardiac myopathy [1].

In particular, NLSD-M patients are characterised by progressive myopathy (100%), cardiomyopathy (44%), elevated serum creatine kinase (100%), hepatomegaly (20%), diabetes (24%), chronic pancreatitis (14%) and short stature (15%) [5–11]. No ichthyosis is observed in patients suffering from NLSD-M, even though the skin of few patients appears to be particularly dry [12].

The onset of NLSD-M is caused by mutations in the *PNPLA2* gene, which codes for ATGL, a member of the patatin-like phospholipase domain-containing proteins [5]. This lipase is a lipid droplet-associated protein and catalyses the first step in the hydrolysis of triacylglycerols (TGs), stored within LDs [1] thereby generating diacylglycerols and free fatty acids.

The human ATGL protein consists of 504 amino acids divided into a N-terminal part containing the patatin domain (amino acids 10 to 178) with catalytic residues S47 and D166 and a C-terminal part containing a hydrophobic lipid binding domain at position 315–360. ATGL's enzymatic activity is co-activated by ABHD5, a 39 kDa protein that associates with LDs, and inhibited by the protein G0G1 switch gene 2 (G0S2). The

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254-N-terminal residues of ATGL are required for TG hydrolysis and for regulatory interactions with its co-activator ABHD5 and its inhibitor G0S2 [13].

To the best of our knowledge, 41 NLSD-M patients have been clinically and genetically characterised [5,14–21]. Twenty-seven different *PNPLA2* mutations, which could differently affect protein function or production, have been described. Here, we report the clinical and genetic findings of a NLSD-M family of Italian origin with three affected siblings. Moreover, in order to highlight the effect that different gene mutations may have on ATGL lipase activity, we have performed a functional characterisation of the novel *PNPLA2* missense mutations identified in our patients.

2. Materials and methods

2.1. Muscle histopathology

Histopathological evaluation of muscle was performed according to the standard procedures described by Dubowitz [22]. Light microscopy of frozen cross sections of muscle biopsies was used for conventional histochemical and histoenzymatic stains, including haematoxylin & eosin (H&E), Gomori trichrome, Oil-Red-O (ORO), NADH-TR, COX, SDH, acid phosphatase, acid and alkaline ATPases.

2.2. Cell culture

Fresh EDTA-treated peripheral blood samples (3 mL) from patients were centrifuged at 3300 *g* for 10 min. Buffy coats were carefully collected by gentle pipette suction, immediately smeared onto slide glasses, completely dried, and fixed with Biofix (Bio-Optica, Milan, Italy). May-Grünwald–Giemsa (MGG) staining was carried out according to the standard procedures. Human skin fibroblasts from patients were obtained from skin biopsies and cultured in Earle's minimum essential media (MEM) with 10% foetal bovine serum (FBS), 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 incubator. Fibroblasts were observed under phase-contrast light microscopy and photographed (40×; I X51, Olympus).

Fibroblasts (200,000 per dish) were also seeded on coverslips in Earle's MEM culture medium and allowed to adhere for 36 h. The medium was then removed and cells were washed with Dulbecco's phosphate-buffered saline (D-PBS), stained with Nile Red dye (NR, 9-diethylamino-5Hbenzo[alpha]phenoxazine-5-one) and examined with a Leica MB5000B microscope equipped with 20×, 40× and 100× oil immersion objectives. Fluorescence images were captured using a Leica DFC480 R2 digital camera and a Leica Application Suite (LAS) software.

2.3. Molecular analysis

Genomic DNA was extracted from peripheral blood using a Puregene DNA Isolation kit (Qiagen, Venlo, Netherlands). Primer sequences and PCR amplification conditions for the analysis of *PNPLA2* coding regions (GenBank NM02376) were previously reported [5]. All PCR products were purified (NucleoSpin Extract II, Macherey-Nagel, Germany) and sequenced on 3730 DNA analyser by the BigDyew Terminator V1.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Informed consent for genetic and histological analysis was obtained from participants of the study. Moreover, written informed consent from the patients was obtained for publication of the article and any accompanying images.

2.4. Generation of site-directed mutagenesis PNPLA2 plasmids and expression of recombinant mutants in HeLa cells

PNPLA2 cDNA has been previously cloned into pEGFP-N1 from Clontech (Mountain View, CA, USA) to produce pEGFPN1-*PNPLA2*, expressing ATGL with GFP at the C-terminus. Point mutations were performed using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific, Waltham, MA, USA). Mutations in pEGFP-*PNPLA2* plasmid were introduced using the following primers: S47A: forward 5′-CACA TCTACGGCGCCGCGGCCGGGGCGCTCACGG-3′ and reverse: 5′-CCGTGA GCGCCCCGGCCGCGGCGCCGTAGATGTG-3′; L56R: forward 5′-GCCACG GCGCGGGTCACCGGGG-3′ and reverse 5′-CCCCGGTGACCCGCGCCGTG GC; I193F: forward 5′-CGGGCGAGAGTGACTTCTGTCCGCAGGAC and reverse 5′-GTCCTGCGGACAGAAGTCACTCTCGCCCG. All constructs were verified by DNA sequencing.

HeLa cells were cultured on glass coverslips in Dulbecco's modified Eagle's medium with 10% FBS. 400 μM oleate complexed to BSA (6:1 molar ratio) was added to the medium and incubated overnight. The cells were then transiently transfected with control and recombinant *PNPLA2* plasmids using the Lipofectamine 2000 transfection reagent, according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). After 24 h, the cells were fixed and stained with ORO, and LDs from immunofluorescent images were examined with a Leica MB5000B microscope. Fluorescence images were captured using a Leica DFC480 R2 digital camera and a LAS software. Moreover, fluorescence images were also captured using a confocal microscope Leica TCS SPE equipped with 63× oil immersion objective.

2.5. Triglyceride quantification in NLSD-M fibroblasts and HeLa transfected cells

NLSD-M and control fibroblasts at the same passage (P8) were seeded onto plates at a density of 1×10^6 cells/plate and cultured as previously described. The day after, the cells were homogenised in 1 mL solution containing 5% TRITON X-100 (Sigma-Aldrich, Saint Louis, MO, USA), incubated at 80 °C for 5 min and centrifuged for 2 min. Cellular triglyceride content was quantified using Triglyceride Quantification Colorimetric Kit (Biovision, Milpitas, CA, USA), according to the instructions. The absorbance was measured at 570 nm with EnVision Multilabel Reader (PerkinElmer, Waltham, MA, USA).

5×10^5 HeLa transfected cells were cultured in DMEM with 10% FBS and supplemented with 400 μM oleate/BSA, and allowed to adhere overnight. The day after, the cells were transiently transfected, as explained in paragraph 2.4. After 24 h, the cells were homogenised and cellular triglyceride content was quantified using Triglyceride Quantification Colorimetric Kit. Intracellular TG content was expressed as nanomoles of TG per milligrammes of cellular protein.

2.6. Statistical and bio-informatic analysis

The statistical analysis of quantitative data on LDs identified from cells (fibroblasts and HeLa transfected cells) by image analysis of immunofluorescence experiments was made using SPSS v.19 package (SPSS, Chicago, IL, USA). The values were compared using x^2-squared test. A P-value of ≤ 0.05 was considered to be statistically significant.

The effect of amino acid substitutions on protein function was predicted using ClustalW, SIFT and PolyPhen software. A multiple sequence alignment of mammalian ATGL proteins was used as input for ClustalW. The NCBI reference sequence (NP_065109.1) of the human ATGL protein was used as the input for SIFT and PolyPhen, with default query options.

3. Results

3.1. Patient description

Family's pedigree is showed in Fig. 1A.

3.1.1. Patient 1

The oldest brother is now 61 years old (Fig. 1B). He worked as a clerk for the rail network, although from the age of 40 he complained of becoming easily fatigued. From the age of 38, he had weakness and