A novel hypomorphic PDX1 mutation responsible for Permanent Neonatal Diabetes with subclinical exocrine deficiency

Running title: PDX1 mutation in permanent neonatal diabetes

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Objective- Genes responsible for monogenic forms of diabetes have proven very valuable to understand key mechanisms involved in β cell development and function. Genetic study of selected families is a powerful strategy to identify such genes. We studied a consanguineous family with two first-cousins affected by neonatal diabetes; their four parents had a common ancestor, suggestive of a fully penetrant recessive mutation.

Research Design And Methods- We performed genetic studies of the family, detailed clinical and biochemical investigations of the patients and the four parents, and biochemical and functional studies of the new mutation.

Results- We found a novel mutation in the pancreatic and duodenal homeobox 1 gene (PDX1; IPF1; MODY4) in the two patients, which segregated with diabetes in the homozygous state. The mutation resulted in an E178G substitution in the PDX1 homeodomain. In contrast to other reported PDX1 mutations leading to neonatal diabetes and pancreas agenesis, homozygosity for E178G mutation was not associated with clinical signs of exocrine pancreas insufficiency. Further, the four heterozygous parents were not diabetic and displayed normal glucose tolerance. Biochemical studies, however, revealed sub-clinical exocrine pancreas insufficiency in the patients and slightly reduced insulin secretion in the heterozygous parents. The E178G mutation resulted in reduced Pdx1 transactivation, despite normal nuclear localization, expression level, and chromatin occupancy.

Conclusions- This study broadens the clinical spectrum of PDX1 mutations and justifies screening of this gene in neonatal diabetes patients even in the absence of exocrine pancreas manifestations.
Although most cases of juvenile-onset insulin dependent diabetes are represented by Type 1 Diabetes (T1D), in a subset of patients diabetes occurs in the neonatal period or very early. A number of monogenic defects have already been recognized to underlie these rare cases, and several genes have been identified. Neonatal diabetes is permanent in approximately half of the patients and may be caused by mutations affecting genes that play a critical role in \( \beta \)-cell development, survival or function. Currently, monogenic causes are identified in more than 50% cases of permanent insulin dependent diabetes occurring before age 6 months (1). Genes responsible for monogenic neonatal diabetes have been identified by candidate gene studies (PDX1, GCK, HNF1B, KCNJ11, ABCC8), by linkage and positional gene identification in neonatal diabetes syndromes (EIF2AK3, FOXP3, PTF1A, GLIS3), or by linkage and candidate gene study in non-syndromic neonatal diabetes (INS) (see (1) and (2) for reviews). While monogenic inheritance is easily suspected in neonatal diabetes occurring in association with other remarkable clinical features (syndromic diabetes), finding new genes responsible for non-syndromic monogenic diabetes may be particularly challenging, as these patients may be misclassified as T1D. The observation that the genetic distribution of HLA class II alleles in patients with permanent insulin-dependent diabetes presenting before age 6 months was similar to that of healthy controls (3)(4) strongly supports the hypothesis that most cases of neonatal or very early onset diabetes have a different disease etiology than T1D.

Genetic study of highly selected families with monogenic inheritance is a powerful alternative to identify these genes. Here we studied a single extended family with two related patients affected by neonatal diabetes with no other clinical features. We showed that a novel homozygous mutation in the PDX1 gene is responsible for diabetes in these patients, and we performed detailed clinical and functional investigations to determine the mechanisms responsible for this unexpected clinical presentation for PDX1 mutation.

**RESEARCH DESIGN AND METHODS**

**Patient and family description.** The family was of Moroccan Caucasian origin and was identified through a diabetic child with neonatal insulin-dependent diabetes (subject 8), whose parents were consanguineous. A first cousin of the proband (subject 4) had similar presenting manifestations and consanguineous parents. Initially eight individuals (subjects 1-8), including the two patients, their parents and unaffected siblings were studied. Another child (subject 9) was born after the initial genetic study and was genetically diagnosed prenatally, and clinically confirmed subsequently, as non-affected. The study was explained to the parents, who agreed to participate in the genetic study and in subsequent clinical and metabolic explorations and signed informed consents. The study protocol was approved by the Hospices Civils de Lyon. Blood samples were obtained on all family members and DNA extracted using standard procedures (Supplementary Methods).

**Metabolic investigations.** Oral Glucose Tolerance Test (OGTT) and Intravenous Glucose Tolerance Test (IVGTT) procedures were performed on the four parents (subjects 1, 2, 5 and 6) using standard protocols (Supplementary Methods).

**Genetic studies: linkage and mutation screening and analyses.** A
genome-wide SNP scan was performed using the Affymetrix 10K microarray panel. We performed multipoint genetic analyses using Merlin software, under a rare disease recessive model (allele frequency: 0.000001) with complete penetrance and no phenocopy.

We performed mutation screening by sequencing genomic DNA of patients and their parents, using Big Dye terminator sequencing on an ABI-3730 sequencer (Applied Biosystems). Primers used for PDX1 sequencing are shown in Supplementary Table 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org. Multiple protein sequence alignments in the region of the mutation were generated using Polyphen (5).

Plasmid constructs. Full-length mouse Pdx1 cDNA was cloned into the pcDNA3 mammalian expression vector, in frame with three HA epitope tags at the N-terminus (HA-Pdx1). The E178G mutation was recapitulated in the mouse sequence by PCR mutagenesis and cloned using the same strategy (HA-Pdx1 E178G). The constructs were confirmed by sequencing.

Immunofluorescence. BHK cells were seeded in plastic chamber slides and transfected with Pdx1 expression plasmids using Lipofectamine 2000 (Invitrogen). Forty-eight hours later cells were fixed with 4% paraformaldehyde, and stained with mouse anti-tubulin (Sigma), rabbit anti-HA (Santa Cruz), and DAPI (nuclei).

Reporter assays. BHK cells were transfected with the indicated combinations of Pdx1 expression plasmids, the Pdx1-responsive somatostatin promoter reporter (TAAT)$_2$-65 SMS-CAT (6), and CMV-beta galactosidase (internal control). Chloramphenicol acetyltransferase (CAT) activity was assessed as described previously (7), and normalized to beta galactosidase activity.

Chromatin immunoprecipitation. Min6 insulinoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 25.5mM glucose (Invitrogen) and nucleofected with 2.5 μg of Pdx1 expression vectors by AMAXA. After 48 hours, cells were harvested in 1xPBS and chromatin immunoprecipitation was performed using rabbit anti HA or purified rabbit IgG (Santa Cruz) essentially as described previously (8). Chromatin occupancy was assessed by quantitative PCR using previously described primers that amplify the Pdx1 autoregulatory region, the insulin promoter, and the albumin promoter (9). Enrichment is presented as a fraction of input chromatin.

RESULTS

Patient and family description: a single extended family with two patients with neonatal diabetes: The family was identified through a patient with neonatal diabetes and extended to include a first cousin of the patient with similar presenting manifestations. The parents of each patient were consanguineous, and both nuclear families were related, with a common ancestor to all patients’ parents (Figure 1). Patient 4, a boy, was born after 39 weeks of pregnancy and was small for gestational age [birth weight, 2030g (< -2 SD); birth length, 45 cm (< -2 SD)]. His glycemia was normal at birth and until day 15 of life, when he presented with hyperglycemia at 24.2 mmol/l. Patient 8, a girl, was born after 36 weeks of pregnancy and was small for gestational age [birth weight, 2030g (< -2 SD); birth length, 45 cm (< -2 SD)]. On the first day of life, her glycemia was 19.6 mmol/l. Anti-GAD, islet cell and human insulin antibodies were negative in both patients. Both patients were
treated with an insulin pump (1.0 unit kg\(^{-1}\) day\(^{-1}\)) and became euglycemic with excellent linear growth thereafter. HbA1C levels were maintained between 7.0 and 9.0% (patient 4) and 7.0 and 8.0% (patient 8). The parents of these two children (subjects 1, 2, 5 and 6) were healthy and non-diabetic.

Based on the family structure (Figure 1), with parental consanguinity, a common ancestor to the four parents and the absence of clinical manifestations in the parents, we considered that recessive inheritance of a monogenic defect was the most likely mode of inheritance for neonatal diabetes in this extended family.

**Genetic study: a homozygous PDX1-E178G mutation responsible for neonatal diabetes:** Based on the family structure available at the time of genetic study (eight individuals), we estimated that the maximum expected LOD score under linkage would be 3.26. We performed a 10K genome scan and linkage analysis under a fully penetrant recessive model. We identified a single region compatible with linkage (LOD score = 3.24) on chromosome 13q12 (Figure 1). This region extends over 4.4 Mb, between SNPs rs943721 and rs723918, and contains 31 genes referenced in NCBI RefSeq, including the \(\text{PDX1} \) gene, encoding pancreatic and duodenal homeobox 1 (also known as Insulin Promoter Factor 1, IPF1). Homozygous or compound heterozygous mutations in \(\text{PDX1} \) have been previously reported in two unrelated patients with neonatal diabetes and exocrine pancreas deficiency due to pancreas agenesis or hypoplasia (10; 11), and heterozygous mutations are responsible for Maturity Onset Diabetes of the Young (\(\text{MODY4} \)) (7; 12). Consequently, and despite the absence of exocrine pancreas deficiency in these patients, we considered \(\text{PDX1} \) as a candidate gene for neonatal diabetes segregating in this family.

We sequenced all exons of \(\text{PDX1} \), exon-intron boundaries, and 2 kb of 5’ flanking regions in this family, and identified an A641G substitution (RefSeq NM_000209.3) homozygous in both patients, heterozygous in the parents and segregating with the disease in the homozygous state, resulting in an E178G non-synonymous change (RefSeq NP_000200.1) (Figure 2A). E178G is located in the second helix of the PDX1 homeodomain, an essential and highly conserved region that mediates DNA binding to TAAT rich motifs in PDX1 target genes (6). The homeodomain also contains a nuclear localization signal (NLS) (13). The E178G substitution was not found in 368 unrelated Caucasian controls. This amino acid is conserved among homologous proteins, including in Echinodermates, Hemichordates, Molluscs and Annelids (Figure 2B).

**Clinical investigation of homozygous patients:** Following the identification of PDX1-E178G homozygous mutations in the patients, we performed further clinical and biochemical investigations and abdominal imaging. Both patients’ weight and length, as well as bone age, were within normal range at age 47 months (patient 4) and 48 months (patient 8), with treatment consisting only of 1.0 unit \(\cdot\) kg\(^{-1}\) \cdot day\(^{-1}\) of insulin (Supplementary Figure 1).

Biochemical investigations of endocrine and exocrine pancreas function are summarized in Table 1. C-peptide secretion following a meal was undetectable, consistent with marked \(\beta\) cell deficiency, and glucagon level was normal or slightly increased, indicating the presence of functional \(\alpha\) cells. Serum lipase levels were low or undetectable, and stool examination revealed slightly increased fecal fat excretion, low chymotrypsin and low elastase levels, indicating biochemical evidence of some exocrine pancreas deficiency. IGF1 levels
were very low and vitamins A, D, E and K levels were at the lower limit of the normal ranges, consistent with some degree of malabsorption. This biochemical evidence of exocrine pancreas deficiency contrasts with the absence of detectable clinical signs.

Abdominal ultrasound imaging of patient 4 revealed a normal sized pancreas, with the presence of a 1.1 cm cyst. For patient 8, ultrasound imaging revealed a well individualized and homogeneous pancreas head, but could not identify the body and the tail (not shown). These results suggest that PDX1-E178G homozygosity is sufficient for pancreatic organogenesis. We note that the apparently reduced-size pancreas observed in patient 8 was correlated with lower levels of serum lipase, and stool chymotrypsin and elastase compared to patient 4, although he remained asymptomatic.

Detailed investigation of the four heterozygous carrier parents: Individuals heterozygous for PDX1 mutations associated with neonatal diabetes and pancreatic agenesis in the homozygous state have been reported to have early onset type 2 diabetes (MODY4); mean ages at onset were 35 years old (range 17-67) (12) and 39-50 years old (11). In contrast, the four parents in our family, heterozygous for the PDX1-E178G mutation, were not diabetic at the age of examination (30 to 38 years old) and family history of type 2 Diabetes (T2D) was unremarkable, based on information provided by the family: the ten brothers and sisters of the four parents were healthy and non diabetic, and none of the obligate carriers of the PDX1 mutation (subjects 11, 14, 15, 18, Figure 1) were reported to be diabetic. These family members were not available for study.

In order to further explore pancreas function in PDX1-E178G carrier individuals, we performed OGTT and IVGTT in the four parents of the two patients (Figure 3). All four parents had normal fasting plasma glucose and normal glucose tolerance, with preserved first phase but reduced late phase insulin secretory responses during OGTT (Figure 3A). The first phase insulin secretory response to IVGTT tended to be low in these parents (25 to 88 mU/l) and was very low in the two fathers (subjects 1 and 5; \( \leq 1^{st} \) percentile) (Figure 3B). Ultrasonography of the pancreas was normal in the four parents (not shown). The levels of serum lipase, vitamins A, D, E and K, and IGF1 were within normal ranges (Supplementary Table 2).

E178G does not affect Pdx1 localization: In order to gain insight into the milder phenotypic and clinical manifestations of homozygous and heterozygous individuals for the PDX1-E178G mutation, compared to previously described homozygous and heterozygous individuals carrying other PDX1 mutations (10-12), we performed functional investigations of this mutation. The homeodomain of PDX1 (AA 144-206), which is 100% conserved between mice and humans, contains the DNA binding domain and NLS (6; 13). The E178G mutation was recreated in the context of the mouse Pdx1 cDNA sequence. Both wild-type Pdx1 and the E178G mutant were then cloned in-frame with an N-terminal HA epitope tag for over-expression and detection in eukaryotic cell lines (HA-Pdx1 and HA-Pdx1 E178G). Upon transfection into BHK cells, both wild-type and mutant Pdx1 localized to the nucleus, with no evidence of cytoplasmic or membrane staining (Figure 4). These findings indicate that the E178G mutation does not result in mislocalization of Pdx1, consistent with its location N-terminal to the NLS contained within the third helix of the homeodomain.

E178G reduces Pdx1 transactivation: To determine whether E178G disrupted the ability of Pdx1 to
transactivate target gene promoters, we assessed its activity using a CAT reporter plasmid harboring the Pdx1-responsive TAAT1 enhancer of the somatostatin promoter (6). BHK cells were transfected with the reporter in combination with HA-Pdx1, HA-Pdx1 E178G, or an empty vector control (Figure 5A). As expected, expression of HA-Pdx1 potently induced reporter activation approximately 12-fold above that of empty vector control. HA-Pdx1 E178G displayed significantly reduced activity compared to the HA-Pdx1 protein (6-fold activation over empty vector, p<0.01 compared to HA Pdx1). This difference in activity was not explained by expression level, as Pdx1 protein level from HA-Pdx1 and HA-Pdx1 E178G transfected cells were equivalent in this system (Figure 5B).

**Pdx1E178G displays normal chromatin occupancy:** Decreased transactivation activity may be explained by the inability of a transcription factor to access chromatin around the promoter or enhancers of its target genes. We addressed this possibility using quantitative chromatin immunoprecipitation (ChIP) in the mouse insulinoma beta cell line Min6 to measure HA tagged Pdx1 protein occupancy of endogenous target gene promoters. HA-Pdx1 occupied two previously established target genes, the proximal promoter of the insulin gene and Area I of the Pdx1 gene itself, but not the albumin promoter which served as a negative control (Figure 6). We observed similar specific enrichment for Pdx1 target sequences in immunoprecipitates from cells expressing HA-Pdx1 and HA-Pdx1 E178G, suggesting that the mutation does not disrupt normal chromatin occupancy or DNA binding.

**DISCUSSION**

PDX1 has been well established as a key factor in pancreas development and function (14; 15), with homozygous mutations resulting in pancreas agenesis associated with neonatal diabetes, intrauterine growth retardation, and exocrine pancreas deficiency in humans and mice (10; 11; 16; 17). Only two patients with homozygous or compound heterozygous PDX1 mutations have been described to date, one with a homozygous frameshift mutation that prevents translation of the homeodomain and C terminus (Pro63fsdelC) (10) and the other one with compound heterozygous mutations (E164D, E178K) affecting the homeodomain (11). Here we identified a novel homozygous PDX1 mutation, PDX1-E178G, which results in a milder syndrome, with complete endocrine pancreas deficiency from birth and prenatally (intrauterine growth retardation), but with no clinical manifestation of exocrine pancreas dysfunction, despite biochemical evidence of subclinical exocrine pancreas deficiency and structural abnormalities detected by ultrasound scan. In our patients, there was visible pancreatic tissue, although quantitatively reduced in patient 8, and pancreatic $\alpha$ cells appeared functional, as indicated by glucagon secretion. The pancreatic cyst observation was discordant between the patients and may be coincidental.

Heterozygous carriers of the PDX1-E178G mutation were asymptomatic and non-diabetic, contrasting with the MODY or early T2D phenotype reported in heterozygous carriers of inactivating PDX1 mutations in human and in mouse (7; 12; 18-20). The two parents heterozygous for PDX1-E164D and E178K mutations studied by Schwitzgebel et al. (11) had high-normal fasting glucose at the time of examination, the mother had gestational diabetes and the family showed a significant history of early-onset T2D in relatives (11). Despite their non-diabetic
status and normal glucose tolerance, PDX1-E178G heterozygous parents showed low insulin secretory response during OGTT, and two of the four parents had very low first-phase insulin response during IVGTT. Interestingly, detailed metabolic explorations performed in heterozygous PDX1-Pro63fsdelC subjects showed that they had increased insulin sensitivity, in addition to impaired insulin secretion (18). The normal phenotype observed in PDX1-E178G heterozygotes may be the result of slightly impaired insulin secretion compensated by slightly increased insulin sensitivity. Based on these findings and the unremarkable history of T2D in the extended family, it is unlikely that heterozygosity for this mutation predisposes to T2D, unless at a late or very late age.

Studies of PDX1 mutations found in neonatal diabetes patients and heterozygous parents suggest that disease severity is variable and correlates with the nature and functional consequences of the mutation. Several rare PDX1 variants have also been identified by sequence screening in patients selected from multiplex T2D families, some of which were reported to co-segregate with early-onset T2D in a dominant mode (21-23). A possible role of rare PDX1 variants has recently been proposed in Ketosis Prone Diabetes (24). Remarkably, exocrine pancreas development and function are only affected in the most severe mutations in the homozygous state, indicating a greater sensitivity of the endocrine compared to the exocrine compartment to PDX1 dysfunction. This concept of differential sensitivity to gene dosage has been well illustrated in mouse models carrying various combinations and gradations of Pdx1 mutations, where homozygosity for hypomorphic Pdx1 mutations also resulted in a milder phenotype, with development of a normal sized pancreas and delayed onset of diabetes (25; 26).

Our functional data indicate a specific effect of E178G on the transactivation function of PDX1, as neither nuclear localization nor chromatin occupancy were affected by the mutation. In agreement with our findings for the E178G mutation, the E178K mutation studied by Schwitzgebel et al. (11) did not alter nuclear localization nor the ability of Pdx1 to bind to DNA target sequences, as assessed by EMSA. Further, in vitro interactions with NeuroD/Beta2, Foxa2 and Pbx1 were unimpaired. Rather, in functional studies conducted in BHK cells, Pdx1 E178K displayed reduced transactivation activity due to a decrease in Pdx1 steady state protein levels resulting from impaired protein stability. In our study, Pdx1 steady state levels were not altered by the E178G mutation when expressed in the Min6 beta cell line, which may more closely mimic the situation of primary beta cells. Taken together, the results support a specific effect of the E178G mutation on the transactivation function of Pdx1 independent of subcellular localization, DNA binding or expression level, suggesting that this mutation may alter Pdx1 interaction with co-factors such as NeuroD, Foxa2, Pbx1, or E47 or a novel factor not yet identified.

Our study extends the phenotypic spectrum of PDX1 mutations and justifies further mutation screening of this gene in non-syndromic neonatal diabetes patients. Based on previous knowledge, such patients are unlikely to have been tested for PDX1 mutations, in the absence of the evocative clinical phenotype (1; 27) and we recommend extending the current practice for molecular diagnosis of neonatal diabetes to include PDX1 screening.

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PDX1 mutation in permanent neonatal diabetes

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Table 1: Follow-up examination of endocrine and exocrine pancreas function in neonatal diabetes patients

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Figure Legends

**Figure 1. Neonatal diabetes family tree with linkage analysis.**
Diabetic patients (neonatal diabetes) are shown in black. A unique region of 4.4 Mb segregates with neonatal diabetes in this family (homozygous red haplotype in patients).

**Figure 2. PDX1 mutation identification in the neonatal diabetes family.**
(A) Sequence of all individuals of the family, including prenatal case (9), showing a homozygous mutation segregating with disease. Positions are given relative to Reference Sequences NM_000209.3 (cDNA) and NP_000200.1 (protein). (B) Multiple protein sequence alignment of the region of Human PDX1-178E, located with helix 2 of PDX1 homeodomain, showing PDX1 and homologous proteins from various organisms.

**Figure 3. OGTT and IVGTT in the four heterozygous parents.** (A) Plasma insulin and plasma glucose levels during OGTT. Normal ranges (higher-lower values) of plasma insulin levels are shown by the black curves. Plasma glucose levels were all within normal ranges (<6.1 mmol/l at time 0 min, and <7.8 mmol/l at time 120 min). Control values for plasma insulin levels were established from 30 normal-weight and non-diabetic Caucasian individuals, 18 to 40 years of age, studied in the same laboratory, using the same procedure. (B) First phase insulin response (FPIR) during IVGTT. *: 5th percentile; **: 1st percentile; ***: <1st percentile compared to a non-diabetic reference population (28). Age of parents was 30 to 38 years and BMI 19.4 to 23.6.

**Figure 4. Pdx1 E178G localizes to the nucleus.** Localization of Pdx1 protein in transfected BHK cells stained for HA (Pdx1 overexpression), tubulin and DAPI (nuclei). 60x.

**Figure 5. Pdx1 E178G has reduced transactivation activity.** (A) Activation of Pdx1-responsive CAT reporter in transfected BHK cells, normalized to beta galactosidase activity. n=3, HA-Pdx1 vs HA-Pdx1 E178G p<0.01. (B) Pdx1 expression level in transfected BHK cells assessed by western blot analysis, visualized with anti-HA.

**Figure 6. Pdx1 E178G does not alter Pdx1 chromatin occupancy.** (A) HA-Pdx1 occupancy of the insulin and Pdx1 promoters was measured by quantitative chromatin immunoprecipitation assay in transfected Min6 cells. n=4 (B) Western blot of protein extracted from transfected Min6 cells.