

# A- $\beta$ - Subtype of Ketosis-Prone Diabetes Is Not Predominantly a Monogenic Diabetic Syndrome

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**OBJECTIVE** — Ketosis-prone diabetes (KPD) is an emerging syndrome that encompasses several distinct phenotypic subgroups that share a predisposition to diabetic ketoacidosis. We investigated whether the A- $\beta$ - subgroup of KPD, characterized by complete insulin dependence, absent  $\beta$ -cell functional reserve, lack of islet cell autoantibodies, and strong family history of type 2 diabetes, represents a monogenic form of diabetes.

**RESEARCH DESIGN AND METHODS** — Over 8 years, 37 patients with an A- $\beta$ - phenotype were identified in our longitudinally followed cohort of KPD patients. Seven genes, including hepatocyte nuclear factor 4A (*HNF4A*), glucokinase (*GCK*), *HNF1A*, pancreas duodenal homeobox 1 (*PDX1*), *HNF1B*, neurogenic differentiation 1 (*NEUROD1*), and *PAX4*, were directly sequenced in all patients. Selected gene regions were also sequenced in healthy, unrelated ethnically matched control subjects, consisting of 84 African American, 96 Caucasian, and 95 Hispanic subjects.

**RESULTS** — The majority (70%) of the A- $\beta$ - KPD patients had no significant causal polymorphisms in either the proximal promoter or coding regions of the seven genes. The combination of six potentially significant low-frequency, heterozygous sequence variants in *HNF-1 $\alpha$*  (A174V or G574S), *PDX1* (putative 5'-untranslated region CCAAT box, P33T, or P239Q), or *PAX4* (R133W) were found in 27% (10/37) of patients, with one additional patient revealing two variants, *PDX1* P33T and *PAX4* R133W. The A174V variant has not been previously reported.

**CONCLUSIONS** — Despite its well-circumscribed, robust, and distinctive phenotype of severe, nonautoimmune-mediated  $\beta$ -cell dysfunction, A- $\beta$ - KPD is most likely not a predominantly monogenic diabetic syndrome. Several A- $\beta$ - KPD patients have low-frequency variants in *HNF1A*, *PDX1*, or *PAX4* genes, which may be of functional significance in their pathophysiology.

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Type 1 and type 2 diabetes are complex, heterogeneous diseases with genetic and environmental components contributing to their pathophysiology, clinical progression, and severity.

Although rapid technological advances in genotyping, such as high-throughput, large-scale, whole-genome approaches, have identified a number of genetic variants linked to both types of diabetes, ma-

ior genetic contributions have been confirmed in relatively small subsets of patients. A significant obstacle to identifying genetic loci that contribute to diabetes is the phenotypic heterogeneity of the disease (1). The lack of specificity in the current classification of diabetes and in clinical criteria that define their “types” has impeded the ability of investigators to circumscribe this diverse illness with sufficient precision in order to make accurate genotype-phenotype correlations.

We and others have identified and prospectively characterized an emerging syndrome that does not fit the current American Diabetes Association (ADA) classification of type 1 and type 2 diabetes (rev. in 2). Termed ketosis-prone diabetes (KPD), this syndrome is characterized by patients who present with diabetic ketoacidosis (DKA), which unequivocally defines the illness and clearly reflects severe  $\beta$ -cell dysfunction as an etiologic factor. Our group has prospectively tested and rigorously validated a classification scheme for KPD that is based on the presence or absence of  $\beta$ -cell autoantibodies (A+ or A-) and the presence or absence of  $\beta$ -cell functional reserve ( $\beta$ + or  $\beta$ -) (3,4). This A $\beta$  system defines KPD patients with high accuracy, distinguishes four phenotypic subgroups, and strongly predicts the natural history of each subgroup with regard to glycemic control, insulin dependence, and  $\beta$ -cell functional reserve (4).

A- $\beta$ - KPD comprises a unique and phenotypically distinct group of patients who have relatively early-onset diabetes and permanent, severe  $\beta$ -cell dysfunction but lack evidence for  $\beta$ -cell autoimmunity. Specifically, they lack circulating autoantibodies to GAD65 or tyrosine phosphatase-like protein (insulinoma-associated protein-2, or IA-2) that are typical of patients with autoimmune type 1 diabetes. Furthermore, the frequencies of class II HLA alleles known to confer susceptibility to autoimmune type 1 diabetes are significantly lower in this subgroup of KPD patients than in the autoimmune-mediated A+ $\beta$ - KPD subgroup (3,5). In our cohort, patients with A- $\beta$ - KPD also have a high frequency (~85%) of

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first-degree relatives with type 2 diabetes, often in multiple generations (3). We hypothesized that A- $\beta$ - KPD patients have a high likelihood of possessing sequence variants in one or more genes associated with  $\beta$ -cell development or regulation of insulin secretion.

Mutations in a number of genes associated with both  $\beta$ -cell development and regulation of insulin secretion have been identified as causes of  $\beta$ -cell dysfunction resulting in diabetes. Maturity-onset diabetes of the young (MODY) is a clinically heterogeneous group of diabetic syndromes characterized by insulin secretory defects, childhood or adolescent onset, and an autosomal dominant inheritance pattern of the disease (6). The six known MODY syndromes (MODY 1 through MODY 6) result from mutations in the following genes: hepatocyte nuclear factor 4A (*HNF4A*), encoding HNF-4 $\alpha$ ; glucokinase (*GCK*); *HNF1A*, encoding HNF-1 $\alpha$ ; pancreas duodenal homeobox 1 (*PDX1*), also known as *IPF1*, *IDX1*, or *STF1*; *HNF1B*, encoding HNF-1 $\beta$ ; and neurogenic differentiation 1 (*NEUROD1*), also known as  $\beta$ -2 ( $\beta$ 2), respectively. A homozygous variant, R133W, in the *PAX4* gene has also been associated with severe  $\beta$ -cell dysfunction in KPD patients of West-African descent (7), likely representing the A- $\beta$ + subgroup of KPD. HNF-4 $\alpha$ , HNF-1 $\alpha$ , *PDX1*, HNF-1 $\beta$ , *NEUROD1*, and *PAX4* form a network of transcription factors in the  $\beta$ -cell that regulates the expression of insulin as well as additional genes involved in glucose transport and metabolism and mitochondrial metabolism (8). The clinical features of the different MODY syndromes vary with the specific genetic etiologies (9).

Here, we set out to examine and characterize genetic variation in minimal promoter, flanking intronic, and exonic regions of *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, and *PAX4* in our A- $\beta$ - KPD patients. We found no significant causal mutation in either the proximal promoter or coding regions of the six MODY or *PAX4* genes, which could be associated with the distinctive diabetic phenotype in the majority of KPD patients. This finding suggests that A- $\beta$ - KPD is predominantly a nonmonogenic diabetic syndrome. Numerous sequence variants were found with an average frequency of 1 in 244 base pairs (bp), and 40% of these were low-frequency variants (i.e., minor allele frequency [MAF] <5%). Comparison of allele frequencies with ethnically matched

control subjects obtained from the Baylor Polymorphism Resource (BPR) identified several low-frequency variants within *HNF1A*, *PDX1*, and *PAX4*, the occurrence of which was greater than fivefold higher in our A- $\beta$ - KPD patient group.

## RESEARCH DESIGN AND METHODS

This study was approved by the institutional review boards for genetic studies of KPD patients at Baylor College of Medicine and the Harris County Hospital District, Houston, Texas, and informed consent was obtained from all subjects. Adult patients admitted to Ben Taub General Hospital with DKA were identified at the time of their hospital stay, recruited to the study, and followed prospectively thereafter as outpatients in a dedicated research clinic between July 1999 and February 2006. DKA was defined by the presence of all of the following: anion gap  $\geq 15$ , blood pH <7.30, serum bicarbonate  $\leq 17$  mmol/L, serum glucose >200 mg/dl, serum ketones  $\geq 5.2$  mmol/L, and urine ketones >13.9 mmol/L. KPD patients were classified as A+ or A- based on the presence or absence of GAD65 or IA-2 autoantibodies, measured in sera by quantitative radioligand binding assays with recent modifications. As described in Maldonado et al. (3), patients were classified as A+ if the autoantibody index for at least one of the autoantibodies exceeded the ethnic-specific 99th percentile or A- if the index for all antibodies tested were below the 99th percentile. Patients were classified as  $\beta$ + or  $\beta$ - based on the presence or absence of  $\beta$ -cell functional reserve, measured by fasting serum C-peptide concentration and C-peptide response to glucagon within 1 week after resolution of ketoacidosis and follow-up visits at 6 and 12 months (3). Only patients with the A- $\beta$ - phenotype of KPD ( $n = 37$ ) were investigated in this study.

## Control group

Genomic DNA samples were obtained from established lymphoblast cell lines from the BPR collection. Healthy adults, recruited in Houston, Texas, were comprised of three, self-declared ethnic groups (African American, Caucasian, and Hispanic). Blood samples were assigned an alphanumeric code, and all identifying information was removed. For this study, PCR and direct DNA sequencing were performed on selected regions from 84 African American, 96 Caucasian, and 95 Hispanic DNA samples.

## Molecular biology

Complete experimental procedures used in this work are available in the online appendix (available at <http://care.diabetesjournals.org/cgi/content/full/dc08-1529/DC1>), including Tables A1-A4.

## RESULTS

The clinical, immunologic, and biochemical features of 37 unrelated A- $\beta$ - KPD patients in this study were found to be similar to those described in the original phenotypic characterization of this syndrome (3). They were 46% Hispanic, 38% African American, and 16% Caucasian and had relatively early-onset diabetes (mean age at diagnosis  $27.8 \pm 12.7$  years), with a slight male predominant sex ratio of 1.6 to 1. The patients were lean (mean BMI  $23.5 \pm 2.7$  kg/m<sup>2</sup>), with a high frequency of family history of type 2 diabetes in first-degree relatives (84%). Noncompliance with insulin treatment was the primary reason for the index presentation with DKA, with only  $\sim 14\%$  of patients presenting with new-onset diabetes at the time of the index episode of DKA. Indexes of  $\beta$ -cell secretory function (fasting C-peptide, glucagon stimulation test using area under the curve of C-peptide, and homeostasis model assessment 2 of  $\beta$ -cell function) showed low  $\beta$ -cell functional reserve, both at the time of the initial DKA episode and on subsequent follow-up after 12 months. The patients were insulin sensitive as measured by the homeostasis model assessment 2 of insulin resistance index. Their glycemic control was poor at baseline and improved (without attaining ADA goals) after 12 months of treatment with insulin (Table 1). None of the A- $\beta$ - KPD patients were able to discontinue insulin therapy without promptly developing ketosis.

The proximal promoter, exons, and flanking intronic regions of *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, and *PAX4* were characterized by DNA sequencing of PCR amplicons for the 37 A- $\beta$ - KPD patients. The seven genes, totaling >24 kilobase pairs (kb), resulted in the identification of 99 sequence variants for the A- $\beta$ - KPD patients (see online appendix Tables A2a and A2b). Forty percent (40 of 99) of the identified variants had a MAF of <5% (see online appendix Table A3). The distribution of sequence variants observed in the intronic and untranslated regions (UTRs) was approximately four- to sevenfold higher than that for the proximal pro-

**Table 1—Clinical characteristics of A-β- KPD patients**

n	37
Demographic	
Age (years)	36.7 ± 12.8
Age at diagnosis of diabetes (years)	27.8 ± 12.7
Duration of diabetes (years)*	6.5 ± 8.7
BMI (kg/m <sup>2</sup> )	23.5 ± 2.7
Male:female	1.63:1
First-degree relative with diabetes (%)	83.8
Glycemic indices	
A1C at index DKA (%)	14.4 ± 2.5
A1C at 12 months (%)	11.4 ± 2.1
β-Cell function indices	
Fasting C-peptide at baseline (ng/ml)	0.28 ± 0.28
Fasting C-peptide at 12 months (ng/ml)	0.11 ± 0.1
GST-AUC C-peptide at baseline (ng/ml)	3.26 ± 3.2
GST-AUC C-peptide at 6 months (ng/ml)	0.9 ± 0
HOMA2-%β at baseline	5.7 ± 4.8
HOMA2-%β at 12 months	6.5 ± 13.7
Insulin therapy discontinued at 12 months	0
Insulin sensitivity	
HOMA2-IR at baseline	0.27 ± 0.29
HOMA2-IR at 12 months	0.17 ± 0.19
Precipitant of index DKA (%)	
Noncompliance	67.6
New onset without clear precipitant	13.5
Acute illness	18.9

Data are expressed as mean ± SD for continuous variables and percentage for categorical variables. \*Five patients had new-onset diabetes diagnosed at the time of presentation with the index DKA. GST area under the curve (AUC) was calculated using the trapezoidal method. HOMA-IR and HOMA2-%B were calculated using a computer program available at <http://www.dtu.ox.ac.uk/>.

moter and exon regions (see online appendix Table A4). The average frequency of sequence variants found in the seven gene regions was 1 in 244 bp.

To focus on sequence variants that might play a functional role in the pathophysiology of severe β-cell failure in A-β- KPD, we selected those that had an MAF of <10% in at least one of the

ethnic groups and resulted in a change in an amino acid residue or a sequence variant in either a known DNA binding element or within the proximal promoter region (Table 2). Seven missense variants, one CCAAT box variant, and one proximal promoter variant were identified and further studied. They were HNF-1α A174V; HNF-1α G574S; PDX1 P33T;

PDX1 P239Q; GCK A11T; HNF-1β N228K; PAX4 R133W; PDX1 (-18 C→T), which we term a putative 5'-UTR CCAAT box variant; and HNF-4α P2 promoter. Several of these variants have been associated with MODY syndromes, type 2 diabetes, or KPD, including HNF-1α G574S (10–12), PDX1 P33T (13), PDX1 P239Q (14), and PAX4 R133W (7), while HNF-1α A174V, HNF-1β N228K, and the HNF4A P2 promoter variants appear novel to this study.

PCR amplicons containing these variants were sequenced in ethnically matched control subjects from the BPR collection to assess allele frequencies within ethnic groups. The allele frequencies for GCK A11T, HNF-1β N228K, and HNF4A P2 promoter variants showed only a modest increase to no difference in A-β- KPD case subjects compared with that of the ethnically matched BPR control subjects. Six variants, however, in either HNF-1α (i.e., A174V or G574S), PDX1 (i.e., putative 5'-UTR CCAAT box, P33T, or P239Q), or PAX4 R133W showed a fivefold or higher allele frequency difference in the A-β- KPD group compared with that of ethnically matched control subjects (Table 2). Although the small number of A-β- KPD patients in this analysis made statistical comparisons unreliable despite the apparent difference in allele frequencies, several observations suggest possible etiological roles for these six variants. HNF-1α G574S and putative PDX1 5'-UTR CCAAT box variants were found in both Hispanic and African American A-β- KPD patient groups, and the PAX4 R133W variant was found in both Caucasian and African American

**Table 2—Sequence variants enriched in A-β- KPD patients**

Exon	Patient ID	Variant	dbSNP	KPD	BPR	Fold difference
HIS group						
HNF4AP2	KPD0115	50bp 5'; G→C		2.9% (1/34)	2.6% (5/190)	1.1
HNF1αEx2	KPD0203	A174V; C→T		2.9% (1/34)	0.0% (0/190)	>5.6
HNF1αEx9	KPD0110	G574S; G→A	rs1169305	2.9% (1/34)	0.5% (1/190)	5.6
HNF1βEx3	KPD0006	N228K; C→G		2.9% (1/34)	3.2% (6/190)	0.9
PDX1Ex1	KPD0216	CCAAT; C→T		2.9% (1/34)	0.5% (1/190)	5.6
AFA group						
GCKEx1a	KPD0123	A11T; G→A		3.8% (1/26)	1.8% (3/168)	2.2
HNF1αEx9	KPD0102; KPD0119	G574S; G→A	rs1169305	7.1% (2/28)	1.2% (2/168)	6.0
PDX1Ex1	KPD0069; KPD0123	CCAAT; C→T		7.1% (2/28)	1.2% (2/168)	6.0
PAX4Ex3	KPD0014; KPD0208	R133W; C→T	rs2233578	7.1% (2/28)	4.2% (7/168)	1.7
CAU group						
PDX1Ex1	KPD0193	P33T; C→A		8.3% (1/12)	0.0% (0/192)	>15.8
PDX1Ex2	KPD0053	P239Q; C→A		8.3% (1/12)	0.5% (1/190)	15.8
PAX4Ex3	KPD0193	R133W; C→T	rs2233578	8.3% (1/12)	0.0% (0/192)	>15.8

KPD groups. Neither the HNF-1 $\alpha$  A174V nor the PDX1 P33T variants were found in their respective ethnic control groups, nor was the PAX4 R133W variant found in the Caucasian BPR group.

**CONCLUSIONS**— In this study, we completely sequenced and analyzed seven genes for variants that might be causative for a monogenic pathophysiology in KPD patients with the carefully circumscribed A- $\beta$ - phenotype of severe, relatively early-onset, nonimmunologic  $\beta$ -cell failure and proneness to ketoacidosis. We found no significant evidence for a role of *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, and *PAX4* mutations in the majority of the A- $\beta$ - KPD patients. Hence, A- $\beta$ - KPD as a whole is unlikely to represent a monogenic syndrome, despite the high frequency of a family history of type 2 diabetes (~85%) in multiple generations and its strong link to  $\beta$ -cell dysfunction. Several potentially significant genetic variants, however, were identified within either *HNF1A*, *PDX1*, and/or *PAX4* that in aggregate represented 30% of case subjects. These variants were located within or near the functional domains of the HNF-1 $\alpha$ , PDX1, and PAX4 proteins or a regulatory region of the *PDX1* gene. In vitro studies (7,12–14) suggest that some of the previously reported mutant variants reduce the production of insulin. Hence, further study of these variants, including their functional effects and the inheritance patterns in the families of the affected patients, are warranted and underway.

Two variants, HNF-1 $\alpha$  G574S and the *PDX1* putative 5'-UTR CCAAT box, were identified in both African American and Hispanic A- $\beta$ - KPD patients. Several studies have identified the HNF-1 $\alpha$  G574S variant exclusively in African American (10,15) or African populations (11,16). HNF-1 $\alpha$  G574S has been associated with "atypical" diabetes in African American (10) and African (11) populations, although this association was not confirmed in other studies (15,16). Recently, Navalón-García et al. found the HNF-1 $\alpha$  G574S variant in two unrelated Mexican type 2 diabetic patients with end-stage renal disease who had no known African ancestry but not in 66 unrelated, nondiabetic Mexican control subjects (12). Cockburn et al. (17) identified the -18 C $\rightarrow$ T variant in the *PDX1* gene, referred to here as a putative 5'-UTR CCAAT box, in one type 2 diabetic patient designated as having mixed African

and East Indian ancestry. This variant was not found in either 60 unrelated nondiabetic Indo-Trinidadian or 60 unrelated nondiabetic Afro-Trinidadian subjects (17). Here, the 5'-UTR CCAAT promoter sequence variant was found downstream of the putative initiation start site in three A- $\beta$ - KPD patients. This places the CCAAT box within the 5'-UTR of the *PDX1* gene; such downstream boxes have been shown to be a functional, regulatory elements (18). The relevance of the recently evolved 5'-UTR CCAAT box in *PDX1* is unknown, although the presence of multiple enhancers in the proximal promoter region and 5'-UTR is congruent with the central role of *PDX1* in the regulation of  $\beta$ -cell development and insulin secretion. Studies are underway to investigate the role of sequence variation within the putative 5'-UTR CCAAT box and its effect on *PDX1* gene expression. Our findings that both HNF-1 $\alpha$  G574S and the *PDX1* putative 5'-UTR CCAAT box variants were identified in African American and Hispanic A- $\beta$ - KPD patients provides direct evidence that these low-frequency variants may not be restricted to specific ethnic groups.

Mauvais-Jarvis et al. (7) reported an association of the homozygous PAX4 W133 variant with KPD in West-African patients. They demonstrated that glucagon-stimulated insulin secretion was markedly lower in four patients who were homozygous for the mutant allele compared with those who were heterozygous ( $n = 11$ ) or homozygous ( $n = 18$ ) for the wild-type allele. Based on our A $\beta$  classification system (3,4), we would assign this West-African cohort to the subgroup of A- $\beta$ + KPD, rather than the subgroup of A- $\beta$ - KPD whom we investigated in the present study. A- $\beta$ - KPD patients are distinct from A- $\beta$ + KPD patients, being predominantly lean with early-onset of diabetes and lacking any  $\beta$ -cell functional reserve. As a group, A- $\beta$ - KPD patients show no recovery of insulin secretory response to glucagon following the index DKA, hence it is not possible for us to ascertain whether presence of the PAX4 R133W variant is specifically associated with markedly reduced  $\beta$ -cell function. Similar to the Mauvais-Jarvis et al. (7) study ( $n = 200$ ), we did not find the W133 variant in any of our 96 Caucasian BPR control subjects. We did, however, find one Caucasian A- $\beta$ - KPD patient as heterozygous for the R133W variant. The differences in KPD subgroups make it difficult to suggest that the PAX4 R133W

variant is either pathogenetically significant or ethnically restricted in patients with the A- $\beta$ - phenotype of KPD.

The remaining three variants, however, were found in specific ethnic groups. The HNF-1 $\alpha$  A174V variant, which has not been reported previously, was found in one Hispanic A- $\beta$ - KPD patient. The A174V variant is located within the B-domain, which confers DNA binding sequence specificity for HNF-1 $\alpha$  (19). Encoded variation within the B-domain could impair the ability of HNF-1 $\alpha$  to properly bind and regulate downstream target genes. Family-based studies, which could determine the role of this variant in KPD, could not be performed given that both parents are deceased. Both PDX1 coding variants were found individually in two unrelated Caucasian A- $\beta$ - KPD patients. The PDX1 P33T variant has been associated with type 2 diabetes and increased susceptibility to gestational diabetes (13), while PDX1 P239Q has been identified in two families with early-onset type 2 disease (14).

Recently, Murphy et al. (9) proposed a classification scheme for monogenic diabetes resulting from mutations that cause  $\beta$ -cell dysfunction, a scheme that is based on specific genetic diagnoses and points to specific therapeutic interventions. Among these monogenic syndromes, the most common genetic mutations among individuals with familial, young-onset diabetes (without extrapancreatic features) are in the *HNF1A* gene. Here, we identified only two low-frequency variants in the *HNF1A* gene in an otherwise well-defined KPD cohort with early, complete, nonautoimmune  $\beta$ -cell dysfunction. While it is plausible that KPD is a monogenic syndrome and we incorrectly chose its corresponding gene(s) to sequence, it is more likely that A- $\beta$ - KPD is a complex genetic syndrome. Thus, this study highlights the difficulty in traditional candidate gene approaches using conventional sequencing methods. The search for genetic etiologies of KPD may not come from the analysis of a handful of candidate genes but rather a more comprehensive and systematic approach. This could be accomplished by expanding the set to hundreds of genes involving numerous pathways such as metabolic and proliferative networks (20) that capture the KPD phenotype by using next-generation sequencing technologies (21). Work is underway to explore this approach.



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