Does the Aspartic Acid to Asparagine Substitution at Position 76 in the Pancreas Duodenum Homeobox Gene (*PDX1*) Cause Late-Onset Type 2 Diabetes?

STEVEN C. ELBEIN, MD[1,2]
MOHAMMAD A. KARIM, MD, PHD[2]

OBJECTIVE — Considerable data support an inherited defect in insulin secretion as one component of type 2 diabetes. Coding variants of the pancreas duodenum homeobox gene (*PDX1*) were proposed to predispose late-onset type 2 diabetes and to decrease transactivation in vitro. We tested the hypothesis that the Asp76Asn (D76N) variant that was identified in several populations predisposed type 2 diabetes and reduced insulin secretion.

RESEARCH DESIGN AND METHODS — We performed a case-control study in 191 control subjects and 190 individuals with type 2 diabetes, all of European descent, then characterized the D76N variant in 704 members of 68 families. We compared the phenotypic characteristics of those with and without the variant by diagnostic status and determined the insulin secretory response to intravenous glucose and tolbutamide among nondiabetic family members.

RESULTS — D76N was not associated with type 2 diabetes, either in our population or when all reported studies in Caucasians were combined. D76N did not segregate with diabetes among the families examined. Among D76N carriers, nondiabetic individuals had a lower waist-to-hip ratio and a trend to lower BMI than their diabetic counterparts. Diabetic carriers of D76N were significantly leaner by BMI ($P = 0.012$) and tended to be younger than diabetic individuals with the D/D genotype. However, insulin secretion in response to oral and intravenous glucose challenge and to intravenous tolbutamide was not reduced in D76N carriers.

CONCLUSIONS — The D76N variant of *PDX1* does not significantly alter insulin secretion or act as a high-risk susceptibility allele for late-onset type 2 diabetes as proposed previously, although we cannot exclude a minor role in increasing risk of diabetes.

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Type 2 diabetes results from combined defects of insulin action (insulin resistance) and impaired insulin secretion (1). In work from our laboratory (2) and others (3,4), indexes of pancreatic β-cell function were strongly heritable, suggesting that genetic factors play an important role in the response of the pancreas to reduced insulin sensitivity (5). Although some common variants may impact this ability among Caucasian families (6,7), most of the genetic propensity to impaired β-cell compensation and to genetically programmed β-cell failure remains undefined.

The failure of the pancreatic β-cell to increase insulin secretion in response to increased demands might result from functional defects (failure of increased insulin biosynthesis and secretion) or defects in β-cell mass (inherited differences in β-cell number or an impaired increase in β-cell mass in response to insulin resistance). Both processes may be controlled by β-cell transcription factors. Defects in hepatocyte nuclear factors 1α (*TCF1*), 4α, and 1β, and in the pancreas duodenum homeobox gene 1 (*PDX1*) all cause autosomal-dominant, insulin-deficient diabetes in humans (5,8,9). Our laboratory previously showed that genetic variants in *TCF1* were present in up to 10% of our cohort of Caucasian familial type 2 diabetic kindreds (10) but were not an important cause of African-American type 2 diabetes (11). Because *PDX1* plays a central role in the development of pancreatic β-cells and preservation of adult β-cell mass, is regulated by transcription factors in the hepatocyte nuclear factor pathway, and in turn, regulates key β-cell genes including the insulin gene, *GLUT2*, and glucokinase (12), this gene has been the focus of considerable attention. Up to 5 or 6% of typical, late-onset type 2 diabetes has been attributed to milder mutations of the *PDX1* gene in French (13) and English families (14), but only a single variant, Asp76Asn (D76N), was common to both studies. This variant showed a 30–40% reduction in binding or transactivation of the insulin gene promoter (13,14), but subsequent studies by Hansen et al. (15), Reis et al. (16), and Silver et al. (17) failed to confirm either the frequency of the previously described *PDX1* variants including D76N or the association with type 2

From the [1]Central Arkansas Veterans Healthcare System, Little Rock, Arkansas; and the [2]University of Arkansas for Medical Sciences, Little Rock, Arkansas.

Address correspondence and reprint requests to Steven C. Elbein, MD, Professor of Medicine, University of Arkansas for Medical Sciences, Endocrinology 111J-1/LR, JLM VA Hospital, 4300 West 7th St., Little Rock, AR 72205. E-mail: elbeinstevenc@uams.edu.

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Abbreviations: AIR_g, acute insulin response to glucose; FSIGT, frequently sampled intravenous glucose tolerance test; IGT, impaired glucose tolerance.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

diabetes. Thus, the role of *PDX1* variants in type 2 diabetes is unclear.

We sought to further examine the hypothesis that the most commonly described *PDX1* mutation, D76N, substantially increased the risk of type 2 diabetes and decreased insulin secretion in nondiabetic individuals. We tested this variant in both case-control and family-based studies from a population of European ancestry.

RESEARCH DESIGN AND METHODS

The case-control study was conducted on 191 unrelated control subjects and included 119 individuals of northern European ancestry ascertained in Utah. Most of these individuals were spouses of families ascertained for multiple diabetic siblings. An additional 72 control subjects were ascertained in Arkansas for mixed Caucasian ancestry. Control subjects underwent a standard 75-g oral glucose tolerance test where possible or had fasting glucose levels <5.6 mmol/l (100 mg/dl) if no glucose tolerance test was available. They had no known history of diabetes in a first-degree relative. Case subjects (190 individuals with type 2 diabetes) included 69 unrelated members of previously ascertained families (18), 1 member of a similarly ascertained family from Arkansas, and 120 individuals ascertained in Utah for type 2 diabetes with onset before 65 years of age and family history of type 2 diabetes. Family studies were conducted on 704 members of 68 families of northern European ancestry that were ascertained for two siblings with type 2 diabetes with onset 65 years of age. The families included 292 individuals counted as affected, as defined previously (18), based on previously diagnosed diabetes treated with medication, diabetic oral glucose tolerance test, or World Health Organization criteria for impaired glucose tolerance (IGT) before 45 years of age. An additional 384 individuals were nondiabetic based on oral glucose tolerance tests. Individuals with type 1 diabetes or who had IGT beyond age 45 years or in whom the diagnosis was uncertain were considered to be of unknown diagnosis (28 individuals). Of those individuals who were not diabetic, 113 family members and 4 unrelated individuals had undergone tolbutamide-modified, rapid frequently sampled intravenous glucose tolerance tests (FSIGTs) for measurement of insulin sensitivity and acute insulin response to glucose (AIR_g), as described in detail elsewhere (2,19), and were also genotyped for the D76N variant. All subjects provided informed consent under protocols approved by the University of Utah Institutional Review Board or the University of Arkansas for Medical Sciences Human Research Advisory Committee.

Genotyping

The D76N results from a change of G (guanine) to A (adenine). We developed a Pyrosequencing assay (Pyrosequencing, Uppsala, Sweden) using the PSQ96 for detection. Amplification primers were CCCCGTACGAGGTGCC (forward) and CGGGAGGTGGTGGTGAAGGT (reverse). In addition, to label the reverse strand, we included a universal "tail" sequence on the 5′ end (TCTGCTGCTCC GGTTCATAGATT), which annealed to a primer of the same sequence with a 5′ biotinylated primer to permit purification using Sepharose beads. Enzymatic amplification was conducted for 60 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Sequencing was performed with the primer ACGAGGTGCCCCCACTCGC. Results were scored automatically using Pyrosequencing software. Duplicate samples showed no discrepancies between calls.

Statistical analysis

Phenotypic measures of individuals carrying the N allele were compared with individuals homozygous for the D allele using the nonparametric Mann-Whitney *U* test or independent Student's *t* test on natural logarithmically transformed values. Because of the small sample size, we performed the analysis both with and without correction for family membership to minimize the number of covariates. Insulin sensitivity (S_i) was calculated from the glucose and insulin data using the minimal model (20) as described previously (2). Insulin secretion was estimated from the AIR_g, the mean insulin response above baseline from 2 to 10 min after the intravenous glucose bolus. Insulin response to tolbutamide was calculated as the mean insulin response above baseline from 22 to 30 min, thus corresponding to 2–10 min after tolbutamide. Insulin secretion was corrected for prevailing insulin sensitivity using the disposition index (S_i*AIR_g), which we have shown to be heritable in these families (2).

Analysis of pooled results from all available studies was performed first by adding all Caucasian individuals without regard to study site; the difference in allelic frequency between case and control subjects was tested using Fisher's exact test. To properly account for differences between studies, we also performed a Mantel-Haenzel test for association. To overcome problems of empty cells, we added 0.5 to each cell in the table for studies with empty cells before calculation, and we removed the studies without both case and control populations.

RESULTS

We identified 3 of 191 (1.57%) carriers among control subjects and 2 of 190 (1.05%) carriers among diabetic individuals in our case-control study; thus, the allele frequency was 0.78% in control subjects and 0.53% among diabetic individuals. Nondiabetic carriers ranged in age from 29 to 50 years and in BMI from 19.7 to 38.0 kg/m^2. The two diabetic individuals had age at diagnosis of diabetes of 55 years (BMI 26.6 kg/m^2) and 64 years (BMI 25.3 kg/m^2). Thus, diabetic individuals were not characterized by early onset of diabetes. Nondiabetic carriers were somewhat younger than the mean age of diabetes onset in this population (52.2 ± 12.4 years) but were similarly obese (mean BMI among 257 diabetic individuals 30.3 ± 9.9 kg/m^2).

The D76N variant segregated in 5 of 68 high-risk families tested (7.3%) and was detected in a total of 16 of 723 people tested. Both diabetic individuals detected in the case-control study were also members of diabetic families. As was reported previously by McFarlane et al. (14) and Hani et al. (13), D76N did not segregate with type 2 diabetes in families (Fig. 1). Among the five families in which the N allele was present in at least one member, we typed 72 individuals, including 23 diabetic family members, 12 individuals with IGT, and 37 individuals with normal glucose tolerance tests (35 individuals) or normal fasting glucose (2 individuals). Among these families, only 6 of 23 diabetic members carried the N allele (26%), 3 of 12 IGT individuals carried the N allele (25%), and 7 of 37 individuals without diabetes carried the N allele (18.9%). An alternative means to judge the significance of the D76N variant among families is to examine sharing among diabetic siblings. The five extended pedigrees in which the D76N variant was detected in-

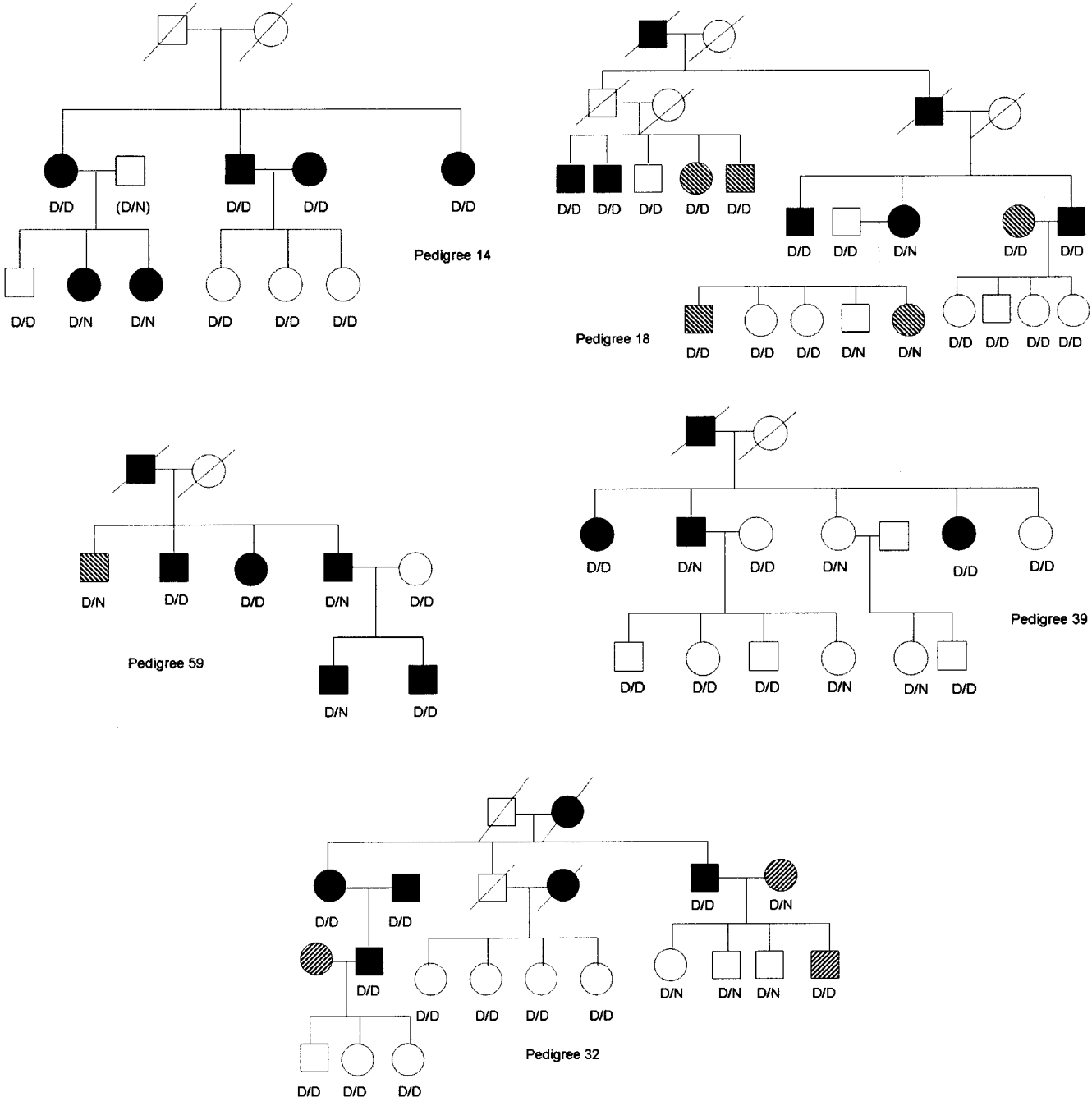

Figure 1—*Pedigree drawings of the five families in which the D76N variant was found. Genotypes are shown below the symbols. Black symbols represent type 2 diabetes, white symbols represent euglycemia (normal), and shaded symbols represent IGT.*

cluded a total of 17 affected sibpairs, only 1 of which shared the D76N variant. Thus, even among those families in which D76N was present, most type 2 diabetes susceptibility could not be attributed to *PDX1*. Indeed, in two of five pedigrees (Fig. 1, Families 32 and 14), the N allele was introduced by a nondiabetic spouse in the second generation.

Table 1 shows the phenotypic characterization of individuals by diagnosis and genotype for members of all families studied. Among nondiabetic family members, individuals heterozygous for D76N were slightly younger and less obese by both BMI and waist-to-hip ratio (a measure of central adiposity), but only waist-to-hip ratio was borderline significant ($P = 0.05$). No measure of insulin or insulin secretion (insulinogenic index) differed between the two genotypes, including an index derived in our laboratory based on the fasting insulin and change in insulin to 60 min that correlates well with the FSIGT-based disposition index (21). Restricting our comparison of carriers and noncarriers to members of families in which the mutation segregated showed the same results; neither insulin levels nor measures of insulin secretion differed be-

tween family members who carried the D76N variant and those with the D/D genotype (data not shown). The same conclusion was reached with individuals who were classified as IGT (Table 1). Among diabetic individuals, carriers of the N allele were significantly leaner (BMI, Table 1; $P = 0.012$) and showed a trend to earlier age of onset. These trends remained when N carriers were compared with diabetic individuals from the same families who carried only the D allele (age at diagnosis 48.7 ± 9.8 vs. 56.4 ± 13.2 years, $P = 0.05$; BMI 25.7 ± 1.6 vs. 29.4 ± 3.9 kg/m^2, $P = 0.02$).

Five family members who had the D76N variant underwent FSIGT for estimation of insulin secretion based on the insulin response to glucose and tolbutamide and insulin sensitivity using the minimal model. Although a trend to decreased insulin secretion in response to both glucose and tolbutamide was observed, none of the differences approached statistical significance (Table 2). These conclusions were the same when we compared the 5 D/N carriers to 13 D/D members of the same families or when a mixed effects regression model was used with BMI, age, sex, family membership, and diagnosis (IGT or normal), included as covariates along with genotype, and with disposition index, AIR_g, or acute insulin response to tolbutamide as measures of insulin secretion ($P > 0.1$ for all).

Given the low frequency of the D76N variant, we examined all published reports to determine the frequency in case and control subjects (Table 3). We included a preliminary report of Brittman et al. (22) as well as the data from the present study. For the study of Hani et al. (13), the number of D76N carriers was calculated from the published allele frequency and the sample size. If all subjects were pooled (Table 3), the frequency of the D76N allele did not differ significantly in the combined 1,466 case (0.68%) and the 1,475 control subjects (0.41%; $P = 0.110$ for the test of allelic association, odds ratio 1.70, 95% CI 0.83–3.48). Using a Mantel-Haenzel test of the odds ratios (ORs) to adjust for study site for the seven studies in which an OR could be calculated (excluding Hansen et al. [15], for which no control group was reported) provided similar results but borderline significance (OR 2.185, 95% CI 1.074–4.445, $P = 0.039$), thus providing some support for an association.

Table 1—*Phenotypic characteristics of D/D homozygotes and D/N heterozygotes by diagnosis*

Diagnosis	Genotype	*n*	Age (years)	Age at diagnosis (years)	BMI (kg/m^2)	Waist-to-hip ratio	Fasting glucose (mmol/l)	60-min glucose (mmol/l)	120-min glucose (mmol/l)	Fasting insulin (pmol/l)	60-min insulin (pmol/l)	Δ insulin/Δ glucose
Normal												
	D/D	366	41.0 ± 14.4	—	27.8 ± 6.1	0.906 ± 0.097*	4.82 ± 0.58	7.57 ± 2.31	5.58 ± 1.39	73 ± 56	507 ± 412	0.96 ± 5.18
	D/N	7	35.8 ± 12.0	—	26.7 ± 5.1	0.831 ± 0.090*	4.55 ± 0.25	7.59 ± 1.16	5.57 ± 1.31	57 ± 33	381 ± 259	0.96 ± 0.60
IGT												
	D/D	81	54.5 ± 13.7	—	28.5 ± 5.7	0.990 ± 0.270	4.99 ± 0.67	10.22 ± 2.01	9.29 ± 0.90	82 ± 76	500 ± 318	0.78 ± 0.53
	D/N	3	60.2 ± 23.9	—	29.2 ± 2.0	0.916 ± 0.122	5.36 ± 1.18	9.41 ± 0.53	9.31 ± 1.18	95 ± 62	449 ± 374	0.75 ± 0.54
Diabetes												
	D/D	245	62.0 ± 12.3	52.3 ± 12.6	30.8 ± 5.7†	0.981 ± 0.081	—	—	—	—	—	—
	D/N	6	57.6 ± 9.7	48.7 ± 9.8	25.7 ± 1.6†	0.963 ± 0.032	—	—	—	—	—	—

Data are means ± SD. Characteristics of individuals carrying the N allele (D/N) and wild-type individuals (D/D) are shown for the full pedigree study. IGT was defined by World Health Organization criteria, normal individuals had normal glucose tolerance tests, and diabetes represents type 2 diabetes by treatment or diabetic glucose tolerance test, using World Health Organization criteria. Δ insulin/Δ glucose, change in insulin from basal to 60 min divided by the change in glucose over the same time. *$P = 0.05$, †$P = 0.012$.

Table 2—*Insulin sensitivity and secretion in individuals with the D/D and D/N genotypes*

Genotype	*n*	BMI (kg/m^2)	$S_i \times 10^{-5}$ min^{-1}/pmol/l	AIR_g (pmol/l)	Disposition index	Acute insulin response to tolbutamide (pmol/l)
D/D	112	27.4 (22.3–33.6)	6.56 (2.621–6.4)	175 (83–369)	0.0114 (0.0032–0.0403)	487 (229–1034)
D/N	5	27.5 (21.6–35.0)	5.08 (1.81–14.3)	133 (38–473)	0.0107 (0.0071–0.0161)	296 (117–751)

Data are geometric means (95% CI). Characteristics of nondiabetic individuals by genotype at D76N. No differences between D/N carriers and all others with the D/D genotype are significant.

CONCLUSIONS — Data from our laboratory and others suggest that defects in insulin secretion are highly heritable and correlate well with type 2 diabetes within high-risk families (2,3). *PDX1*, which is central to the transcription of key β-cell genes, including glucokinase, *GLUT2*, and insulin, as well as pivotal to the development and maintenance of β-cell mass, is an attractive candidate for defective insulin secretion. A key role for this gene is supported by studies of humans and mice lacking *PDX1* activity (8,23). Based on these data, multiple investigators have looked for *PDX1* mutations in early-onset diabetes (maturity-onset diabetes of the young) and typical (late)-onset type 2 diabetes (13–17,24, 25). *PDX1* mutations rarely cause early-onset, dominant diabetes, but six missense variants and one CCG insertion were identified in English (14), French (13), and Scandinavian (25) populations. Initial reports suggested that together these variants were more common among diabetic than control individuals and that in aggregate *PDX1*, variants might account for up to 6% of common type 2 diabetes (13), with an OR of 3 (14) to 11 (13). Of these variants, only the D76N variant has appeared in multiple studies and multiple populations. However, the impact of D76N on *PDX1* transactivation activity (13,14), the prevalence (16,17), and the functional importance (15) (Table 3) have been uncertain.

We undertook the present study to reexamine this variant using a combination of approaches, including a case-control study, a family-based analysis, and an examination of careful phenotypic measures of insulin secretion in nondiabetic family members with and without the variant. In general, our data do not support an important role for the D76N variant in susceptibility to diabetes. We were unable to reproduce the high ORs reported by Hani et al. (13) and Mcfarlane et al. (14), either within our population or in the combined analysis of all available studies. When all available data from eight studies with more than 1,400 total case subjects and 1,400 control subjects were examined, the frequency of the D76N carriers was <1.5% and did not differ between case and control subjects. If the allele frequencies were 1% in case subjects and 0.5% in control subjects (approximately the number observed in all studies combined; OR 2.0), we would have 61% power to detect a difference at $\alpha = 0.05$, but the power increases to 97% for an OR of 3.0 (1.5% in case subjects, 0.5% in control subjects). Thus, our results seem to be inconsistent with an OR of ≥3. Furthermore, the trend to an association (Table 3) is driven primarily by the very high frequency of the N76 allele (2.3%) observed in the study of Hani et al. (13), which is double that observed by Macfarlane et al. (14) and over threefold higher than the average across all cases. Additional evidence against a major role for D76N comes from the lack of segregation of D76N with type 2 diabetes in families (13,14) (Fig. 1), the lack of sharing among affected sibpairs in our study, and our inability to replicate the findings of Hani et al. (13) that D76N reduced insulin response to secretagogues, whether measured using the oral glucose tolerance test or measures of insulin secretion derived from the FSIGT. These data are consistent with the failure of Hansen et al. (15) to demonstrate reduced transactivation in vitro.

Our study was clearly limited by the unexpectedly low frequency of the D76N variant, despite testing of families that were comparable in size and ascertainment to those studied by Hani et al. (13). In contrast to the high OR previously reported for D76N, the OR of the three well-replicated diabetes genes, calpain 10, the P12A variant of peroxisome proliferator–activated receptor-γ, or the E27K variant of the β-cell potassium channel, are all in the range of 1.2–1.4 (26). Despite the much higher allelic fre-

Table 3—*Summary of all reported studies of D76N*

Study	Case subjects	Control subjects	Reference no.
Macfarlane et al., England	5 of 206	7 of 675	14
Hani et al., France	9 of 192	1 of 231	13
Reis et al., France	0 of 296	0 of 147	16
Hansen et al., Denmark	1 of 200	—	15
Weng et al., Botnia (Finland)	2 of 183	0 of 92	25
Silver and Shetty., U.S., Caucasian	0 of 110	1 of 107	17
Brittman et al., Italy	1 of 89	0 of 50	22
Elbein and Karim., Utah, Caucasian	2 of 190	3 of 191	—
Total	20 of 1,466 (1.36)	12 of 1,475 (0.81)	—

Data show the number of individuals who carry the N allele in each study. The frequency (percentage) of heterozygous individuals for all combined studies is shown in parentheses for case and control subjects.

quency of these variants, the effects on diabetes risk are difficult to detect without very large populations. Because of its low prevalence, even an effect of D76N as great as an OR of 2.0 would be very difficult to detect in populations of fewer than 3,000 case subjects and 3,000 control subjects. The power to detect a small impact on insulin secretion is thus very low in the population sizes that are practical to characterize for insulin response to intravenous glucose or tolbutamide. However, if the effect of D76N is indeed this small, it is unlikely to have a significant impact on the health of an individual patient or much importance in clinical settings.

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