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IPF-1/MODY4 gene missense mutation in an Italian family with type 2 and gestational diabetes

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Abstract

Maturity-onset diabetes of the young (MODY) is a monogenic autosomal-dominant form of diabetes mellitus with onset before 25 years of age. Genetic variation in insulin promoter factor-1 (*IPF1*) (MODY4) is uncommon but may contribute to early- or late-onset diabetes as part of a polygenic background. IPF1 is a homeodomain transcription factor required for pancreas development. Our aim was to identify whether *IPF1* gene mutations play a role in Italian early-onset type 2 diabetic (T2D) patients and what functional impact mutations may have in the beta cell. We screened 40 Italian early-onset type 2 diabetic probands for *IPF1* mutations, performed oral glucose tolerance tests in the unaffected family members, and performed in vitro functional studies of the mutant variant. In an extended family (Italy-6) of 46 members with clinical phenotypes of gestational diabetes, MODY, and T2D, a single nucleotide change of CCT to ACT was identified at codon 33 resulting in a Pro to Thr substitution (P33T) in the IPF1 transactivation domain that also contributes to an altered metabolic status in the unaffected NM subjects. Of the 22 genotyped Italy-6 members, 9 carried the P33T allele (NM), of whom 5 have either T2D or elevated fasting glucose levels. Oral glucose tolerance tests showed higher glucose levels at 90 minutes in unaffected NM compared with unaffected NN subjects. Of the 5 female pregnant carriers of the *IPF1* mutation, 4 had pregnancies complicated by reduced birth weights, miscarriages, or early postnatal deaths. In studies in vitro, the IPF1 mutant protein (P33T) showed a reduction in DNA-binding and transcriptional activation functions as compared to the wild-type IPF1 protein. Our findings suggest that the P33T *IPF1* mutation may provide an increased susceptibility to the development of gestational diabetes and MODY4 in the Italy-6 pedigree.

1. Introduction

Insulin promoter factor-1 (IPF1) is a homeodomain transcription factor required for pancreas development and for the transcriptional regulation of genes in pancreatic beta cells such as insulin, glucose transporter-2, and glucokinase [1]. The absence of *IPF1* results in pancreatic agenesis in mice [2]. A homozygous frameshift mutation in *IPF1* resulting in a premature stop codon or a compound heterozygous mutation in the *IPF1* homeodomain causes human pancreatic agenesis [3,4]. To date, multiple mutations have been identified in the *IPF1* gene in patients with diabetes [5,6]. *IPF1* mutations cause maturity-onset diabetes of the young (MODY4) [3,5] and may contribute to susceptibility to late-onset type 2 diabetes mellitus (T2D) in selected populations [7-11]. Our goal was to identify additional genetic variations in the *IPF1* gene that may be potentially responsible for T2D or MODY4 in the Italian population. Here we report the identification of a new *IPF1* mutation, P33T, present in an extended Italian family (Italy-6) in which patients presented with MODY, T2D, or gestational diabetes phenotypes. The P33T mutation is located in the transactivation

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domain of *IPF1* and impairs both DNA-binding and transcriptional activation activities in vitro.

2. Research design and methods

2.1. Subject volunteers

Forty Italian families with a diagnosis of early-onset (age at diagnosis <40 years) T2D (22 men and 18 women) and 50 healthy Italian subjects (26 men and 24 women) were recruited from the region around Rome. The subjects gave informed consent for the study. The study was approved by an institutional review committee, following the guidelines of the Helsinki Declaration. Patients were diagnosed with T2D according to the National Diabetes Data Group (1979). Genomic DNA was extracted from 20 mL whole blood by phenol/chloroform standard procedures [12]. Among the 40 probands, 28 were diagnosed with T2D before 35 years of age and 12 were diagnosed between 35 and 39 years of age. Four of the probands had mutations in the *HNF-1α*/MODY3 (promoter −58nt A→C, G42P43fsCC→A154X, W113X, H514R) [13,14] and 2 had mutations in the *GCK*/MODY2 (IVS4nt +7C→T, G44S) [13].

2.2. Genomic sequencing

The 40 probands were screened for mutations in the *IPF1* gene. The 2 *IPF1* exons and flanking intron regions were amplified using the Advantage GC Genomic PCR kit (Clontech, Palo Alto, Calif) and the primers F5′AACGCCACACAGTGCCAAATC3′ and R5′ TTAGTCCGACCCGGGATAATC3′ for exon 1 and F5′ TGA-AGGGGTTGGGCTGCGTG3′ and R5′GAGTGG-TTGAAGCCCCTCAG3′ for exon 2. Polymerase chain reaction products were purified using the QIAquick kit (Qiagen, Valencia, Calif), directly sequenced on both strands using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit, and processed on an ABI Prism 377 sequencer (Applied Biosystems, Foster City, Calif). The mutated polymerase chain reaction products were subcloned into the pGEM-4Z plasmid (Promega, Madison, Wis), and the clones were sequenced to confirm the mutation on both DNA strands. Fifty unrelated Italian healthy subjects with a negative family history for diabetes and normoglycemia were screened for mutations in exon 1 of *IPF1*. We have previously screened the proband of family Italy-6 for mutations in exons 1a and 2 to 10, flanking introns and promoter regions of *HNF-4α* and *GCK,* in exons 1 to 10, flanking introns and promoter region of *HNF-1α*, and in exons 1 to 2 and flanking introns of *HNF-6* and in exon 1 and flanking region of neurogenin-3 (*NEUROG*3) gene.

2.3. Oral glucose tolerance tests

Clinical data were collected for the available Italy-6 family members. All available unaffected family members were tested with an oral glucose tolerance test (OGTT) (standard 75 g for adults and 1.75 g/kg of body weight up to 75 g for children). Mean and SD of plasma glucose, insulin, C-peptide, and insulin/glucose and C-peptide/glucose ratios were determined at the times 0, 30, 60, 90, and 120 minutes in the heterozygous carriers (NM) (n = 3) and wild-type homozygous subjects (NN) (n = 5) and were subjected to an analysis of variance.

2.4. Data analyses

Beta-cell function was estimated for the nondiabetic NM and NN subjects by the homeostasis model assessment (HOMA) index [15,16], derived from the following formula: 20 × basal insulin (mU/L) / basal glucose (mmol/L) − 3.5 [17]. Insulin sensitivity was calculated for the unaffected family members according to the formula: HOMA-IR = basal insulin (mU/L) × basal glucose (mmol/L)/22.5 [18], where HOMA indices of 12.01 are considered to be in the nondiabetic range, 16.14 in the impaired fasting glucose or impaired glucose tolerance range, and 26.99 in the diabetic range [19]. Both HOMA index and HOMA-IR, and age, cholesterol, triglyceride, hemoglobin A_{1c} (HbA_{1c}), and body mass index (BMI) (kg/m^2) were tested for significant differences in the NM and NN groups using an unpaired 2-tailed *t* test.

To test for a relationship of the P33T mutation with the birth weight of the NM subjects, we divided the subjects into groups consisting of NM/NM (maternal genotype/fetal genotype) (n = 5), NM/NN (n = 5), and NN/NN groups (n = 2) and tested by 1-sample *t* test for significant differences in the birth weight averages in the NM/NM and NM/NN, as compared with the mean normal birth weight (3.5 kg) of healthy Italian newborn infants [20].

2.5. In vitro functional studies of the IPF1 P33T mutation

We analyzed the effects of the P33T mutation of *IPF1* on DNA-binding and transcriptional activation functions in vitro. The P33T mutation was introduced by Quikchange site-directed mutagenesis (Stratagene, La Jolla, Calif) into an expression plasmid encoding human *IPF1* cDNA, pcDNA3*IPF1*. Proteins were transcribed and translated in vitro in a TNT-coupled reticulocyte lysate system (Promega, Madison, Wis) [21], assessed for equivalency of IPF1 expression by Western blot analysis detected by a carboxy-terminal anti-*IPF1* antibody, and used in EMSA with a [^{32}P]-labeled double-stranded human insulin promoter oligonucleotide probe (CT2) (5′ GATCCCCCTGGTTAA-GACTCTAATGACCCGCTGG3′) [22]. Both the Western blot and the EMSA data were analyzed with densitometric scans. To determine the transcriptional activation functions of P33T IPF1 in beta cells, Gal4(1-147)/IPF1(1-121) wild-type and P33T fusion protein expression constructs were generated in pM vectors (Clontech). Clonal beta cells (INS-1) were transiently transfected with 250 ng of pM-IPF1(1-121), pM-IPF1(1-121) P33T, or the empty vector pM and 250 ng of a Gal4-luciferase reporter construct (pFR-Luc, Stratagene) using lipofectamine (Invitrogen, Carlsbad, Calif).

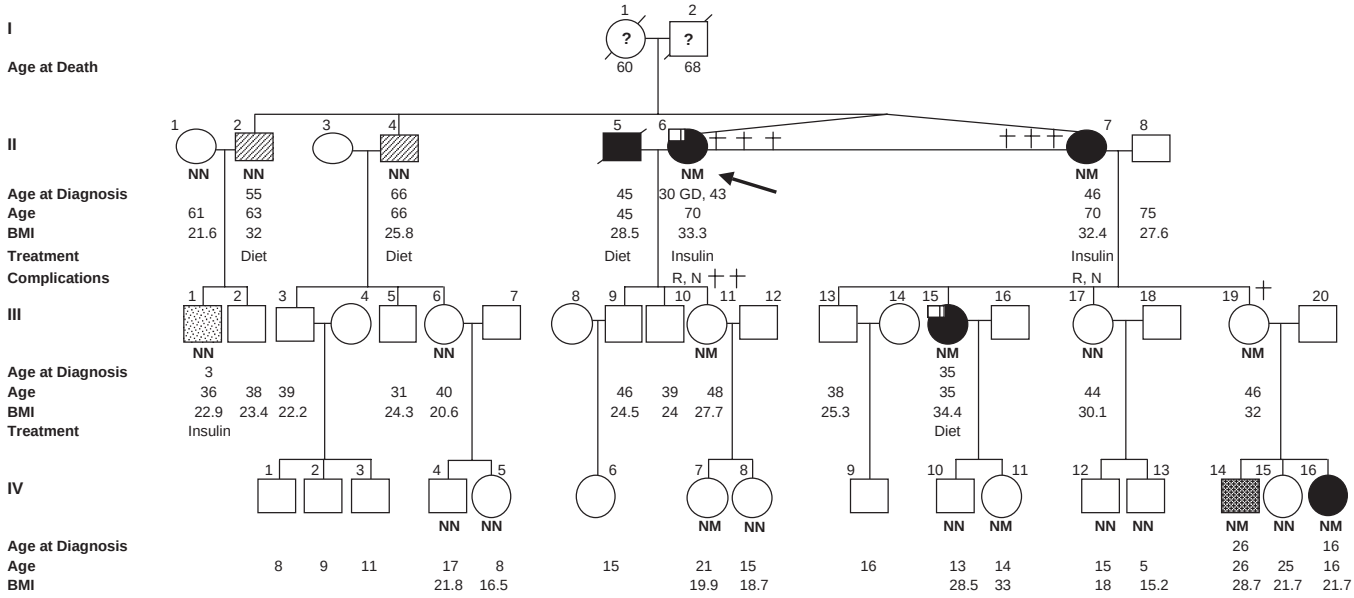

Fig. 1. Pedigree of the Italy-6 Family: type 2 diabetes ■; IGT, ▨; IFG, ▩; type 1 diabetes, ⬚; unaffected, □; type 2 diabetes and gestational diabetes, ◔; deceased, indicated by slash (/). M indicates mutant allele; N, wild type (normal allele), R indicates retinopathy, N, nephropathy; and †, prenatal or postnatal death.

Five separate experiments were performed, each in duplicate. Luciferase assays were performed as described previously (Promega) [23]. Luciferase activity was normalized to protein concentrations of the cellular extracts.

3. Results

3.1. The IPF1 P33T mutation contributes to MODY4, T2D, and gestational diabetes phenotypes

We identified a single nucleotide change of CCT to ACT at codon 33 resulting in a Pro to Thr substitution (P33T) in the transactivation domain (exon 1) of *IPF1* in 9 of 22 genotyped members of the Italy-6 family. The mutation was not present in 50 healthy Italian subjects. In the proband of the family Italy-6 we excluded mutations in the *HNF-4α*, *GCK*, *HNF-1α*, *HNF-6*, and *NEUROG3* genes. The *IPF1* P33T mutation is shared by 2 T2D identical twins of generation II, II.6 (the proband) and II.7, diagnosed at 30 years of age with gestational diabetes and at 46 years of age with T2D, respectively, by the patient III.15 diagnosed at 35 years of age with gestational diabetes and T2D at 35 years of age, and by the patient IV.16 at 16 years of age (Fig. 1). The clinical data for the 4 carriers with diabetes are shown in Table 1. Of the 4 P33T carriers with diabetes, 3 had a BMI greater than 32. Because subject IV.16 was diagnosed at 16 years of age with T2D, we conclude that Italy-6 represents a family with MODY4. Subject IV.14 has recently been diagnosed with impaired fasting glucose. Subject IV.11 recently had 2 fasting glucose levels above 100 mg/dL but below 110 mg/dL. Subjects II.2 and II.4 were diagnosed with impaired glucose tolerance and they are wild type (NN). Subject III.1 is affected with type 1 diabetes mellitus and is wild type (NN). To date, we have identified 4 unaffected carriers of the P33T *IPF1* mutation in the Italy-6 family.

3.2. Nondiabetic carriers of the P33T mutation have elevated glucose levels on OGTT

The mean and SD of the parameters tested (age, cholesterol, triglyceride, BMI, HOMA index, HOMA-IR) in the 3 available NM and 5 NN unaffected family members were not significantly different by performing a

Table 1
Clinical data for T2D and MODY (NM) Italy-6 members[a]

Parameter	II.6	II.7	III.15	IV.16	Reference value
Age at diagnosis (y)	30 GD, 43	46	35 GD	16	–
Diabetes duration (y)	37	26	<1	<1	–
BMI (kg/m^2)	33.3	32.4	34.4	21.7	<24
Treatment					
Diet (y)	20	10	<1	<1	–
OHA (y)	4	4	–	–	–
Insulin (y)	13	7	–	–	–
HbA_{1c} (%)	9	6.4	5.2	4.5	<6.4
Fructosamine (μmol/L)	428	327	–	–	<285
Cholesterol (mg/dL)	231	243	193	174	<240
Triglyceride (mg/dL)	190	158	122	80	<145
Basal C-peptide (ng/mL)	1.2	1.3	3.0	1.3	1.1-5.0
Neuropathy	Yes	Yes	Yes	No	–
Retinopathy	Yes	Yes	No	No	–
Nephropathy	No	No	No	No	–
Hypertension	Yes	Yes	No	No	–
Carpal tunnel syndrome	Yes	Yes	No	No	–

OHA indicates oral hypoglycemic agents.
[a] In addition to the 4 NM members with either T2D or MODY, 1 other NM member has elevated fasting glucose levels.

t test. The mean and SD values, in the unaffected NM and unaffected NN subjects, respectively, were 38.33 ± 15.04 and 20.80 ± 14.77 years for age, 26.52 ± 6.19 and 20.71 ± 5.75 kg/m^2 for BMI, 185.00 ± 13.00 and 164.60 ± 34.31 mg/dL for cholesterol, 71.33 ± 9.07 and 95.20 ± 43.32 mg/dL for triglycerides, 4.60% ± 0.82% and 4.38% ± 0.39% for HbA_{1c}, 173.95 ± 50.62 and 356.92 ± 159.36 mU/mmol for HOMA index, and 12.61 ± 10.88 and 8.87 ± 4.58 mU × mmol for HOMA-IR. The HOMA-index *t* test analysis resulted in a *P* value of .06, showing a trend toward reduced beta-cell function in the NM subjects compared with the NN subjects. We noted trends toward higher plasma glucose and lower plasma insulin and C-peptide levels, lower insulin/glucose ratios and C- peptide/glucose ratios in OGTTs of the NM unaffected subjects compared with the NN subjects, although these differences were not significant by an analysis of variance. In the unaffected NM and unaffected NN subjects, respectively, the mean and SD values were 113.93 ± 20.93 and 103.28 ± 19.71 mg/dL for plasma glucose, 45.16 ± 23.93 and 48.53 ± 24.96 (μU/mL) for plasma insulin, 4.98 ± 2.09 and 5.28 ± 2.51 nmol/L for C-peptide, 45.86 ± 21.42 and 54.46 ± 24.70 (pmol/L)/(mmol/L) for insulin/glucose ratios, and 0.74 ± 0.24 and 0.86 ± 0.36 (nmol/L)/(mmol/L) for C-peptide/glucose ratios.

3.3. The P33T mutation predisposes to reduced birth weight, miscarriage, and early postnatal death

Interestingly, 4 of 5 NM female family members have a history of miscarriages or pregnancies complicated by early postnatal deaths, a feature not noted in the 2 NN female carriers who have been pregnant. Subject II.6 has had 2 miscarriages (fifth month each) and 1 postnatal death at the 31st hour of life. Subject II.7 has had 1 miscarriage (third month) and 2 postnatal deaths during the second day of life, 1 of which was probably due to trauma

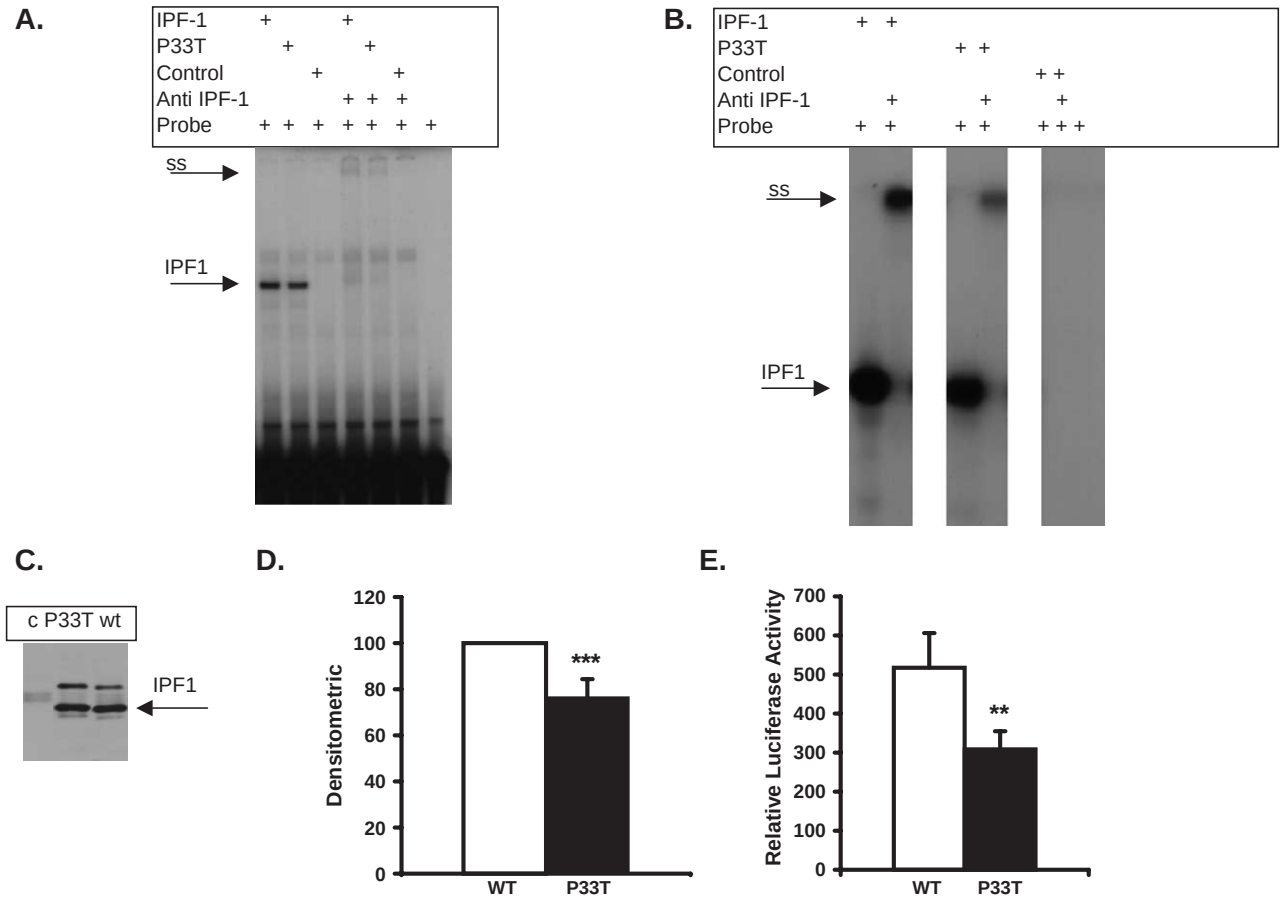

Fig. 2. Functional studies of the P33T *IPF1* mutation. (A) DNA-binding activity of WT and P33T IPF1 proteins to a radiolabeled human insulin promoter element CT2 oligonucleotide probe. A representative autoradiogram is shown. The migration positions of the IPF1 complex bound to probe (IPF1) and of the supershifted complexes (ss) are designated. Control is represented by the empty vector TNT lysate. (B) A longer exposure autoradiogram from another representative DNA-binding experiment highlights the supershift of both the IPF1 WT and P33T proteins by preincubation with anti-IPF1 antiserum. (C) Western blot of P33T and WT proteins. The higher molecular weight band above the arrow likely represents a posttranslationally modified form of *IPF1*, for example, sumoylation, glycosylation, phosphorylation. (D) Densitometric analysis of the gel shift assay WT and P33T bands (mean and SD in %) (n = 6). Triple asterisk indicates *P* = .0007. (E) Transcriptional activation of Gal4-*IPF1*(1-121) (WT) and Gal4-*IPF1*(1-121) P33T of a Gal4-luciferase reporter in transient transfections of INS-1 cells. Luciferase activity normalized to the empty Gal4 expression vector pM is shown (n = 5 separate experiments, each conducted in duplicate). Double asterisk indicates *P* = .005; white bar, wild type (WT); black bar, P33T *IPF1*.

during delivery. Subject III.11 has had 2 miscarriages (third and fifth month) and subject III.19 has had 1 miscarriage (third month).

There was a significant difference (P = .017; 95% CI, 2.91-3.40) between the 5 NM/NM maternal/fetal genotype birth weights (3.16 ± 0.19 kg, mean ± SD) as compared with the normal newborn birth weight of 3.5 kg in Italian infants, whereas there was no significant difference (P = .50, 95% CI, 2.47-4.09) between the 5 NM/NN maternal/fetal genotype birth weights (3.29 ± 0.65 kg, mean ± SD) and the normal birth weight of Italian infants. The 2 NN/NN maternal/fetal genotype birth weights were 3.49 ± 0.02 kg (mean ± SD), consistent with normal birth weights.

3.4. Blast Search for amino acid P33 conservation across species

We performed a Blast Search (www.ncbi.nlm.nih.gov/blast) of the human IPF1 protein across multiple species to identify species in which the amino acid 33 was conserved. The Blast Search demonstrated that the proline residue at position 33 of the IPF1 protein is conserved in human, mouse, rat, hamster, frog, and fish species, thus strongly supporting the biologic relevance for conservation of amino acid P33.

3.5. The P33T mutation impairs IPF1 DNA-binding and transcriptional activation functions

Both the DNA-binding and transcriptional activation functions were impaired in the mutant P33T IPF1 protein in vitro assays. In gel shift experiments, in vitro translated mutant P33T IPF1 proteins consistently demonstrated a reduced capacity to bind a known *IPF1* binding site in the human insulin promoter enhancer (CT2) compared with wild-type IPF1 proteins (Fig. 2A, B). By densitometric analysis of 6 independent experiments, the DNA-binding activity of the mutant P33T IPF1 protein was significantly reduced to 76.4% relative to that of wild-type IPF1 (Fig. 2D) in experiments in which the mutant protein input was equivalent or greater than the wild-type protein input as assessed by Western blots (Fig. 2C). To assess the transcriptional activation functions of IPF1, fusion protein constructs encoding the yeast Gal4 DNA-binding domain and the amino-terminal transcriptional activation domain of human IPF1 (amino acids 1-121) were generated with or without the P33T mutation. In transfections of INS-1 clonal beta cells the mutant Gal4-IPF1(1-121) P33T fusion protein had a statistically significant 40% reduction in its ability to activate a Gal4-luciferase reporter as compared with the wild-type Gal4-IPF1(1-121) fusion protein (Fig. 2E).

4. Conclusions

Our studies suggest that the *IPF1* P33T allele (NM) predisposes to phenotypes of gestational diabetes, MODY, or T2D. However, *IPF1* P33T is not 100% penetrant and metabolic phenotypes may be exacerbated by advanced age, pregnancy-related metabolic stress, or high BMI as observed in P33T carriers with diabetes. The P33T gene variant may contribute to higher glucose levels on OGTT at 90 minutes.

These findings suggest that a prediabetic state develops in the NM carriers compared with the NN subjects. This is the second family showing gestational diabetes as a phenotype resulting from a mutation in the *IPF1* gene. A Swedish patient with gestational diabetes had a P239Q *IPF1* mutation [24]. Our data suggest that the P33T newborn carriers have lower birth weights compared with the NN subjects of the family Italy-6 and to the subjects from the general population. This difference in birth weights is significant when compared with the average normal birth weight of the healthy Italian newborn population. Fetuses that carry the P33T mutation (NM) may have a lower birth weight due to diminished insulin secretion as compared with the NN fetuses due to the decreased DNA-binding and transactivation activities of the P33T variant IPF1 on the insulin gene, but there may be other potential explanations because IPF1 is known to regulate pancreas development. Of note, mutations in the *GCK*/MODY2 gene are reported to result in reduced birth weights [25]. It is known that birth weight is inversely associated with risk for T2D during adulthood [26]. Furthermore, it has recently been shown that exendin-4, a long-acting analog of glucagon-like peptide-1, prevents the onset of diabetes and restores the expression of PDX1 (rat IPF-1) in intrauterine growth-retarded rats [27]. Therefore, reduced PDX1 expression may be responsible for T2D and pancreatic growth retardation in these rats [27].

It is possible that the P33T *IPF1* mutation may have contributed to the multiple miscarriages and postnatal deaths present in the female mutant carriers of this family in combination with other factors in their genetic background. This phenotype of reduced birth weights, miscarriages, or prenatal/postnatal deaths with *IPF1* mutations has not been reported to date. It would be of interest to study female carriers of additional *IPF1* mutations to confirm or exclude a similar pattern of correlations of the mutations with miscarriages and reduced birth weights.

Our findings demonstrate that the P33T mutation impairs both the DNA-binding and transcriptional activation functions of IPF1. The mutation site occurs within the previously defined amino-terminal transactivation domain of IPF1 [28,29]. The reduction in transcriptional activation of the mutant protein implies that the conserved amino acid proline 33 may play an important function in the transcriptional activation of IPF1. The apolar amino acid proline is replaced by the polar amino acid threonine so that a resulting conformational change is possible. The reduction of DNA-binding activity may be explained by either a conformational change in the secondary structure of the mutant protein and/or a change in the interaction of the mutant protein with other proteins required for DNA-binding activity.

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