HHS Public Access

Author manuscript

Hum Mol Genet. Author manuscript; available in PMC 2018 September 19.

Published in final edited form as:

Hum Mol Genet. 2004 December 15; 13(24): 3151–3159. doi:10.1093/hmg/ddh341.

PAX4 gene variations predispose to ketosis-prone diabetes

Franck Mauvais-Jarvis[1,3,4,*], **Stuart B. Smith**[3], **Cédric Le May**[1], **Suzanne M. Leal**[2], **Jean-François Gautier**[4], **Mariam Molokhia**[5], **Jean-Pierre Riveline**[6], **Arun S. Rajan**[1], **Jean-Philippe Kevorkian**[7], **Sumei Zhang**[3], **Patrick Vexiau**[4], **Michael S. German**[3], and **Christian Vaisse**[3]

[1]Division of Diabetes, Endocrinology and Metabolism, Department of Medicine [2]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA [3]Diabetes Center, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143, USA [4]Department of Endocrinology and Diabetes, Saint-Louis Hospital, University of Paris VII School of Medicine, Paris 75010, France [5]Epidemiology Unit, London School of Hygiene and Tropical Medicine, London, WC1E 7HT, UK [6]Department of Diabetes and Metabolic Diseases, Sud Francilien Hospital, Corbeil-Essonnes 91100, France and [7]Department of Internal Medicine B, Lariboisiere Hospital, Paris, 75010, France

Abstract

Ketosis-prone diabetes (KPD) is a rare form of type 2 diabetes, mostly observed in subjects of west African origin (west Africans and African-Americans), characterized by fulminant and phasic insulin dependence, but lacking markers of autoimmunity observed in type 1 diabetes. PAX4 is a transcription factor essential for the development of insulin-producing pancreatic β-cells. Recently, a missense mutation (Arg121Trp) of PAX4 has been implicated in early and insulin deficient type 2 diabetes in Japanese subjects. The phenotype similarities between KPD and Japanese carriers of Arg121Trp have prompted us to investigate the role of PAX4 in KPD. We have screened 101 KPD subjects and we have found a new variant in the *PAX4* gene (Arg133Trp), specific to the population of west African ancestry, and which predisposes to KPD under a recessive model. Homozygous Arg133Trp PAX4 carriers were found in 4% of subjects with KPD but not in 355 controls or 147 subjects with common type 2 or type 1 diabetes. *In vitro*, the Arg133Trp variant showed a decreased transcriptional repression of target gene promoters in an alpha-TC1.6 cell line. In addition, one KPD patient was heterozygous for a rare PAX4 variant (Arg37Trp) that was not found in controls and that showed a more severe biochemical phenotype than Arg133Trp. Clinical investigation of the homozygous Arg133Trp carriers and of the Arg37Trp carrier demonstrated a more severe alteration in insulin secretory reserve, during a glucagon-stimulation test, compared to other KPD subjects. Together these data provide the first evidence that ethnic-specific gene variants may contribute to the predisposition to this particular form of diabetes and suggest that KPD, like maturity onset diabetes of the young, is a rare, phenotypically defined but genetically heterogeneous form of type 2 diabetes.

[*]To whom correspondence should be addressed at: Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, BCMA 700B, Houston, TX 77030, USA. Tel: +1 7137987224; Fax: +1 7137983810; fmjarvis@bcm.edu.

INTRODUCTION

Rare genetic defects in transcription factors controlling the expression of the insulin gene and other proteins critical for normal pancreatic β-cell metabolism/function impair insulin secretion and lead to diabetic syndromes characterized by a rapid evolution toward insulin deficiency, such as maturity onset diabetes of the young (MODY) (1–5). PAX4 is a transcription factor that plays a critical role in the differentiation of embryonic pancreatic progenitors into insulin-producing β-cells. Mice with targeted disruption of the *pax4* gene show absence of mature insulin-producing β-cells, and die in the first days of life from severe insulin deficient diabetes (6). Although *PAX4* gene mutations have not been found in MODY families, a rare missense mutation in PAX4 (Arg121Trp) has recently been described in the Japanese type 2 diabetic population. Japanese carriers of the Arg121Trp variant are characterized by either a transient insulin dependence at diabetes onset or a rapid evolution toward insulin deficiency, suggesting that PAX4 mutations lead to severe β-cell dysfunction in humans (7).

Ketosis-prone diabetes (KPD) belongs to a rare subgroup of type 2 diabetes with severe insulin deficiency, mostly observed in subjects of sub-Saharan African ancestry, such as west Africans, Caribbeans and African-Americans (8–14). Its phenotype is distinct from the common type 2 and type 1 diabetes. It is characterized by a fulminant initial insulin dependence, without the immunological markers observed in classical type 1 diabetes, followed by a subsequent clinical course which varies from non-insulin treated type 2 diabetes to insulin-dependent idiopathic type 1 diabetes (14). A severe dysfunction of the insulin-producing β-cells is attested to by the observation that 25% of the subjects are insulin dependent at diabetes onset, whereas the remaining 75% will develop permanent insulin dependence within 10 years (14).

The clinical similarities between Japanese type 2 diabetic carriers of the Arg121Trp PAX4 mutation and west African subjects with KPD suggest that mutations in PAX4 could also predispose to the latter.

We studied a cohort of west African subjects with KPD for mutations in the *PAX4* gene. Our results provide the first evidence that an ethnic-specific variant of PAX4 (Arg133Trp), associated with functional alterations *in vivo* and *in vitro*, predisposes to KPD, and represents a marker of severe insulin deficiency in the population of west African descent.

RESULTS

Screening of west African ketosis-prone diabetic subjects for variants in PAX4

We sequenced the coding region of the *PAX4* gene in 101 unrelated west African subjects with KPD. The results are summarized in Table 1. We found two new missense variants, one common variant already described, and three silent polymorphisms.

Four subjects (4%) carried a new homozygous missense variant resulting in the conversion of arginine (R) to tryptophane (W) at position 133 (R133W). The heterozygous R133W variant was found in 27 subjects. One subject (1%) carried a new missense mutation R37W.

The P321H variant has already been described in the Caucasian and Japanese populations (15). The P321H and the three silent variants were not associated with KPD in our population (Table 1). As was expected, owing to their close physical proximity there was a strong linkage disequilibrium ($P < 0.00001$) between variants R133W and P321H in both the control ($D' = 0.97$) and ketosis-prone diabetic subjects ($D' = 0.82$).

Most patients studied are immigrants from west African countries and their first and second degree relatives were not accessible.

The R133W variant is not found in Caucasians

To investigate the ethnic specificity of the R133W variant, we evaluated its prevalence by sample genotyping a Caucasian control population. In this population, *PAX4* was monomorphic at the 133R site and the 133W allele was not found in a total of 200 individuals (Fig. 2).

R133W is found at the homozygous state in KPD only

To evaluate the specificity of the R133W variant to KPD, we genotyped an extended population of diabetic and controls of west African origin (see Materials and Methods) (Table 2). All groups were in Hardy–Weinberg equilibrium.

The homozygous R133W variant was found in ketosisprone diabetic subjects only (4%), and was not found in 147 type 2 diabetic, type 1 diabetic and 355 controls of west African origin (Table 2). The frequency of homozygous R133W carriers was compared between ketosis-prone diabetic subjects and west African non-diabetic controls by separating homozygous (W/W) individuals and wild-type (R/R)/heterozygous (R/W) individuals into two different groups. Under these conditions, the homozygous R133W carriers were associated with an increased risk of KPD with an odds ratio (OR) of 23.5 [95% confidence interval (CI), 2.7–203.6, $P = 0.001$].

The frequency of the heterozygous R133W variant was increased in ketosis-prone diabetic subjects compared with common type 2 diabetic subjects ($\chi^2 = 4.91$; $P = 0.026$) (Table 2). The increased frequency of heterozygous R133W in ketosis-prone diabetic subjects did not reach significance compared with west African non-diabetic controls ($\chi^2 = 1.54$, $P = 0.21$), owing to small sample size, but reached significance when compared with African-Americans ($\chi^2 = 8.04$, $P = 0.04$). However, in this latter group, the genetic admixture with Caucasians certainly lowers the 133W allele frequency. In any case, the 133W allele frequency was higher in ketosis-prone diabetic subjects than in other west African diabetic and control groups (Table 2) with an OR > 1.5, suggesting that the heterozygous R133W variant may also increase the risk of KPD.

Interaction between R133W and P321H

As R133W and P321H are both frequent variants from the same gene, we assessed whether the 321H allele could modify the diabetic risk associated with the 133W allele by comparing the haplotypes frequencies associated with these alleles between ketosis-prone diabetic subjects and controls (Table 3). The haplotypes frequencies were different in subjects and

controls ($\chi^2 = 9.74$; $P = 0.02$), further suggesting that the *PAX4* locus predisposes to KPD. As expected, when comparing the haplotypes R133/P321 and R133/321H (effect of the P321H variant alone) there was no increased risk of diabetes [OR: 0.97 (CI: 0.63–1.47); $P = 0.85$]. However, when comparing the haplotypes 133W/321H to 133W/P321 (effect of P321H on R133W), we observed a trend toward an increased diabetic risk associated with the haplotype 133W/P321 [OR: 4.48 (CI: 0.82–24.32), $P = 0.079$]. Together, these data suggest that the 321H allele may partially reverse the diabetes risk associate with the 133W allele (Table 3). Owing to the small sample size we cannot draw any conclusion.

PAX4 variants show diminished ability to repress gene expression

PAX4 is expressed in the early embryonic pancreas and is not expressed in adult islets (16). It is required to specify and maintain the islet β-cell fate while repressing non-β-cell fate. PAX4 is a transcription factor that represses gene expression through a paired domain and homeodomain (16). Figure 1A shows the position of the R37W and R133W mutations in the PAX4 molecule. We assessed the functional significance of the R37W and R133W variants by looking at their ability to repress gene expression, following transfection in α-TC1.6 cells. We chose the a-cell because of previous work demonstrating that PAX4 repression of target promoters *in vitro* is most fully achieved in α-cells, whereas it is weak in β-cells (16,17). Similarly, we chose the insulin promoter in our functional assay because wild-type PAX4 binds to it with a higher affinity than other promoters tested (16). α-TC1.6 cells were co-transfected with an insulin promoter reporter construct along with each human PAX4 variant (Fig. 1B). Wild-type PAX4 represses promoter activity by 70% compared with the control (compare lane 3 with lane 2). On the other hand, both PAX4 variants R37W and R133W repress reporter activity by only 45 and 37% of control, respectively (compare lanes 4 and 5 with lane 2).

PAX4 (R37W) binds target genes with lower affinity than wild-type PAX4

The ability of a transcription factor to stimulate or inhibit gene expression is partly secondary to its ability to bind DNA sequence on the target gene promoter. We studied the binding of R133W and R37W to the insulin promoter and to the PAX4 promoter in EMSA conditions (Fig. 1C). Both wild-type PAX4 and R133W mutants showed similar binding activities to specific DNA sequences. On the other hand, the binding of the R37W variant to DNA sequences was decreased by 50% of that of the wild-type PAX4.

Clinical features of the homozygous R133W and R37W PAX4 variant carriers

Table 4 shows the individual clinical and metabolic features of ketosis-prone diabetic patients 1–4 carrying the homozygous R133W variant and patient 5 carrying the R37W variant.

All patients originated from west African countries, and all had a family history of diabetes, except one for which family history was unknown. All patients showed very low C-peptide levels and major hyperglucagonemia despite hyperglycemia at initial admission, consistent with severely altered pancreatic α- and β-cell function during diabetic ketoacidosis.

Among homozygous R133W carriers, patients 2 and 4 were younger and leaner at diabetes onset than the other subjects with KPD (14). All homozygous R133W carriers experienced remission of insulin dependence within the first 6 months of initial admission, allowing the maintenance of good blood glucose control with oral hypoglycemic agents (OHA). However, the clinical course of patients 3 and 4 was characterized by a new episode of acute insulin deficiency with development of permanent insulin dependence in <2 years after diabetes onset (14). Patients 1 and 3 were still being treated with OHA 3 years after diabetes onset. Other clinical and metabolic characteristics at diabetes onset were not different between ketosis-prone diabetic carriers of the R133W and wild-type PAX4 (Table 4).

Patient 5 (R37W) did not experience remission of insulin dependence and remained insulin dependent from diabetes onset up to the present time, consistent with a severe β-cell insulin secretory defect (14).

PAX4 variant carriers show severe alterations of insulin secretion *in vivo*

Insulin secretory reserve was assessed in patients 1–5, using a glucagon-stimulation test as described (9,14,18).

As previously described (14), we observed a relative restoration of insulin secretion following resolution of hyperglycemia in patients 1–4, the homozygous carriers of the R133W mutation. However, the insulin secretory reserve was more severely altered than in other ketosis-prone diabetic subjects with heterozygous R133W or wild-type PAX4 variant (Fig. 2). In patient 5 (R37W), the glucagon-stimulation test was consistent with total insulin deficiency [C-peptide (ng/ml) basal: 0.1; 8 min: 0.6] and insulin secretory reserve remained low for up to 10 years following admission (data not shown). It should be noted that although three out of the four R133W homozygote carriers were from Senegal, the country of origin did not act as a confounder and we did not detect any difference in insulin secretion assessed by glucagon-stimulation, when comparing ketosis-prone diabetic wild-type carriers from Senegal with those originating from other west African countries (data not shown).

Thus, both the homozygous R133W and R37W variants are associated with severe alteration of pancreatic β-cell function and insulin secretion *in vivo*.

DISCUSSION

Separating the common form of type 2 diabetes into phenotypic subgroups has led to the identification of MODY, due to mutations in at least five genes, including glucokinase and β-cell transcription factors (1,2,4,19) and mitochondrial diabetes, a maternally inherited diabetic syndrome, secondary to a mutation in the mitochondrial genome (20).

KPD is a rare subtype of type 2 diabetes characterized by a severe β-cell dysfunction and mostly found in populations of west African ancestry (8–10,12,14,18). Although genetic susceptibility to KPD is very likely (8–10,12,14,18), it is not known whether we are facing a polygenic model or a model with a major gene influence. The observation that targeted disruption of the *PAX4* gene in mice leads to absence of pancreatic β-cells along with the

recent finding that *PAX4* is a candidate gene associated with insulin deficient type 2 diabetes in Japanese subjects, prompted us to screen this gene in west Africans with KPD.

Stringent phenotyping has allowed us to identify a population of diabetic patients of west African ancestry with well defined KPD. Using this population, we identified a novel functional variant in the *PAX4* gene (R133W) that is specific to this population and predisposes to KPD under a recessive model.

The heterozygous R133W variant is present in 7–10% of the population of west African descent and is not found in Caucasians. Similarly, other investigators failed to find this variant in Japanese (7,15). The high frequency of the heterozygous R133W variant within the west African population could be due to population history, but could also reflect a selective advantage conferred to carriers of at least one copy of the variant, for a disease exposure that is endemic to this region.

The homozygous R133W variant of PAX4 is present in 4% of subjects with KPD, is specific to that subtype of diabetes and dramatically increases the risk of disease. Thus, it could be a marker of severe insulin deficiency in type 2 diabetic populations of west African descent. We observe an increased frequency of the heterozygous R133W carriers in the KPD group compared with other individuals of the same west African ancestry, suggesting that the heterozygous state may also predispose to KPD.

The R133W mutation is located between the paired domain and homeodomain, and leads to functional alterations *in vitro*. Studies using a luciferase assay show that R133W leads to a reduction by half of normal PAX4 function as a transcriptional repressor. Surprisingly, the R133W variant did not affect DNA-binding affinity as measured *in vitro* by EMSA. As this variant does not lie within the defined repressor domain of PAX4 (16), the decreased transcriptional repression observed in the R133W variant may result, not from decreased DNA-binding affinity for the sites that we tested, but possibly from altered binding specificity, impaired interaction with other proteins or from a decreased functional stability *in vivo*.

Consistent with functional studies, clinical investigation in ketosis-prone diabetic carriers of the homozygous R133W variant show a more severe alteration of insulin secretion than heterozygous and wild-type ketosis-prone diabetic carriers. Together, the genetic study along with the *in vitro* and *in vivo* functional characterization, suggest that the homozygous R133W variant predisposes to KPD.

We have also identified a R37W variant in one patient. R37W affects the DNA-binding domain of the molecule and *in vitro* shows a 50% reduction of PAX4-binding activity to target gene promoters resulting in a similar decrease of normal PAX4 transcriptional repression activity. This more severe biochemical phenotype of R37W compared with R133W may help explain why it is clinically apparent even in its heterozygous form. However, because the R37W variant was found in only one case, we cannot ascertain its role in diabetes without family studies, and these have not been performed.

A variant in PAX4 (R121W) has been described previously in the Japanese type 2 diabetic population. Interestingly, the Japanese R121W diabetic carriers and west African homozygous R133W diabetic carriers share some clinical similarities: half the type 2 diabetic carriers of the R121W variant showed initial insulin dependence (14). In addition, the homozygous R121W carrier had early-onset diabetes (29 years old) and early insulin dependence (7), and half of the west African homozygous R133W ketosis-prone diabetic carriers were under 25 years of age, at diabetes onset. *In vitro* functional studies of the R121W Japanese variant reveal a more severe alteration of transcriptional repression of target genes and DNA-binding activity compared with the west African R133W variant. This is consistent with the observation that the 121W allele is absent from the background Japanese population (strong effect in heterozygotes), whereas the 133W allele is present in 7–10% of the population of west African ancestry (weak effect in heterozygotes). Taken together, these studies suggest that *PAX4* is a candidate gene for severe insulin deficiency in non-white populations.

Evidence from mice with targeted disruption of the *PAX4* gene demonstrates a role for PAX4 in β-cell formation during fetal development (21). In these mice, loss of PAX4 decreases the expression of β-cell specific genes and results in a dramatic decrease in β-cell mass at birth (22). Thus, homozygous R133W carriers may have fewer β-cells at birth, or may have difficulty replacing β-cell mass as they get older. Alternatively, inadequate PAX4 function could lead to the formation of dysfunctional β-cells.

This study presents two limitations: One is the lack of access to family members at inclusion to demonstrate the segregation of the mutations with KPD. The other is the lack of power of our sample size. As west Africans are the genetic founders of African-Americans, a large study is needed to explore the role of PAX4 variants in African-Americans with KPD at the level of the US population.

In summary, we describe a novel functional variant of PAX4 (R133W) that is specific to west Africans. Homozygous carriers of the PAX4 R133W variant have an increased risk of KPD. These data provide the first evidence that ethnic-specific gene variants may contribute to the predisposition to this particular form of diabetes and suggest that KPD, like MODY, is a rare phenotypically defined but genetically heterogeneous form of type 2 diabetes.

MATERIALS AND METHODS

Patient and control populations

The diabetic populations used in this study have been described previously (14,23). Briefly, KPD was defined as new onset diabetes, with the presence of strong ketosis (urine ketones >80 mg/dl) or DKA, without precipitating illness (infection, stress), and in the absence of auto-antibodies (ab) to islet cells (ICA) and to glutamic acid decarboxylase (GAD) 65. Ketosis-prone diabetic patients did not show the classical HLA-DR haplotypes encountered in type 1 diabetes (18). Type 1 diabetic subjects were defined by the presence of ICAs and GAD 65 ab. Type 2 diabetic subjects were selected for diabetes treated by diet or OHA and were resistant to ketosis despite the presence of precipitating illness (14,23).

The control populations come from either the Lariboisiere/St Louis Medical Center, outpatient clinic, Paris, France (west Africans) or the Bermondsey and Lansdowne Medical Mission, the Clapham Manor Health Centre, and from two churches in south-east London, UK (west Africans, Caucasians). These patients were normoglycemic, and without family history of diabetes. The diabetic and control populations were comparable with regard to their west African countries of origin. Other controls (Caucasians) come from the human variation collection of the NIGMS repository (Coriell Institute for Medical Research, Camden, NJ). Informed consent was obtained from all subjects. Genomic DNA was extracted from peripheral lymphocytes for all subjects as previously described (23). This protocol was approved by all the local ethics committees.

Insulin secretion

Pancreatic insulin reserve was assessed at least 48 h after resolution of ketosis and normalization of blood glucose by measuring the C-peptide level before and 8 min following the i.v. injection of 1 mg glucagon as previously described (14). Patients were studied 12 h after an overnight fast and before taking the morning insulin injection.

Glucagon assay

Blood was drawn during the initial admission for diabetic ketoacidosis in tubes containing aprotinin as protease inhibitor. Serum glucagon level was assessed using a glucagon RIA kit (Linco Research, St Charles, MO, USA) and a glucagon-specific antibody with a sensitivity of 20 pg/ml. A standard curve was generated with purified recombinant glucagon over a range of 20–400 pg/ml.

PAX4 mutation screening

The coding sequence of the human *PAX4* gene (nine exons) was amplified in three different PCR reactions: Exon 1, 2, 3: PAX4-1F (AGG TGG TGT GTG GAT ACC TC) and PAX4-3R (GAT TTG GCT GTG ATT AGC CC); Exon 4, 5, 6, PAX4-4F (CTG ACC AGA GGA ATC ACC ATC) and PAX4-6R (GAT GAC TGA GCG GGC AGA TG); Exon 7, 8, 9: PAX4-7F (AGT GGC TGA CTT TCC TAG AAC) and PAX4-9R (TGG GCA GGA TGG TAT TAG ATC TTC TCT ATG). Sequencing reactions were performed with the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA) under the standard manufacturer's conditions. Sequencing was performed on an ABI PRISM® 3700 automated DNA sequencer (Applied Biosystems). Sequences were analyzed using Sequence Navigator (Applied Biosystems).

PAX4 variants genotyping

The C397T [R133W] mutation creates a restriction site for the enzyme Hsp92 II that we used to create a restriction length fragment polymorphism (RFLP) assay. We amplified *PAX4* exon 3 from genomic DNA by PCR, using primers PAX4-3F (AGCCCTGAGTCTGAGCACCA) and PAX43R2 (GGAGAGAATGAGACTCCCT). Restriction digest of the 260 bp PCR product by the enzyme Hsp92 II (Promega, Wisconsin, MA, USA) was performed as recommended by the manufacturer and lead to 110 and 150 bp products in presence of the mutant allele. Homozygous mutations were confirmed by direct sequencing.

Other PAX4 variants were genotyped by TaqMan allelic discrimination assay system after designing flanking primers and fluorogenic probes, both of which were specific to target alleles.

Cloning of wild-type and mutant PAX4

A PCDNA3.1 plasmid expressing human PAX4 (hPAX4) was used to clone the mutations C109T (Arg37Trp) and C397T (Arg133Trp) by site-directed mutagenesis. Both mutations are flanked by the unique restriction sites *Afl*II and *Bste*II, respectively. We designed the mutagenesis primers (PAX4R133W—CAGCTGGTGACCTGAGCCaTGTGCA, PAX4R37W—GATACCTTAAGGATCCaTGAGAT) encompassing these respective restriction sites and used these primers to amplify large PCR products that were cloned back into the wild-type PCDNA3.1 backbone.

Electromobility shift assays

For *in vitro* expression, the mutant and wild-type hPAX4 were cloned into the plasmid pSP72 downstream of the SP6 promoter. The entire PAX4 sequence of all constructs was verified by direct sequencing. The wild-type and mutant PAX4 proteins were produced *in vitro* using the TNT Quick Coupled Lysate System® (Promega) and 1 μl of the 50 μl total reaction volume was used per binding mix.

Single stranded oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (16). An excess of complementary strand was then annealed to form a duplex strand that was column purified. For EMSA buffers and electrophoresis conditions, we used 500 ng of poly(dI–dC): poly(dI–dC) per 10 μl binding mix as described previously (16). Oligonucleotides used were as follows (coding strand shown from each double-stranded pair): Rat insulin I C2 element, bp −328 to −304 5′-ctgggaaatgaggtggaaaatgctc-3′; Human PAX4 promoter, bp −4164 to −4116 (P4 4.2) 5′cccaattgtcaaaggtggaataatttgatcaacaaaataatgtattg-3′. EMSA results are representative of at least two independent experiments.

Cell culture and transient transfections

α-TC1.6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% fetal bovine serum and 15% horse serum. Twenty-four hours prior to transfection, cells were split into 6-well plates and 1 million cells per well were used for transfection. The reporter construct used (−410Rins-pfoxluc) was created using the −410 bp of the rat insulin promoter cloned upstream of luciferase in the pFoxluc1 backbone reporter vector. An aliquot of 2 μg of the reporter construct was used per well, 50 ng of any cotransfected transcription factor cDNA was used per well. Transfast lipid agent (Promega) was used for all transfections according to the manufacturer's instructions. Cells were harvested 48 h after transfection and luciferase assays performed as previously described (24). Luciferase activity was corrected for protein concentration; at least three independent sets of transfections were performed. Data are expressed as mean ± SE.

Statistical analysis

For association studies of the R133W variant, each diabetic and control group was tested for deviations from Hardy–Weinberg Equilibrium using χ^2 tests. The minor allele frequency was estimated for each group and the 95% CI was calculated on the basis of the binomial distribution. In order to test whether there was a significant difference in frequency of homozygous R133W individuals in KPD compared with control individuals, the ketosis-prone diabetic subjects were compared with the west African control groups and a Fisher-exact test was performed. The OR was also calculated using a Sheehe correction (25) owing to the presence of a cell with 0 observation in the non-ketosis-prone diabetic groups.

Owing to ambiguity of heterozygous individuals at both loci, haplotypes were constructed for ketosis-prone diabetic subjects and west African controls using the SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt) program which implements the Expectation Maximization (EM) algorithm. Using the estimated haplotype frequencies under linkage equilibrium and disequilibrium, D' was estimated for both the cases and controls (26). A likelihood ratio test was performed to determine if there was a difference in the haplotype frequencies between cases and controls. Briefly, the log-likelihood of the cases and controls estimated separately was compared to the log-likelihood of the entire dataset [2(ln(L, cases) + ln(L, controls)) 2 ln(L, cases + controls together)]. Differences in haplotype frequencies (R133W/P321H) were evaluated between the ketosis-prone diabetic cases and the other groups by calculating ORs and their 95% CI using the Sheehe correction. The χ^2 test was used to evaluate if the differences in haplotype frequencies between the ketosis-prone diabetic cases and controls were statistically significant.

For *in vitro* and *in vivo* studies, each variable was analyzed using the unpaired Student's test. For all analyses, a *P*-value of <0.05 was considered significant. Results are given as mean ± SE.

ACKNOWLEDGEMENTS

We gratefully acknowledge F. Andreelli, M. Assayag, F. Bosquet, C. Carette, B. Charbonnel, G. Charpentier, A. Colombel, N. D'Escrivain, C. Deybach, F. Duron, A. Grimaldi, P.J. Guillausseau, H. Hanaire-Broutin, J. SamuelLajeunesse, N. Vigier, M.L. Virally, A. Warnet and the many nurses who assisted them, for participating in patient collection. We thank Jean-Marie Villette for DNA extraction. F.M.-J. was supported by the Assistance Publique-Hoˆpitaux de Paris, an unrestricted grant from Bristol-Myers Squibb and Novo-Nordisk, and by the Division of Diabetes, Endocrinology and Metabolism at Baylor College of Medicine. C.V. was supported by NIH RO1 DK60540 and an American Diabetes Association Career Development Award.

REFERENCES

1. Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ,Fajans SS, Signorini S, Stoffel M and Bell GI (1996) Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). Nature, 384, 458–460. [PubMed: 8945471]
2. Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H,Vaxillaire M, Southam L, Cox RD, Lathrop GM, Boriraj VV et al. (1996) Mutations in the hepatocyte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3). Nature, 384, 455–458. [PubMed: 8945470]
3. Stoffers DA, Ferrer J, Clarke WL and Habener JF (1997) Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. Nat. Genet, 17, 138–139. [PubMed: 9326926]

4. Horikawa Y, Iwasaki N, Hara M, Furuta H, Hinokio Y,Cockburn BN, Lindner T, Yamagata K, Ogata M, Tomonaga O et al. (1997) Mutation in hepatocyte nuclear factor-1 beta gene (TCF2) associated with MODY. Nat. Genet, 17, 384–385. [PubMed: 9398836]
5. Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T,Saad M, Warram JH, Montminy M and Krolewski AS (1999) Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. Nat. Genet, 23, 323–328. [PubMed: 10545951]
6. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G and Gruss P(1997) The *Pax4* gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature, 386, 399–402. [PubMed: 9121556]
7. Shimajiri Y, Sanke T, Furuta H, Hanabusa T, Nakagawa T,Fujitani Y, Kajimoto Y, Takasu N and Nanjo K (2001) A missense mutation of *Pax4* gene (R121W) is associated with type 2 diabetes in Japanese. Diabetes, 50, 2864–2869. [PubMed: 11723072]
8. Banerji MA, Chaiken RL, Huey H, Tuomi T, Norin AJ,Mackay IR, Rowley MJ, Zimmet PZ and Lebovitz HE (1994) GAD antibody negative NIDDM in adult black subjects with diabetic ketoacidosis and increased frequency of human leukocyte antigen DR3 and DR4. Flatbush diabetes. Diabetes, 43, 741–745. [PubMed: 8194658]
9. Umpierrez GE, Casals MM, Gebhart SP, Mixon PS, Clark WSand Phillips LS (1995) Diabetic ketoacidosis in obese AfricanAmericans. Diabetes, 44, 790–795. [PubMed: 7789647]
10. Pinero-Pilona A, Litonjua P, Aviles-Santa L and Raskin P (2001) Idiopathic type 1 diabetes in Dallas, Texas: a 5-year experience. Diabet. Care, 24, 1014–1018.
11. Sobngwi E, Mauvais-Jarvis F, Vexiau P, Mbanya JC andGautier JF (2002) Diabetes in Africans. Part 2: Ketosis-prone a typical diabetes mellitus. Diabet. Metab, 28, 5–12.
12. Maldonado M, Hampe CS, Gaur LK, D'Amico S, Iyer D,Hammerle LP, Bolgiano D, Rodriguez L, Rajan A, Lernmark A et al. (2003) Ketosis-prone diabetes: dissection of a heterogeneous syndrome using an immunogenetic and beta-cell functional classification, prospective analysis, and clinical outcomes. J. Clin. Endocrinol. Metab, 88, 5090–5098. [PubMed: 14602731]
13. Kitabchi AE (2003) Ketosis-prone diabetes—a new subgroup of patientswith atypical type 1 and type 2 diabetes? J. Clin. Endocrinol. Metab, 88, 5087–5089. [PubMed: 14602730]
14. Mauvais-Jarvis F, Sobngwi E, Porcher R, Riveline JP,Kevorkian JP, Vaisse C, Guillausseau PJ, Charpentier G, Vexiau P and Gautier JF (2004) Ketosis-prone type 2 diabetes in patients of sub-Saharan African origin: clinical pathophysiology and natural history of beta-cell dysfunction and insulin resistance. Diabetes, 53, 645–653. [PubMed: 14988248]
15. Dupont S, Vionnet N, Chevre JC, Gallina S, Dina C, Seino Y, Yamada Y and Froguel P (1999) No evidence of linkage or diabetesassociated mutations in the transcription factors BETA2/NEUROD1 and PAX4 in Type II diabetes in France. Diabetologia, 42, 480–484. [PubMed: 10230653]
16. Smith SB, Ee HC, Conners JR and German MS (1999) Pairedhomeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol. Cell. Biol, 19, 8272–8280. [PubMed: 10567552]
17. Smith SB, Watada H, Scheel DW, Mrejen C and German MS(2000) Autoregulation and maturity onset diabetes of the young transcription factors control the human PAX4 promoter. J. Biol. Chem, 275, 36910–36919. [PubMed: 10967107]
18. Sobngwi E, Vexiau P, Levy V, Lepage V, Mauvais-Jarvis F,Leblanc H, Mbanya JC and Gautier JF (2002) Metabolic and immunogenetic prediction of long-term insulin remission in African patients with atypical diabetes. Diabet. Med, 19, 832–835. [PubMed: 12358870]
19. Froguel P, Zouali H, Vionnet N, Velho G, Vaxillaire M, Sun F,Lesage S, Stoffel M, Takeda J, Passa P et al. (1993) Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. N. Engl. J. Med, 328, 697–702. [PubMed: 8433729]
20. Kadowaki T, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y,Tanabe Y, Sakura H, Awata T, Goto Y et al. (1994) A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. N. Engl. J. Med, 330, 962–968. [PubMed: 8121460]
21. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B and Sussel L (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc. Natl Acad. Sci. USA, 101, 2924–2929. [PubMed: 14970313]

22. Wang J, Elghazi L, Parker SE, Kizilocak H, Asano M, Sussel Land Sosa-Pineda B (2004) The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev. Biol, 266, 178–189. [PubMed: 14729487]

23. Mauvais-Jarvis F, Boudou P, Sobngwi E, Riveline JP, Kevorkian JP, Villette JM, Porcher R, Vexiau P and Gautier JF (2003) The polymorphism Gly574Ser in the transcription factor HNF-1alpha is not a marker of adult-onset ketosis-prone atypical diabetes in Afro-Caribbean patients. Diabetologia, 46, 728–729. [PubMed: 12743700]

24. German MS, Wang J, Chadwick RB and Rutter WJ (1992) Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix–loop–helix protein: building a functional insulin minienhancer complex. Genes Dev, 6, 2165–2176. [PubMed: 1358758]

25. Sheehe PR (1966) Combination of log relative risk in retrospectivestudies of disease. Am. J. Public Health Nations Health, 56, 1745–1750. [PubMed: 5951494]

26. Lewontin RC (1964) The interaction of selection and linkage: I. Generalconsiderations. Genetics, 49, 49–67. [PubMed: 17248194]

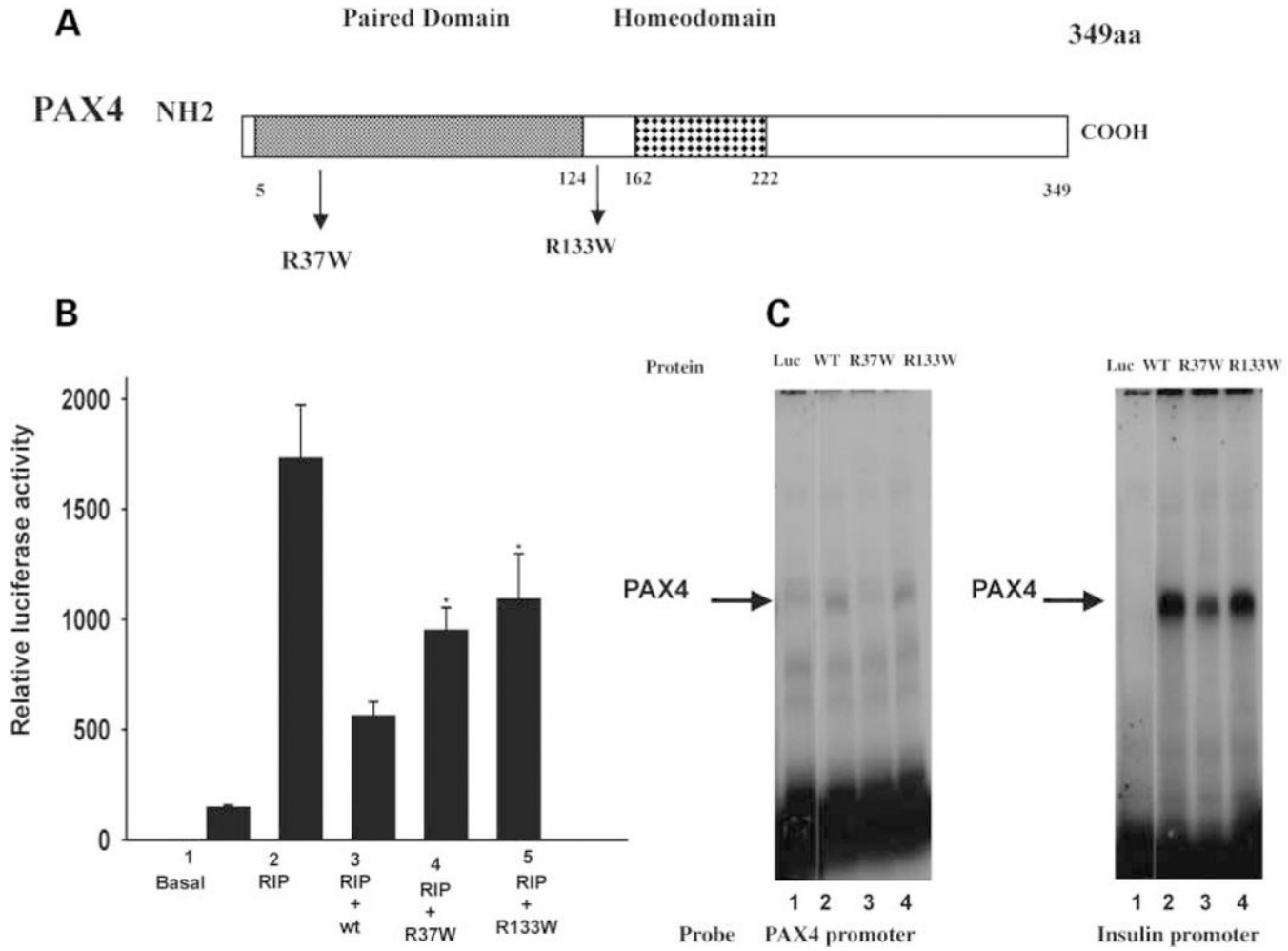

Figure 1.

(A) Structure of the PAX4 molecule and position of the R37W and R133W variants. **(B)** Effect of the PAX4 mutations on gene transcription activity in α-TC1.6 cells. The wild-type and mutant hPAX4 (WT, R133W, R37W) were co-transfected with an insulin promoter reporter construct (−410Rins-pfoxluc) in α-TC1.6 cells. The amount of reporter plasmid used was 2 μg/lane (lanes 1–5). The amount of co-transfected hPAX4 constructs (wt and variants) was 50 ng/lane(lanes 3–5). Data are expressed as mean ± SE. *P < 0.01 comparing R133W or R37W to WT. **(C)** Effect of PAX4 mutations on PAX4 binding to target genes.An EMSA using control (lane 1), wild-type PAX4 (lane 2), R37W variant (lane 3) and R133W variant (lane 4) is shown. The arrow indicates binding of PAX4 proteins to the probe. The oligonucleotide probes were the human PAX4 promoter (left) and the rat insulin promoter (right). Identical results were obtained in at least two independent experiments.

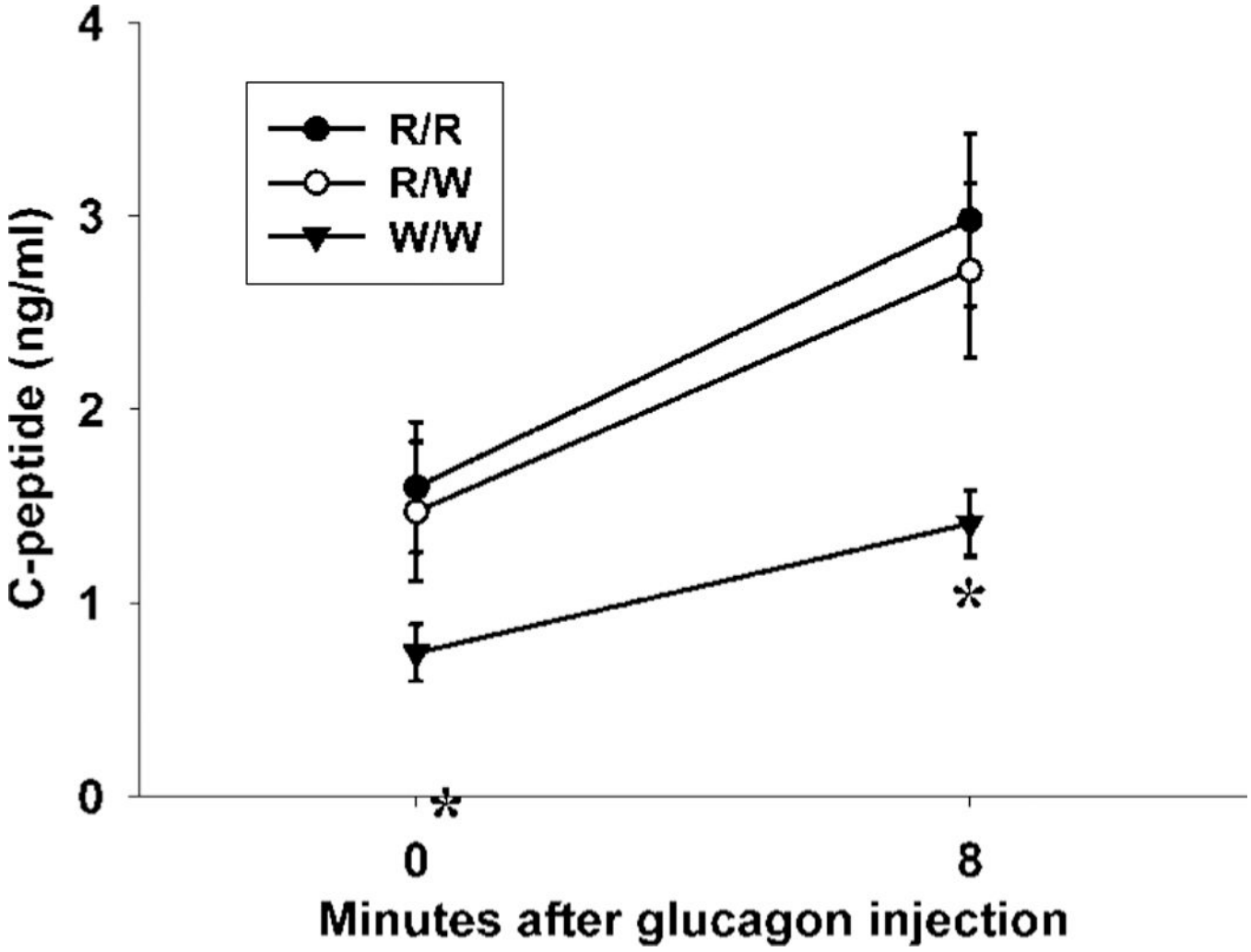

Figure 2.

β-cell insulin secretory reserve was assessed in a cohort of ketosis-prone diabetic subjects carrying the wild-type PAX4 (R/R, $n = 18$), the heterozygote (R/W, $n = 11$) and the homozygous mutation (W/W, $n = 4$), by measuring basal C-peptide (0 min) and C-peptide response following IV glucagon injection (8 min) after correction of hyperglycemia. $*P < 0.05$, R/R versus W/W.

Table 1.

Variants of the *PAX4* gene in ketosis-prone diabetes

Nucleotide change	Amino acid change	Designation	Genotype frequency Ketosis-prone diabetic subjects	Controls	*P*-value[a]
C109T	Arg > Trp	R37W	C/T: 1 (1%) C/C: 100 (99%)	0 (0%) 255 (100%)	–
C397T	Arg > Trp	R133W	T/T: 4 (4%) C/T: 27 (26.7%) C/C: 70 (69.3%)	0 (0%) 55 (21.6%) 200 (78.4%)	See Table 2
C450T	Gly > Gly	G150G	C/T: 2 (2%) C/C: 99 (98%)	6 (2.4%) 249 (97.6%)	0.83
A519G	Gln > Gln	Q173Q	A/A: 74 (73%) G/A: 21 (21%) G/G: 6 (6%)	184 (72%) 56 (22%) 15 (6%)	0.97
C612T	Asp > Asp	D204D	C/T: 1 (1%) T/T: 100 (99%)	2 (0.8%) 253 (99.2%)	0.85
C962A	Pro > His	P321H	A/A: 10 (10%) C/A: 44 (44%) C/C: 47 (46%)	19 (7.5%) 113 (44.3%) 123 (48.2%)	0.14

[a]Differences in genotype frequencies between ketosis-prone diabetic subjects and controls of west African origin were tested using the χ^2 test.

Table 2.

Frequency of the R133W variant of PAX4 in west African populations

Population	Genotypes R/R	R/W	W/W	133W Allele Frequency (95% CI)	OR (95% CI)[a] χ^2, *P*-value[b]
West African diabetic subjects Ketosis-prone diabetes[c] (*n* = 101)	70 (69.3%)	27 (26.7%)	4 (4%)	0.173 (0.124, 0.233)	–
Type 2 diabetes[c] (*n* = 106)	90 (84.9%)	16 (15.1%)	0 (0%)	0.076 (0.044, 0.120)	2.57 (1.37, 4.66) $\chi^2 = 9.14$, $P = 0.0025$
Type 1 diabetes[c] (*n* = 41)	33 (82.9%)	8 (19.5%)	0 (0%)	0.110 (0.052, 0.198)	1.94 (0.85, 4.05) $\chi^2 = 2.59$, $P = 0.108$
West African controls Non-diabetic west African controls (*n* = 255)	200 (78.4%)	55 (21.6%)	0 (0%)	0.106 (0.060, 0.162)	1.77 (1.12, 2.79) $\chi^2 = 5.90$, $P = 0.015$
African-American controls (*n* = 100)	86 (86%)	14 (14%)	0 (0%)	0.070 (0.039, 0.115)	2.72 (1.44, 5.16) $\chi^2 = 9.99$, $P = 0.0016$
Caucasian controls (*n* = 200)	200 (100%)	0 (0%)	0 (0%)	–	–

[a] Odds ratios and confidence intervals evaluate the calculated risk of KPD when comparing the frequency of the R133W allele between the ketosisprone diabetic subjects and each diabetic and control groups.

[b] χ^2 test was used to evaluate if the OR in ketosis-prone diabetic subjects compared with each diabetic and control group was significant.

[c] Ketosis-prone diabetic, diabetic and type 1 diabetic subjects are west African natives.

Table 3.

R133W/P321H haplotypes

Allele at R133W	Allele at P321H	Haplotypes frequencies Ketosis-prone diabetes	Controls
R133	P321	0.649583	0.704452
R133	321H	0.170236	0.188126
133W	P321	0.021588	0.002579
133W	321H	0.158592	0.104842

Table 4.

Clinical characteristics of the R37W and R133W variant carriers

	Homozygous R133W (*n* = 4)					Heterozygous R133W (*n* = 27)	Heterozygous R37W (*n* = 1)	Wild-type PAX4 (*n* = 70)	
	Patient 1	Patient 2	Patient 3	Patient 4	Mean ± SD[a]	Mean ± SD	Patient 5	Mean ± SD	*P*-value[b]
Origin	Senegal	Senegal	Ivory Coast	Senegal	–	–	Cameroon	–	–
Family history of diabetes	Father	Unknown	Mother	Father	–	–	Father	–	–
ICA, GAD 65 ab	Negative	Negative	Negative	Negative	–	–	Negative	–	–
Gender (M/F)	M	M	M	M	M	–	M	–	–
Age at onset (years)	47	22	38	20	31.8 ± 12.6	36.9 ± 6.3	39	41.2 ± 8.6	0.04
BMI (kg/m^2) at admission	26.5	16.2	25.4	21.6	22.4 ± 4.6	25.8 ± 4.5	28.7	25.2 ± 5.0	0.29
BMI (kg/m^2) before symptoms	29.1	18.5	28.3	26.5	25.8 ± 4.3	29.0 ± 4.8	30.4	28.6 ± 5.3	0.31
Weight loss (kg)	8	10	8	15	10.3 ± 3.3	9.5 ± 4.9	5.0	10.5 ± 6.6	0.94
Blood glucose (mM)	25	45	28	35	32.2 ± 13.2	26.1 ± 7.4	20	27.8 ± 12.0	0.24
HbA1c (%) at admission	13.8	12.2	14.1	12.5	12.7 ± 1.6	13.5 ± 2.2	11.6	13.7 ± 2.0	0.34
Bicarbonates (mM)	6	14	4	15	13.7 ± 5.7	15.2 ± 7.2	15	15.5 ± 6.5	0.17
Arterial pH	7.15	7.1	7.09	7.12	7.19 ± 0.8	7.19 ± 0.9	7.25	7.20 ± 0.5	0.82
C-peptide at admission (ng/ml)	0.2	0.6	0.4	0	0.3 ± 0.25	0.2 ± 0.35	0.1	0.3 ± 0.21	0.99
Glucagon at admission (pg/ml)[c]	>400	>400	>400	>400	>400	>400	>400	>400	–
Remission of insulin dependence	Yes	Yes	Yes	Yes	–	65%	No	74%	–
Duration of initial	22.5	16	20	14	18.0 ± 3.7	15.8 ± 19	Permanent	11.7 ± 17	0.70
Insulin therapy (weeks)									
Treatment after remission of insulin-dependence	OHA	OHA	OHA	OHA	–	–	Insulin	–	–
HbA1c (%) at Remission	6.6	5.1	6.2	7.3	6.3 ± 0.9	6.0 ± 1.1	8.2	6.1 ± 0.5	0.75
Relapse in insulin dependence (weeks)	No	No	64	20	–	–	–	–	–

[a] Mean + SD for R133W homozygous carriers.

[b] Difference between R133W homozygous carriers and wild-type carriers (*t*-test). Normal HbA1c is <6%. OHA, oral hypoglycemic agent.

[c] Serum glucagon values in non-diabetic individuals: 40–130 pg/ml.

Hum Mol Genet. Author manuscript; available in PMC 2018 September 19.