SHORT COMMUNICATION

Characterisation of non-obese diabetic patients with marked insulin resistance identifies a novel familial partial lipodystrophy-associated *PPARγ* mutation (Y151C)

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Abstract

Aims/hypothesis Familial partial lipodystrophy (FPLD) is a rare metabolic disorder with clinical features that may not be readily recognised. As FPLD patients require a specific therapeutic approach, early identification is warranted. In the present study we aimed to identify cases of FPLD among non-obese patients with type 2 diabetes mellitus and marked insulin resistance.

Methods We searched the databases of three diabetic outpatient clinics for patients with marked insulin resistance, arbitrarily defined as the use of ≥100 U insulin/day, and BMI ≤27 kg/m^2. In all patients, metabolic variables and anthropomorphic measurements were evaluated and DNA was sequenced for mutations in the genes encoding lamin A/C (LMNA), peroxisome proliferator-activated receptor γ (PPARγ) and cell death-inducing DFFA-like effector c (CIDEC).

Results Out of 5,221 diabetic individuals, 24 patients fulfilled all criteria. Twelve patients were willing to participate, of whom five showed clinical features of lipodystrophy. In three of these patients the clinical diagnosis of FPLD was confirmed by the presence of mutations in *LMNA* or *PPARG*; one patient harboured a novel heterozygous mutation (Y151C) in *PPARG*. The Y151C mutant displayed impaired DNA-binding capacity and hence reduced transcriptional activity compared with wild-type PPARγ. Dominant-negative activity was absent.

Conclusion/interpretation The combination of BMI ≤27 kg/m^2 and the use of >100 U insulin/day increases the chance of identifying lipodystrophy. Thus careful assessment of clinical features of FPLD should be considered in these patients, allowing earlier therapeutic interventions.

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List of abbreviations

CIDEC	Cell death-inducing DFFA-like effector c
DBD	DNA-binding domain
FPLD	Familial partial lipodystrophy
PPARγ	Peroxisome proliferator-activated receptor -γ
PPRE	Peroxisome proliferator response element
RXRα	Retinoid X receptor, α

Introduction

Familial partial lipodystrophy (FPLD) is an autosomal dominant disorder characterised by abnormal distribution of subcutaneous fat accompanied by a variable degree of metabolic derangements, including marked insulin resistance and severe hypertriacylglycerolaemia, with recurrent episodes of pancreatitis. FPLD is a heterogeneous disorder and diagnosis is predominantly based on clinical features. A number of genetic defects underlying these clinical entities have been described, but the majority of mutations are rare coding-sequence variants in either *LMNA* or in *PPARG* [1–3].

Although the exact numbers for estimation of prevalence are lacking, FPLD has always been considered to be rare. But whereas generalised lipodystrophies can be readily detected because of the characteristic features from birth onwards, lipodystrophy in FPLD may be overlooked because of the overlap of clinical phenotype with the more common presentation of type 2 diabetes mellitus.

The identification of FPLD patients carries distinct therapeutic consequences. Patients with FPLD require intensive measures to prevent pancreatitis and early cardiovascular disease and may benefit from leptin-replacement therapy [4]. Furthermore, identification of FPLD patients may lead to the discovery of novel causative genes that may lead to new treatment targets and improve our understanding of the physiological mechanisms underlying lipodystrophy.

In the present study we aimed to identify novel cases of FPLD in non-obese patients (BMI $\leq$27 kg/m^2) with type 2 diabetes mellitus and marked insulin resistance, arbitrarily defined as total insulin dose $\geq$100 U/day.

Methods

In the databases of three diabetic outpatient clinics ($n=$5,221) we searched for patients >18 years old with type 2 diabetes mellitus, a normal to moderately elevated BMI ($\leq$27 kg/m^2) and marked insulin resistance, defined as the use of insulin $\geq$100 U/day. Medical records from patients selected from the databases were first screened for eligibility. Patients unable to visit the study centre because of a poor medical condition as well as patients with any other explanation for severe insulin resistance (e.g. patients with HIV-induced lipodystrophy) were excluded from the study. Patients fulfilling all inclusion and exclusion criteria were invited to our research clinic. Here, the participant's complete medical history was evaluated and fasting blood samples were drawn for biochemical analysis and DNA extraction. All participants underwent a complete physical examination with careful assessment of the presence of FPLD stigmata. Upper arm fat was assessed by skinfold thickness measurement and interpreted using normal ranges, adjusting for age and sex [5]. The combination of marked insulin resistance together with skinfold thickness <10th percentile was considered to be compatible with the phenotypical diagnosis of lipodystrophy [6].

The study was approved by the institutional review board of the Academic Medical Centre in Amsterdam, the Netherlands. All participants gave written informed consent.

Genomic DNA was isolated from blood samples containing EDTA. The coding exons of *PPARG*, *CIDEC* and *LMNA* (exon 8–9) plus intron–exon boundaries were sequenced using a BigDye Terminator v 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3730 DNA analyser (Applied Biosystems). Primer sequences are available upon request. Functional analysis of the novel variant was performed as described previously by our group [7] (for details, see data presented as electronic supplementary material [ESM]).

Results

From a total of 5,221 patients with a diagnosis of diabetes, 24 patients met all the inclusion and exclusion criteria. Of these 24 patients, 12 were willing to participate (ESM Fig. 1). Five of the 12 non-obese patients with marked insulin resistance had a lipodystrophic phenotype (Table 1). Thus our selection criteria increased the likelihood of identifying FPLD >400-fold, from 5/5,221 to 5/12.

The characteristics of the study participants are described in Table 1. Participants S2 and S5 were included in the study despite having a BMI >27 kg/m^2 at the centre visit, as eligibility was based on data from the patient's medical records which, for both participants showed a BMI $\leq$27 kg/m^2. Two of the patients with a lipodystrophic phenotype (S11 and S12) had previously been diagnosed with FPLD confirmed by mutations in *PPARG* (R194W) [7] and *LMNA* (R482W) (E. S. Stroes, unpublished observations). The third participant (S8) had a history of systemic lupus erythematosus and chronic corticosteroid use, which in itself may explain the severe insulin resistance and abnormal skinfold thickness measurement [8]. The fourth patient (S9) had clinical features of FPLD, including lipodystrophy, but no mutations in *PPARG*, *LMNA* or *CIDEC*. DNA analysis of the fifth patient (S10) resulted in the identification of a novel functional mutation in *PPARG*, establishing the diagnosis of FPLD. This 61-year-old woman had a history of type 2 diabetes since the age of 49 years, severe hypertriglyceridemia with pancreatitis, hypertension and cardiovascular disease (see ESM). Physical examination

Table 1 Patients' characteristics

Characteristic	Patients without a lipodystrophic phenotype							Patients with a lipodystrophic phenotype				
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Age (years)	54	64	74	67	51	67	70	52	58	61	34	39
Sex	M	M	M	M	M	M	F	F	F	F	F	F
Ethnicity	SEA	Af	NeE	NeE	EE	Turk	Af	NeE	SEA	NeE	Turk	UK
BMI (kg/m^2)	26	30	27	25	28	26	27	25	25	23	24	25
Insulin (U/day)	104	120	102	120	106	126	114	104	176	142	125	400
Pancreatitis	–	–	–	–	–	–	–	+	–	+	+	+
Hypertension	–	+	+	+	+	+	+	+	–	+	+	+
CVD	+	–	+	–	+	–	–	+	–	+	–	+
Triacylglycerol (mmol/l)[a]	7.4	9.9	1.3	3.3	2.4	2.0	3.8	6.0	3.4	43.3	44.7	94.0
WHR	1.02	0.99	1.05	1.05	1.03	1.03	1	0.97	1	0.95	0.91	0.92
Leptin (ng/ml)	3.8	10.5	13.7	11.0	9.0	8.9	23.2	9.9	27.7	6.1	–	21.6
Free androgen index[b]	–	62.9	1.1	47.2	35.6	45.2	3.5	0.4	1.8	2.3	8.2	4.6
Gynaecological history	–	–	–	–	–	–	–	–	Ovariectomy after torsion	Two successful pregnancies	PCOS, one successful pregnancy	PCOS
Triceps skinfold thickness (percentile)	<50th	<75th	<75th	<90th	<90th	<90th	<25th	<5th	<10th	<5th	<10th	<5th
Arm fat area (percentile)	<75th	<90th	<75th	<90th	<90th	<90th	<25th	<10th	<25th	<5th	<25th	<5th
Buffalo hump	–	–	–	–	–	–	+	–	+	–	+	+
Muscular hypertrophy	–	–	–	–	–	–	–	–	–	+	+	+
Cushingoid appearance	–	–	–	–	–	–	–	–	–	+	+	–
Hirsutism (grade 0–4)	0	0	0	0	0	0	0	0	0	0	2	0
Acanthosis nigricans	–	+	–	–	–	–	+	–	+	–	+	–
Mutation *LMNA/PPARG/CIDEC*	–	–	–	–	–	–	–	–	–	*PPARG*	*PPARG*	*LMNA*

[a] Highest triacylglycerol levels documented

[b] Reference values: for women 0.0–8.0; for men 20.0–90.0

Af, African; CVD, cardiovascular disease; EE, Eastern European; F, female; M, male; NeE, north-eastern European; PCOS, polycystic ovary syndrome; SEA, south-east Asian; Turk, Turkish

showed a clear excess of subcutaneous fat on the face, neck, trunk and abdomen with a lack of subcutaneous fat on the extremities. Triceps skinfold thickness and upper arm fat area were <5th percentile. MRI of tissue confirmed these findings (ESM Fig. 2). No other abnormalities related to FPLD were observed in this patient, and there was no acanthosis nigricans or hirsutism. Clinical features of lipodystrophy were also found in the patient's sister and son (see ESM).

S10 had a heterozygous A-to-G mutation in exon 5 of the *PPARG* gene (Fig. 1a), resulting in a Y151C substitution in the PPARγ2 isoform. DNA sequence analysis of family members of the index patient showed the same mutation in the patient's son, sister and nephew (Fig. 1b).

Tyrosine 151 is located in the DNA-binding domain (DBD) of PPARγ and is completely conserved amongst species and well conserved among nuclear receptors (ESM Fig. 3).
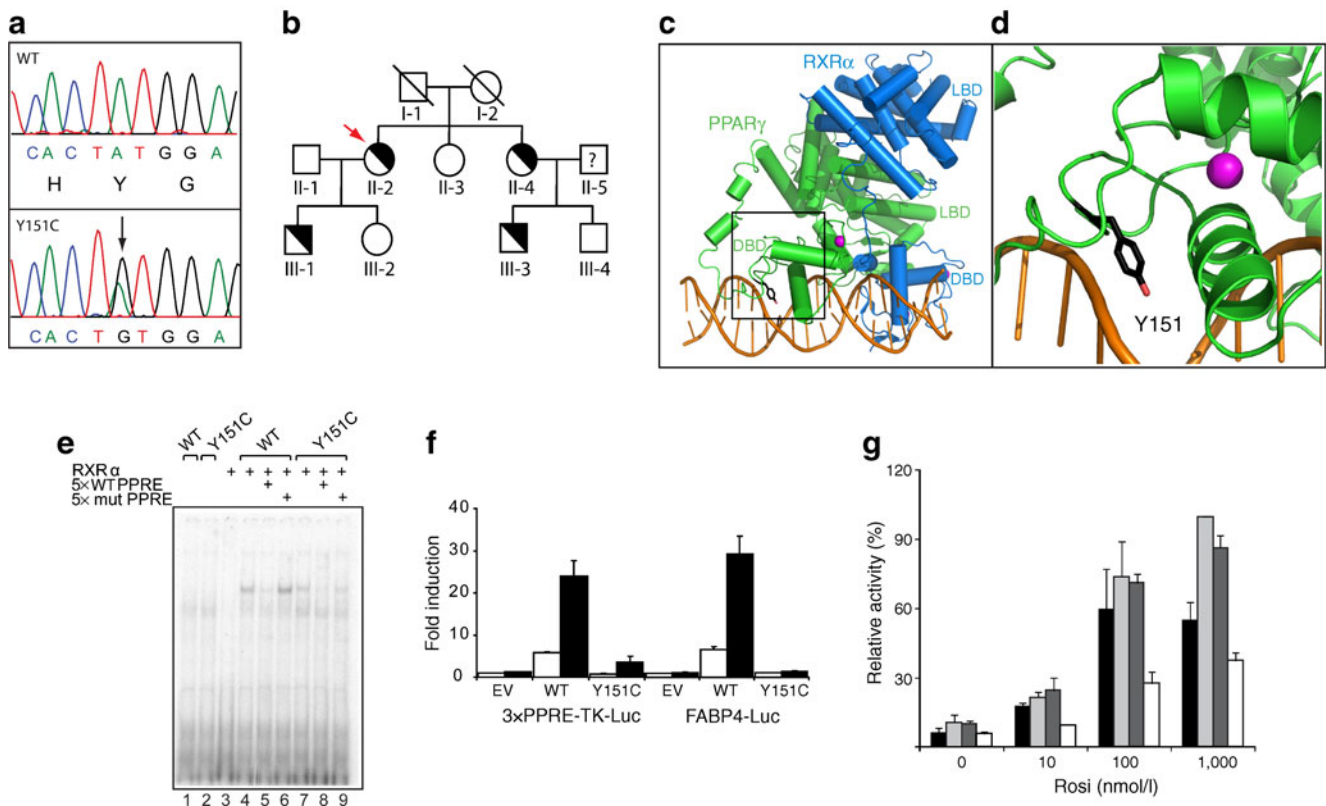

Fig. 1 The FPLD-associated Y151C mutation reduces DNA binding, and hence transcriptional activity of PPARγ. **a** The electropherogram tracing shows both alleles from the proband (Y151C; PPARγ2 numbering) compared with corresponding genomic DNA sequence from a healthy participant. The position of the mutation in the DBD is indicated by the arrow. Nucleotide and amino-acid sequence is shown below the electropherogram tracings. **b** Family pedigree. A diagonal indicates 'not genotyped'. Half-filled symbols indicate individuals with the heterozygous Y151C mutation. Unaffected individuals are indicated by unfilled symbols. The proband is indicated by an arrow. **c** Global structure of the PPARγ–RXRα–DNA complex. The DBDs and ligand-binding domains (LBDs) of PPARγ and RXRα are shown, with the zinc ions in the PPARγ DBD. The square box indicates the region magnified in **d**. **d** PPARγ–DNA interaction. Magnified view of helices of PPARγ–DBD localised around the zinc ions, with Y151 indicated in stick format. Starting structure of PPARγ was adapted from the Protein Data Bank entry 3DZY. Figures were generated using PyMOL 0.99rc6 open source software (www.pymol.org). **e** Impaired DNA binding of Y151C. In vitro translated RXRα or PPARγ2 (wild-type [WT] or mutant) proteins were incubated with ^{32}P-labelled probe in absence or presence of 5× unlabelled probe (WT or mutant [mut]) as indicated. Protein–DNA complexes were separated from unbound

DNA on non-denaturing SDS-polyacrylamide gels and visualised by autoradiography of dried gels. **f** The FPLD3-associated Y151C mutation reduces the transcriptional activity of PPARγ2. U2OS cells were transfected with expression vector encoding PPARγ2 WT or PPARγ2 Y151C respectively, and 3× peroxisome proliferator response element (PPRE)-Tk-Luc reporter or a FABP4-Luc reporter. Activation of the luciferase reporter, in the absence or presence of 1 μmol/l rosiglitazone, is expressed as fold induction over that with empty vector (EV) in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± SEM. **g** The FPLD3-associated Y151C mutation fails to display dominant-negative activity. U2OS cells were transfected with 3× PPRE-Tk-Luc reporter and incubated with different concentrations of rosiglitazone (Rosi), as indicated. WT PPARγ2 (black bars), WT PPARγ2 plus an equal amount of WT PPARγ2 (light grey bars), WT PPARγ2 plus the Y151C mutant (dark grey bars), or WT PPARγ2 plus the dominant-negative L496A/E499A mutant (white bars), incubated with different concentrations of rosiglitazone, as indicated. Reporter activities are presented relative to the activity of WT PPARγ2 in the presence of 1 μmol/l rosiglitazone. Results are averages of at least three independent experiments assayed in duplicate ± SEM

Crystal structure studies of the PPARγ–retinoid X receptor, α (RXRα) heterodimer bound to DNA indicate that Y151 is in direct contact with the DNA backbone (Fig. 1c). In vitro experiments showed the Y151C mutant to display reduced DNA binding and transcriptional activity (Fig. 1d–e). As shown in Fig. 1f, PPARγ2 Y151C failed to display dominant-negative activity.

Discussion

In the present study, we show an increase in the incidence of FPLD in non-obese patients with type 2 diabetes mellitus and marked insulin resistance. Characterisation of 12 participants fulfilling these criteria resulted in the identification of five patients with lipodystrophic features. In three patients the diagnosis of FPLD was supported by mutations in *PPARG* or *LMNA*, with one patient harbouring a novel heterozygous *PPARG* mutation (Y151C). These data indicate that thorough evaluation for FPLD should be considered in non-obese patients with marked insulin resistance. However, the numbers in our study may deviate from the actual incidence of FPLD. First, the small number of eligible patients willing to participate in the study may have resulted in a positive 'selection' bias. Second, our stringent inclusion criteria may have increased the false-negative rate. The aim of the present study was to find an effective method to select rare cases of FPLD from a large group of individuals with the common type 2 diabetes mellitus. To effectively increase the chances of identifying FPLD, we aimed to select patients with an unusual phenotype, namely non-obese patients with marked insulin resistance. Our inclusion criteria may, however, have been too stringent as neither absence of marked insulin resistance nor the presence of obesity excludes the presence of FPLD.

It should be noted that lipodystrophy may be more easily recognised in patients with increased BMI because of the accentuated differences in subcutaneous fat in extremities vs trunk when patients become obese. Thus, although our selection criteria may serve as a guideline, the presence of other typical features of FPLD, in any patient, should still alert clinicians to the possibility of lipodystrophy.

To date, 15 different FPLD-associated mutations in the coding region of *PPARG* have been reported [9]. Here we identified a novel heterozygous tyrosine-to-cysteine mutation located in the region of the *PPARG* gene encoding the DBD (Y151C). The mutation was shown to result in reduced DNA binding and reduced transcriptional activity, while direct dominant negative activity was absent. Tyrosine 151 is located in the DBD of PPARγ and is completely conserved amongst species and within the nuclear receptor superfamily. Interestingly, mutation of the analogous position in the androgen receptor (Y571) to histidine or cysteine has been associated with various forms of the androgen insensitivity syndrome (Androgen Receptor Gene Mutations Database; http://androgendb.mcgill.ca). Like the PPARγ Y151C mutant reported in this study, the androgen receptor Y571H mutant displayed impaired DNA-binding capacity and hence reduced transcriptional activity [10]. These findings underscore the importance of this conserved tyrosine residue in DNA binding by nuclear receptors.

In summary, our results indicate that careful assessment of the clinical features of FPLD should be considered in non-obese patients with marked insulin resistance, allowing earlier therapeutic interventions.

Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

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