Genes affecting triglyceride metabolism: from steatosis to lipodystrophy
Monajemi, H.

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Chapter 6

Familial partial lipodystrophy phenotype resulting from a single-base mutation in DNA binding domain of peroxisome proliferator-activated receptor gamma

Houshang Monajemi, Lin Zhang, Gang Li, Ellen H. Jeninga, Henian Cao, Mario Maas, C.B. Brouwer, Eric Kalkhoven, Erik Stroes, Robert A. Hegele, Todd Leff

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ABSTRACT

Context: Familial partial lipodystrophy (FPLD) results from coding sequence mutations either in LMNA, encoding nuclear lamins A/C, or in PPARG, encoding peroxisome proliferator-activated receptor gamma (PPARγ). The LMNA form is called FPLD2 (MIM 151660) and the PPARG form is called FPLD3 (MIM 604367).

Objective: To investigate whether the clinical phenotype of this proband is due to mutation(s) in PPARγ.

Design: Case report

Setting: Academic medical center

Patient: A 31-yr-old female with the clinical phenotype of FPLD3; i.e lipodystrophy and early childhood diabetes with extreme insulin resistance and hypertriglyceridemia leading to recurrent pancreatitis.

Results: The proband was heterozygous for a novel C>T mutation in PPARG gene that led to the substitution of arginine 194 in PPARγ2 isoform, a conserved residue located in the zinc finger structure involved in DNA binding, by tryptophan (R194W). The mutation was absent from the genomes of 100 healthy Caucasians. In vitro analysis of the mutated protein showed that R194W (and R166W in PPARγ1 isoform) could not bind to DNA and had no transcriptional activity. Furthermore, R194W had no dominant negative activity.

Conclusions: The R194W mutation in PPARG disrupts its DNA binding activity and through haploinsufficiency leads to clinical manifestation of FPLD3 and the associated metabolic disturbances.
INTRODUCTION

Dunnigan-type familial partial lipodystrophy results from rare coding sequence mutations either in \textit{LMNA}, encoding nuclear lamina A/C, or in \textit{PPARG}, encoding peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) (1;2). The \textit{LMNA} and \textit{PPARG} forms are called FPLD2 (MIM 151660) and FPLD3 (MIM 604367), respectively. These mutations underlie profound redistribution of fat stores, characterized by lipoatrophy of the extremities and gluteal region in combination with lipohypertrophy in face, neck, trunk and central adipose stores. This redistribution can be accompanied by a variety of clinical characteristics, including severe insulin resistance, often with acanthosis nigricans, and hypertriglyceridemia, sometimes associated with pancreatitis and eruptive xanthomata (3). The core clinical phenotype is fat loss with subsequent development of secondary metabolic disturbances that are characteristic of the insulin resistance syndrome.

The presence of lipodystrophy in subjects with dysfunctional \textit{PPARG} missense mutations, such as R425C, F388L, E138fs$\Delta$AATG, V290M, P467L and Y355X (4-9) and in PPAR$\gamma$-deficient murine models (10;11) has confirmed the central role of PPAR$\gamma$ in adipogenesis. PPAR$\gamma$ interacts with retinoid X receptor (RXR), binds DNA as a heterodimer and subsequently regulates transcription of PPAR$\gamma$-responsive genes. Heterozygous loss of function or haploinsufficiency is clinically important when gene dosage is strictly regulated. Here, we show that a heterozygous mutation of a conserved arginine residue into tryptophan in the PPAR$\gamma$ (referred as R166W in PPAR$\gamma$1 and R194W in PPAR$\gamma$2 isoform) zinc finger II region disrupts DNA binding and transcriptional activity and thus underlies FPLD3.

METHODS

Study subject

\textit{The study was approved by the University of Western Ontario Ethics Review Panel (protocol \textcolor{red}{07920E}) and the subject gave informed consent to participate.}

Magnetic Resonance Imaging

MRI was performed using a 1.5 Tesla scanner (Signa, GE Medical Systems, Milwaukee, WI, USA) with a neck coil and body coil. Axial and sagittal T1 weighted images of the c-Spine, Axial T1 weighted images of the abdomen and the lower leg were acquired according to the procedure described earlier.

DNA sequence analysis

After DNA sequencing showed no mutation in \textit{LMNA}, we amplified and sequenced the 6 exons of \textit{PPARG} plus $>$100 bp at intron-exon boundaries and $\sim$700 bp of the promoter (5;7). The R194W mutation was genotyped by scoring the electropherogram tracing of exon 4 sequences from the Applied Biosystems 3730 Automated DNA Sequence Analyser (ABI, Mississauga, ON). Genomic DNA from 100 healthy Caucasian subjects was studied,
permitting 70% power to exclude a mutation with frequency >2% in the healthy population (two-tailed alpha<0.05).

**PPAR gamma clones**

A cDNA encoding full length human PPARγ1 was cloned into the pTRE-shuttle2 eukaryotic expression vector (Clontech, Palo Alto, CA). A double-FLAG epitope tag (MDYKDHDGDYKDHD) was added to the N-terminus of the clone. The pCDNA3-PPARγ1 and pCDNA3-PPARγ2 constructs were kind gifts from Dr. V.K.K. Chatterjee. The R194W mutation was introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by sequencing.

**Electrophoretic mobility shift assays (EMSA)**

EMSA experiments were performed as described (12). In short, a radiolabeled double-stranded DNA oligomer, containing the PPRE from the rat acyltransferase-coenzyme A oxidase promoter, was incubated with *in vitro* translated PPARγ (wild type or mutants) and/or *in vitro* translated RXRα proteins. For supershift experiments 1 μg of α-RXR (sc553; Santa Cruz Biotechnologies), α-PPARγ (sc7273) or α-Gal4 (sc510) antibodies were added. Receptor-DNA complexes were separated from unbound DNA on native gels and visualized by autoradiography. At least three independent experiments were performed. The complete probe sequences used for binding and competition analysis were as follows: PPRE-wild-type, 5'-CCG GGG ACC AGG ACA AAG GTC ACG AAG CT-3' and PPRE-mutant, 5'-CCG GGG GAC CAG CAC AAA GCA CAC GAA GCT-3'. Western blot analyses of the different *in vitro* translated PPARγ proteins was performed as described (13). α-PPARγ antibody (sc7196) was used to probe for PPARγ protein and ECL (Amersham Biosciences) was used for detection.

**Cell culture, reporter assays and dominant negative assays**

NIH 3T3 mouse fibroblasts and human U2OS osteosarcoma cells were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), 100 μg of penicillin/ml and 100 μg streptomycin/ml (Gibco Life Technologies). NIH 3T3 mouse fibroblasts were grown in 24-well plates (1.0×10⁵ cells/well) in DMEM+10% fetal calf serum. Cells were transfected with 25 ng WT or R194W expression plasmid, 6 ng of pTET-off, 25 ng of pRXR, 2 ng of a β-galactosidase control plasmid and 200 ng of the PPAR-dependent luciferase reporter pFATP-Luc (i.e. three copies of the mouse FATP gene PPRE inserted upstream of the minimal thymidine kinase promoter). Cells were transfected for 4 h with Lipofectamine-plus and then treated with DMSO or increasing doses of rosiglitazone for 16 h. Transfections were performed in triplicate. Mixing experiments examining dominant negative activity (Fig. 4E) were conducted as described above except with the amounts of PPARγ plasmids: NIH 3T3 cells that were transfected with the combination of 5 ng of WT PPARγ and increasing amounts of WT, the R194W mutant, or the dominant-negative mutant P467L (5, 10, or 20 ng; indicated in the figure as 1:1, 1:2 and 1:4 respectively), in the presence or absence of rosiglitazone (Fig. 4E and 4F).
U2OS were also seeded in 24-wells plates and transiently transfected using the calcium-phosphate precipitation method. Each well was cotransfected with 1 μg reporter construct, 10 ng pCDNA-PPARγ expression constructs and 2 ng pCMV-Renilla (Promega). After washing, cells were maintained in medium in presence or absence of rosiglitazone (1 μM) for 24 h. Activities of luciferase plus β-galactosidase (NIH-3T3) or luciferase plus renilla (U2OS) were measured with the Dual-light assay system (ABI, Foster City, CA) or Dual-Luciferase Reporter Assay System (Promega), respectively, using a 96-well luminometer (Berthold Technologies, Bad Wildbad, Germany).

RESULTS

Patient medical history and clinical evaluation
The proband was a 31-yr-old Turkish female living in the Netherlands. Menarche occurred at age 11, followed by regular menstrual cycles. At age 15, she was diagnosed with diabetes with severe insulin resistance.

Figure 1 Clinical aspects of the proband. (A and B) showing the masculine appearance with a clear trunk-sparing lipodystrophy; (C-E), acanthosis nigricans on her neck, axilla and feet; (F), eruptive xanthoma.

Despite insulin therapy, she developed severe hypertriglyceridemia, with plasma concentration > 50 mmol/L, leading to eruptive xanthomas on her trunk and extremities. At age 17, her menstrual cycle became irregular and her extremities and face developed excessive hair.
growth, leading to the diagnosis of polycystic ovarian syndrome (PCOS). At age 19, she became pregnant after in vitro fertilization and gave birth to a healthy son. Subsequently, she was hospitalized twice more for pancreatitis at ages 20 and 22. During outpatient follow-up, her insulin dose was increased >300 U per day. At the end of 2005, she was referred to the Academic Medical Center, Amsterdam for management of refractory hypertriglyceridemia despite fibrate and insulin treatment. On examination, she was mildly obese (weight 68 kg; height 167 cm and body mass index [BMI], 25 kg/m²). Her resting blood pressure was 130/70 mm Hg. She had excess subcutaneous (sc) fat on the face, neck, trunk, and abdomen, with lack of sc fat on the gluteal region and extremities (Figure 1). This was confirmed with magnetic resonance imaging (Figure 2), which showed excessive and relatively symmetrical deposition of sc fat on the face, neck, and upper trunk, with disproportionate depletion of sc fat in the lower body. Furthermore, she had acanthosis nigricans on her feet, axillae and neck. She was also hirsute. Measurements from fasting plasma: glucose 8.8 mmol/L; HbA1c 8.2%; insulin 1074 pmol/L (reference 34-172); C-peptide 950 pmol/L (reference 176-664); total cholesterol 9.42 mmol/L; HDL cholesterol 1.33 mmol/L; and triglyceride 35.0 mmol/L. APOE genotype was E3/E3. Lipoprotein lipase (LPL) activity was normal and no genomic DNA sequence changes were seen in the LPL gene (data not shown). The free androgen index was 134 (normal ratio 0-8). At the time of these measurements, she was being treated with multiple daily insulin injections totaling 300 U/day, ciprofibrate 100 mg and cyproterone 50 mg daily.

Figure 2 Magnetic resonance imaging (MRI) scans. T1 weighted images were obtained: (A and B), scans of the neck showing a layer of sc fat measuring 2.52 cm; (C), cross section at the abdomen, showing a symmetrical layer of sc fat measuring 1.09 cm; (D), cross section at the gluteal region, showing sc fat measuring 0.80 cm; (E), cross section at the level of upper leg region, showing a dorsal layer of sc fat measuring 0.64 cm; (F) cross section at the level of lower leg region, showing a dorsal layer of sc fat measuring 0.56 cm;
Her 53 year-old father had a history of type 2 diabetes and dyslipidemia, but was not lipodystrophic clinically. Her mother died at age 20 from meningitis. The proband’s sister, half-sister and two half-brothers were healthy; none had criteria for metabolic syndrome diagnosis. None of the family members were willing to participate in additional clinical or genetic testing.

**DNA sequence analysis**

In the genome of the proband, we found a heterozygous nucleotide substitution C>T at position 1762 in the PPAR\(\gamma\) isoform 4 (Figure 3). All other regions analysed were free of DNA sequence changes. This mutation was absent from the genomes of 100 normal Caucasian controls. This mutation causes an amino acid substitution R194W in PPAR\(\gamma\) isoform 2 (R166W in PPAR\(\gamma\) isoform 1).

**R194W mutant PPAR\(\gamma\) does not bind DNA and is transcriptionally inactive**

The location of the mutation within the DNA binding domain of PPAR\(\gamma\) suggested that it might influence DNA binding. To investigate this possibility, the binding of the R166W as well as R194W mutant to a standard PPRE sequence was assessed using an electrophoretic mobility shift assay (EMSA). While PPAR\(\gamma\) wild type in the presence of RXR\(\alpha\) was capable of binding to PPAR\(\gamma\) response element, the R166W mutant had no detectable DNA binding.
activity (Figure 4A). As expected, lack of DNA binding was also observed in the PPARγ2 isoform (Figure 4B).

The transcriptional activity of the R194W mutant PPARγ was assessed by transient transfection of PPARγ expression plasmids into NIH 3T3 cells and analysis of luciferase activity from a PPAR responsive reporter. The R194W mutant receptor was inactive at all doses of the ligand rosiglitazone (Figure 4C). In addition, U2OS cells were transfected with PPARγ1 (WT and R166W) or PPARγ2 isoform (WT and R194W). Whereas both WT isoforms had a slight basal expression level that was highly induced by rosiglitazone, both mutant isoforms displayed no transcriptional activity in the absence or presence of exogenous ligand (Figure 4D).

**R194W mutant PPARγ displays no dominant-negative activity**

To investigate if the R194W receptor had dominant-negative activity against WT PPARγ, a mixing experiment was performed in which an increasing amount of mutant or wild-type receptor were mixed with a fixed amount of WT PPARγ (Figure 4E). While simply increasing the amount of the WT receptor caused a significant increase in transcriptional activity (WT+WT), the addition of increasing amounts of R194W PPARγ to a fixed amount of WT receptor resulted in no change in total PPARγ transcriptional activity (WT+R194W). For comparison, the same experiment was conducted with the P467L that has dominant-negative activity(4). Increasing amounts of P467L PPARγ caused a dose-dependent decrease in WT PPARγ transcriptional activity (WT+P467L).
transcriptional activity (WT+P467L). When the cells were treated with high concentration of rosiglitazone (Figure 4F), the dominant negative activity of P467L was abolished as described earlier(4). Together, these findings indicate that the R194W mutant does not possess any dominant-negative activity against the WT PPARγ receptor.

DISCUSSION

The principal findings of this study are: 1) association of a novel heterozygous PPARγ missense mutation, R194W (R166W in gamma1 isoform), with FPLD3, including fat redistribution, severe insulin resistance, hypertriglyceridemia, hirsutism and acanthosis nigricans; and 2) functional analysis showing that the R194W mutant is transcriptionally inactive, independent of PPARγ isoform (γ1 and γ2) and cell type (NIH-3T3 and U2OS)

The substitution of a hydrophilic arginine to a hydrophobic tryptophan within an α-helix would predict disrupted structure and decreased DNA binding, as was seen with EMSA. The importance of the conserved arginine residue is underscored by natural mutations in other nuclear receptors causing hormone resistance. For instance, a R614H mutation and deletion of this amino acid (Δ614) in the androgen receptor (AR) have been reported in two patients with complete androgen insensitivity (14). Furthermore, mutation of the analogous residue (R477H) in the glucocorticoid receptor (GR) was detected in a patient with primary cortisol resistance (15). In addition, mutation of this conserved arginine residue in the photoreceptor-specific nuclear receptor PNR into tryptophan (R104W) (16) or glutamine (R104Q) (17) were found in patients with enhanced S-cone syndrome. DNA binding of the AR Δ614 and R614H mutants and the GR R477H mutant was impaired (14;15) analogous to the PPARγ R194W mutant, emphasizing the importance of this conserved arginine residue in nuclear receptor signaling.

R194W brings the number of reported PPARG mutations associated with clinical phenotypes to fourteen. Only the PPARγ2 P115Q mutation was not associated with FPLD3 (18). Two PPARγ missense mutations (P467L and V290M), along with the recently published subjects by Agostini et al (C114R, C131Y, C162W, F5315X and R357X) act via a dominant negative mechanism (19), while five (-14A>G, F388L, E138fsΔAATG, Y355X and R194W) caused FPLD3 through haploinsufficiency (5;7;20) (Figure 3). The R425C mutation (9) also lacks dominant negative activity (E.H. Jeninga et al., submitted). All patients with PPARG haploinsufficiency mutations were ascertained based upon a diagnosis of FPLD; almost every patient with a PPARG mutation had partial lipodystrophy as a core phenotype. FPLD3 has proven to be a useful and appropriate clinical designation; the term acknowledges the centrality of lipodystrophy, while concurrently distinguishing FPLD3 from phenotypically similar but molecularly distinct forms of lipodystrophy, such as FPLD2 due to LMNA mutations.

Mutations can lead to disease through either i) loss of function; ii) gain of function; or iii) dominant negative activity. According to the “classical” dominant negative hypothesis, the mutant allele eliminates the WT function by direct interference. For instance, in the case of nuclear receptors the mutant receptor competes with the WT for binding DNA. However,
there is some evidence that nuclear receptors can also have indirect dominant negative activity by affecting the bioavailability of other components of the transcriptional machinery, such as coactivators, and hence could interfere with the WT allele. We have shown that R194W has neither direct (figure 4 A) nor indirect (Figure 4E) dominant negative activity under our experimental conditions. With haploinsufficiency, 50% reduced gene expression results from one nonfunctional allele, whereas dominant negative mutations induce even greater reduction in gene expression. How do these two mechanisms underlie the same phenotype? One possibility is that subjects with either mutation type might have slightly different clinical phenotypes that are not easily discerned using current methods. For instance, hypertension in human subjects with dominant negative mutations seems to be more severe than in subjects with haploinsufficiency mutations (21). Additional pedigrees with PPARG would allow for better comparisons of these two mechanisms in vivo.

Since the first publication on familial partial lipodystrophy by Dunnigan and Kobberling (22), awareness of this condition by clinicians has increased. Several mutations both in LMNA and PPARG have been described. Yet, many such patients are probably overlooked, because of clinical similarities with the common obesity-related metabolic syndrome that currently is endemic to westernized societies, largely due to lifestyle changes. Careful physical examination of patients with insulin resistance and hypertriglyceridemia could help identify partial lipodystrophy. In summary, in a proband with FPLD3 we found a novel PPARG mutation that fails to bind DNA and is transcriptionally inactive. Human PPARG mutations will improve our understanding of mechanisms involved in lipodystrophy and insulin resistance.
REFERENCE LIST

FPLD phenotype resulting from a single-base mutation in DNA binding domain of PPARγ

