Brief Genetics Report

Cosegregation of MIDD and MODY in a Pedigree

Functional and Clinical Consequences

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The aim of this study was characterization of a family carrying two mutations known to cause monogenic forms of diabetes, the M626K mutation in the *HNF1α* gene (MODY3) and the A3243G in mtDNA. β-Cell function and insulin sensitivity were assessed with the Botnia clamp. Heteroplasmy of the A3243G mutation and variants in type 2 diabetes susceptibility genes were determined, and transcriptional activity, DNA binding, and subcellular localization of mutated *HNF1α* were studied. Thirteen family members carried the mutation in mtDNA; 6 of them also had the M626K mutation, whereas none had only the M626K mutation. The protective Ala12 allele in peroxisome proliferator–activated receptor (PPAR)γ was present in two nondiabetic individuals. Carriers of both mtDNA and *HNF1α* mutations showed an earlier age at onset of diabetes than carriers of only the mtDNA mutation (median 22 vs. 45 years) but no clear difference in β-cell function or insulin sensitivity. In vitro, the M626K mutation caused a 53% decrease in transcriptional activity in HeLa cells. The mutated protein showed normal nuclear targeting but increased DNA binding. These data demonstrate that several genetic factors might contribute to diabetes risk, even in families with mtDNA and *HNF1α* mutations. *Diabetes* 53:1894–1899, 2004

Over the last decade, the molecular causes of several monogenic forms of diabetes have been described, including maturity-onset diabetes of the young (MODY) and maternally inherited diabetes and deafness (MIDD) (1). Both MODY and MIDD lead to impaired insulin secretion, which, especially in the case of MODY, causes diabetes at a young age (2). MODY is characterized by autosomal-dominant inheritance, whereas MIDD is maternally transmitted.

In Finland, the most common form of monogenic diabetes is MODY3, which results from mutations in the hepatocyte nuclear factor (HNF)-1α gene (3). HNF1α is a transcription factor expressed in several tissues, including liver, kidney, pancreas, and gut (4–6). The protein regulates a number of liver-specific genes and genes involved in glucose metabolism (e.g., GLUT2, L-type pyruvate kinase) (7,8). HNF1α is composed of three functional domains: an NH_2-terminal dimerization domain, a DNA-binding domain, and a COOH-terminal transactivation domain (9). Two distinct regions within the transactivation domain are required for a high level of transcription, AD I (from amino acid 547 to 628) and AD II (from 281 to 318) (10). MODY3 is characterized by postprandial hyperglycemia, low urine threshold for glucose, and absence of macrovascular complications (11).

The A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene associated with MIDD is also associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) in some pedigrees (12). Differences in heteroplasmy, the fraction of the total mitochondrial DNA (mtDNA) carrying the mutation, in different tissues could explain part of the phenotypic variation (13). Common clinical features of MIDD are diabetes neurosensory hearing loss, a normal or low BMI, short stature, and presence of macular dystrophy. High prevalence of neuromuscular and psychiatric disturbances has also been reported (14). These defects have been ascribed to mitochondrial defects in oxidative phosphorylation (15).

The aim of this study was to investigate the pathophysiology of diabetes in a family carrying a mutation in both the ADI region of *HNF1α* (M626K) and the A3243G mutation in mtDNA.

Thirteen family members had detectable amounts of the mtDNA mutation (individuals I:1 and I:3–III:1) (Fig. 1); 6 of them carried both the mtDNA mutation and the HNF1α mutation (I:1, I:4, II:3, II:4, II:5, and II:6). The family shows a clear maternal transmission of the 3243 mutation in mtDNA. Although one woman (I:2) did not have detectable amounts of the mtDNA mutation in her peripheral blood lymphocytes (PBLs), we cannot exclude that she carried small amounts of the mutation because heteroplasmy levels have been shown to decrease faster in PBLs than in

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HNF, hepatocyte nuclear factor; MIDD, maternally inherited diabetes and deafness; MODY, maturity-onset diabetes of the young; PBL, peripheral blood lymphocyte.

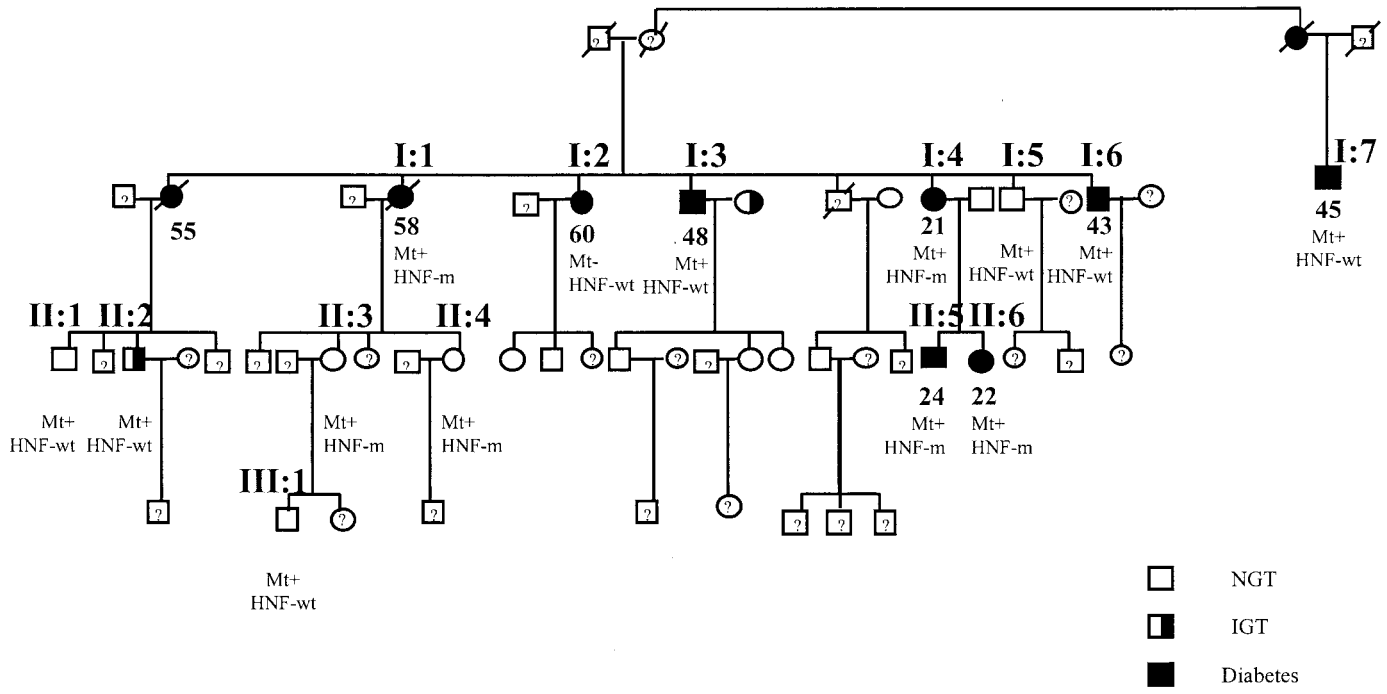

FIG. 1. Pedigree of the family with mutations in mtDNA and HNF1α gene showing data for sex (males in squares, females in circles), presence of diabetes (filled symbols for diabetes, open symbols for normal glucose tolerance, and question mark for glucose tolerance unknown). Age at onset of diabetes and genotypes (Mt+ indicates a carrier of the mtDNA mutation, mt- indicates a noncarrier of the mtDNA mutation, HNF-m indicates a M626K/HNF1α mutation carrier, and HNF-wt indicates a noncarrier of the HNF1α mutation) are shown underneath the symbol. In addition, the following subjects also had other signs and symptoms: I:3, hearing loss, hypertension, and coronary heart disease; I:4, short stature, hearing loss, hypertension, cardiomyopathy and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS); I:5, myocardial infarction and asthma; I:6, myocardial infarction; I:7, hypertension; II:1; glaucoma; II:2, hypertension and asthma; II:3, sporadic headache; II:4, short stature, hearing loss, sporadic headache, impaired cognitive function, and epilepsy; II:6, short stature, hearing loss, mild mental retardation, pigment epithelium degeneration, facial palsy, and muscle spasticity; and III:7, severe mental retardation.

other tissues upon aging (16). In β-cells, this decline is thought to enhance cell death and thereby contribute to the reduction in insulin secretion (17,18). Heteroplasmy correlated strongly with age at onset of diabetes in our pedigree ($r = 0.9$, $n = 7$; $P = 0.02$).

Three of the six patients with both mutations showed a very low degree of heteroplasmy (5% or less); two of them were healthy at the age of 44–48 years and the third had onset of diabetes at age 58 years. The other three carriers of both mutations had a higher degree of heteroplasmy (median 42%) and an earlier age at onset of diabetes (median 22 years). It is unlikely that the degree of heteroplasmy per se would determine the development of diabetes, as three nondiabetic family members with only the mtDNA mutation had a similar degree of heteroplasmy. The finding that among insulin-treated diabetic individuals, those with only the mtDNA mutation developed diabetes later than those with both mutations (45 vs. 22 years, $P = 0.05$) suggests that the effect of the two mutations might be additive. Caution is, however, warranted in this interpretation given the small number of subjects available. Unfortunately, none of the family members had only the *HNF1α* mutation, so we could not evaluate its separate effect. Individual III:7 showed marked sensory neural symptoms despite a low degree of heteroplasmy. This could be explained by a difference in the degree of heteroplasmy in PBLs and the neural tissue. Alternatively, the symptoms might solely be due to asphyxia during delivery (emergency cesarean section) and not MIDD.

Variants in the genes encoding peroxisome proliferator–activated receptor *PPARγ* (*P12A*), *Calpain10* (SNP43/44), and *Kir6.2* (E23K) were genotyped to study whether they potentially would modify the phenotype. It is of interest that the protective Ala allele at codon 12 of the *PPARγ* gene was observed in two of the nondiabetic carriers of the mtDNA mutation. The risk allele Lys at codon 23 of *Kir6.2* was found in 3 of 14 family members, of whom 1 was nondiabetic. The GG risk genotype of SNP 43 was seen in 5 of the 14 family members, and 4 of them had diabetes. The C allele of SNP 44 was seen in 13 family members, 7 of them with diabetes. Family member I:2 neither carried the HNF1α mutation nor had detectable amounts of the mtDNA mutation, but she developed diabetes at the age of 60 years. However, she carried risk variants in two type 2 diabetes susceptibility genes, *PPARγ* and *Kir6.2*, in addition to being obese. Compared with the healthy control subjects, she had relatively normal glucose uptake and insulin response to intravenous glucose (Table 1).

In general, the carriers ($n = 6$) of both mtDNA and HNF1α mutations tended to have higher fasting insulin concentrations but lower first-phase insulin response and lower peak insulin levels during an intravenous glucose tolerance test than those carrying only the mtDNA mutation ($n = 8$). This could indicate a decreased ability to upregulate their insulin secretion in response to glucose. In support of this, individuals with both mutations tended to have a lower C-peptide response to glucagon (serum C-peptide at 6 min = 0.66, 0.10, and 0.59 nmol/l in individuals I:4, II:6, and II:5, respectively) than those with

TABLE 1
The clinical characteristics and genotypes of individuals included in the study

Individual no.	I:2	I:6	I:7	I:3	I:5	II:1	II:2	III:1	I:4	II:6	II:5	I:1	II:4	II:3	Control subjects (n = 20)
HNF1α-M626K	MM	MM	MM	MM	MM	MM	MM	MM	MK	MK	MK	MK	MK	MK	—
Heteroplasmy (%)	<5	17	13	16	19	28	34	8	19	42	45	<5	<5	5	—
Age at onset (years)	60	43	45	48	—	—	—	—	21	22	24	58	—	—	—
Age at investigation (years)	65	51	52	61	55	46	41	17	56	30	26	65	44	48	54 ± 9
Treatment	Diet	Insulin	Oral + insulin	Insulin	—	—	—	—	Insulin	Insulin	Insulin	Diet	—	—	—
BMI (kg/m^2)	31	27	28	22	29	20	20	16	21	23	19	31	22	22	25 ± 1
Fasting plasma glucose (mmol/l)	6.0	10	6.2	6.6	6.9	5.7	5.4	—	7.6	8.8	7.9	7.0	5.1	5.5	5.6 ± 0.9
Fasting serum insulin (mU/l)	8.9	22		12	15	6.8	7.3	—	33	26	9.6	13	8.4	30	6.0 ± 2.7
Glucose uptake (mg · kg lean body mass^{-1} · min^{-1})	6.9	2.3	—	4.1	4.2	14	8.1	—	4.1	3.6	—	—	11	22	10 ± 2.7
CGO (mg · kg lean body mass^{-1} · min^{-1})	2.8	1.2	—	2.6	0.5	4.6	3.9	—	—	2.2	—	—	3.6	6.2	4.2 ± 1.2
CLO (mg · kg lean body mass^{-1} · min^{-1})	0.3	1.7	—	0.5	1.6	0.5	0.5	—	—	1.0	—	—	0.6	−0.3	0.2 ± 0.4
Glucose storage (mg · kg lean body mass^{-1} · min^{-1})	4.0	1.1	—	1.5	3.7	9.2	4.1	—	—	1.4	—	—	7.3	16	7.6 ± 2.6
FPIR (μU^2/ml^2)	303	—	—	—	413	451	302	—	—	—	—	—	275	104	461 ± 338
Peak insulin (mU/l)	70	—	—	—	83	98	75	—	—	—	—	—	62	22	99 ± 60
Disposition index (glucose uptake/FPIR)	0.02	—	—	—	0.01	0.03	0.03	—	—	—	—	—	0.04	0.21	0.05 ± 0.04
Fasting serum C-peptide (nmol/l)	0.86	1.0	0.51	0.46	1.4	0.57	0.51	—	0.37	0.06	0.58	0.59	0.44	0.43	0.39 ± 0.13
Serum C-peptide (6 min) (nmol/l)	—	—	0.80	0.80	—	—	—	—	0.66	0.10	0.59	—	—	—	0.76 ± 0.22*
Δserum C-peptide	—	—	0.29	0.34	—	—	—	—	0.29	0.04	0.01	—	—	—	0.43*
Allele data:															
Kir6.2-E23K	KK	KK	EK	KK	KK	KK	KK	EK	KK	EK	KK	KK	KK	KK	24/51/26†
PPAR-P12A	PP	PP	PP	PP	PP	PA	PA	PP	PP	PP	PP	PP	PP	PP	73/24/2†
Calpain-SNP43	AG	AA	GG	AG	AA	GG	GG	GG	AG	AG	GG	AG	AG	AG	50/42/8†
Calpain-SNP44	CT	CT	TT	CT	CT	CC	CT	CT	CT	CT	CC	CT	CT	CT	62/32/6†

Serum C-peptide (6 min) is the concentration 6 min after an intravenous injection of 0.5 mg glucagon. Δserum C-peptide is calculated as (serum C-peptide [6 min] − fasting serum C-peptide). CGO, clamp glucose oxidation; CLO, clamp lipid oxidation; FPIR, first-phase insulin response. *n = 9; †n = 2,293.

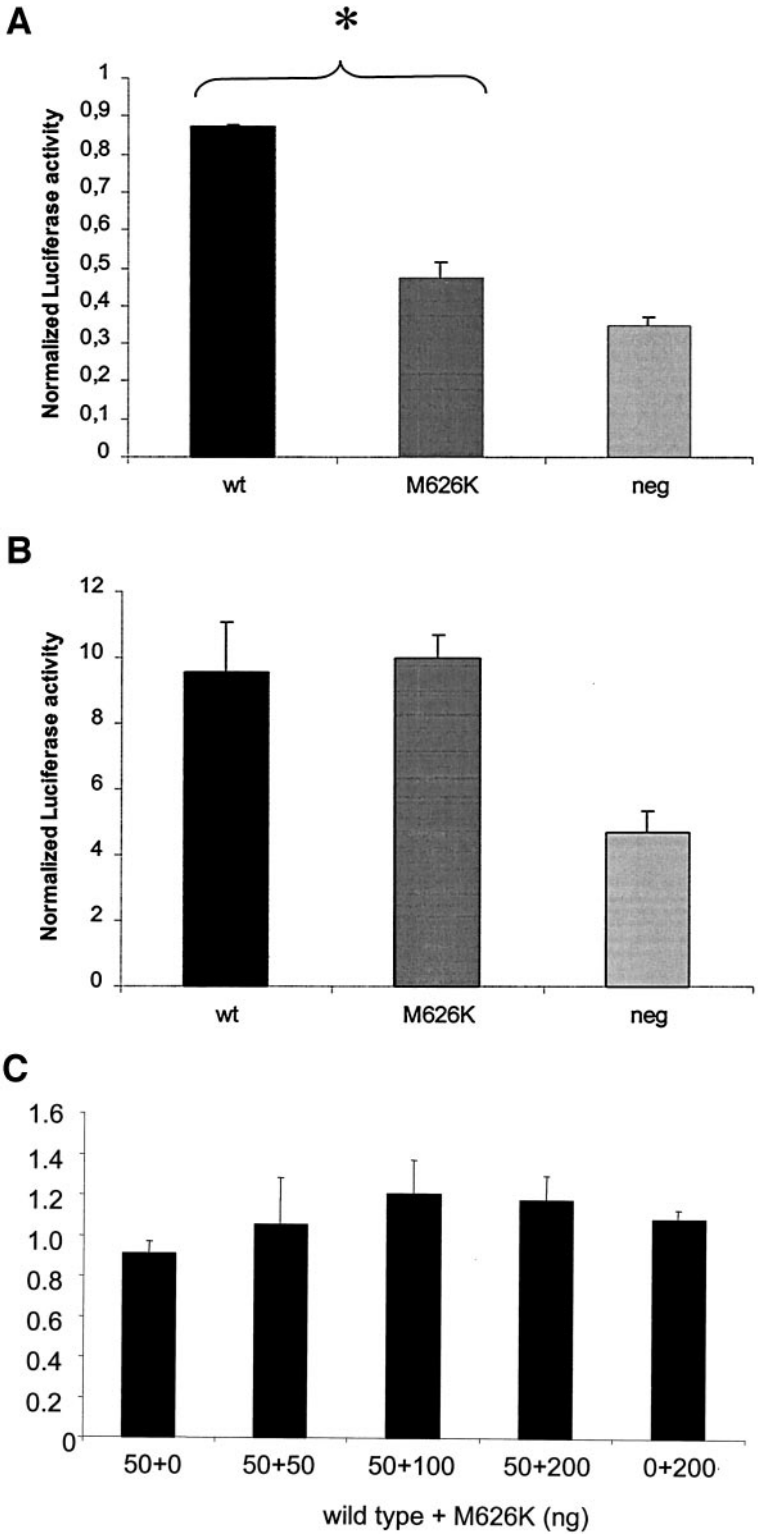

FIG. 2. Transcriptional activation by wild-type (wt) and mutant HNF1α. Luciferase activity was normalized by the activity of the internal control pRL-TK and the reporter construct containing the GLUT2 promoter. The results presented are from three independent experiments (mean ± SEM). *$P < 0.05$. *A*: HeLa cells were transfected with 50 ng wild type, M626K-HNF1α/pcDNA3.1, or empty vector (neg). *B*: MIN6 cells were transfected with 100 ng wt, M626K-HNF1α/pcDNA3.1, or empty vector (neg). *C*: Wild-type HNF1α-pcDNA3.1 (50 ng) and M626K-HNF1α/pcDNA3.1 (0, 50, 100, and 200 ng) were cotransfected to examine a possible dominant-negative effect by the mutated protein. Total amount of DNA added was adjusted to 250 ng with empty vector.

only the mtDNA mutation (0.80 and 0.80 nmol/l in individuals I:7 and I:3). Due to the small number of individuals, it is difficult to apply any statistical tests to the data.

To characterize the functional consequences of the M626K/*HNF1α* mutation in vitro, we studied the ability of the mutated protein to activate the human GLUT2 promoter. Transcriptional activity of the mutated *HNF1α* in HeLa cells (lacking endogenous HNF1α) was reduced by 53% compared with the wild type (0.23 ± 0.04 vs. 0.51 ± 0.08; $P < 0.001$) (Fig. 2*A*). On the other hand, transcriptional activity was normal when tested in MIN6 cells (Fig. 2*B*) that express the corresponding mouse HNF1α, suggesting that the endogenous protein can rescue the observed defect of the mutation. To directly test whether the mutation could have a dominant-negative effect on wild-type human HNF1α, increasing amounts of the M626K/*HNF1α* were transfected together with wild-type *HNF1α* in HeLa cells. No dominant-negative effect was observed

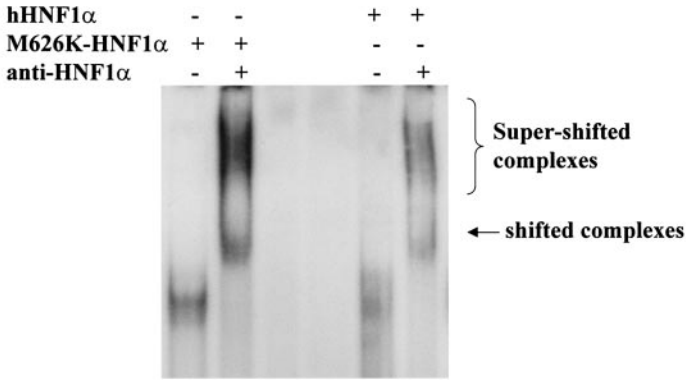

FIG. 3. Electrophoretic mobility shift assay demonstrating DNA-binding ability of wild-type and mutated HNF1α to the binding sequence of the GLUT2 promoter.

(Fig. 2*C*), suggesting a loss of function effect of the mutation. The result is supported by earlier findings (10) that the ADI region is necessary for transactivation, accounting for 50% of the full activity in an in vitro system.

The mutant protein showed increased DNA binding (Fig. 3), which theoretically could compensate for decreased transcriptional activity. Alternatively, the increased DNA binding could downregulate the transcription of target gene(s). Indeed, a gain-of-function mutation in HNF1α has been associated with increased transcriptional activity but impaired insulin secretion, explained by negative regulation of the expression of HNF4α- or insulin promoter factor-1–dependent genes (19).

Finally, the M626K mutation did not affect nuclear translocation of the HNF1α protein as determined by immunofluorescence in transfected HeLa cells (equal expression of both wild-type and mutant protein was confirmed by Western blot) (data not shown).

In conclusion, the M626K mutation in *HNF1α* results in decreased transcriptional activity and, together with the A3243G mutation in mtDNA, might influence the phenotypic expression of diabetes. The presence of other type 2 diabetes susceptibility variants may further modify this risk. The data from this single family emphasize the need to screen patients, even in families that appear to have a monogenic inheritance, for other genetic risk factors.

RESEARCH DESIGN AND METHODS

Fourteen members of a Finnish family were studied (20). Minor details in Fig. 1 have been altered to protect anonymity. The local ethics committee approved the study, and the subjects received genetic counseling by a clinical geneticist (B.L.). Nondiabetic unrelated individuals served as control subjects for the clamp studies ($n = 20$) and for the glucagon test ($n = 8$) (Table 1), whereas 2,293 nondiabetic individuals (1,051 men and 1,242 women, age 45 ± 14 years, BMI 26 ± 4 kg/m^2, and fasting plasma glucose 5.6 ± 0.6 mmol/l) provided information on genotype frequencies for the Kir6.2-E23K, PPAR-P12A, and Calpain-SNP43/44 polymorphisms (V. Lyssenko, M. Orho-Melander, M. Sjögren, unpublished observations) (Table 1).

Genotyping. The degree of mtDNA 3243 heteroplasmy was determined by PCR in DNA extracted from PBLs. The primers used were 5′-AAGGTTCGTT TGTTCAACGA-3′, 5′-CCGATGATGTTGGGAAGCGA-3′, and the restriction enzyme *Apa*I. The amount of DNA produced was determined by direct correlation to the intensity of fluorescence in a 3% agarose gel, containing 150 μg/ml ethidium bromide, by a Kodak camera using the Kodak 1D2.0 ds computer program. The heteroplasmy was used as a measure of the amount of mutated mitochondrial DNA and estimated by dividing the amount of mutated (cleaved) by wild-type (uncleaved) DNA fragments. The sensitivity for heteroplasmy detection was <5%. The M626K/HNF1α mutation was determined by sequencing. Variants in known diabetic susceptibility genes were genotyped: PPARγ (P12A) using restriction fragment–length polymorphism with primers 5′-GATAGAGACAAAATATCAGTG-3′ and 5′-TTACCCT TACATAAATGCC-3′ with the restriction enzyme *Bst*UI; Kir6.2 (E23K) using Applied Biosystems 7900HT (Foster City, CA), primer sequences VIC-CCTGC-CAAGCCCAG-MGBNFQ and 6FAM-CTGCCGAGCCCAG-MGBNFQ, and probes CAGTTGCCTTTCTTGGACACAAA and CCGAGGAATACGTGCTGACA; and Calpain10 (SNP43/44) was genotyped as previously described (21).

Metabolic studies. The Botnia clamp, an intravenous glucose tolerance test followed by an euglycemic-hyperinsulinemic clamp, was performed to obtain independent estimates of insulin secretion and action (22). The rate of glucose uptake was calculated from the glucose infusion rates during the last 60 min of the clamp. Indirect calorimetry was used in the basal state and during the last 45 min of the clamp to estimate the net rates of substrate oxidation. For patients requiring insulin therapy, β-cell function was assessed as C-peptide response 6 min after intravenous injection of 0.5 mg glucagon (23). Plasma glucose was measured with a glucose oxidase method using the Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA), and insulin was measured with an enzyme-linked immunosorbent assay (Dako, Cambridgeshire, U.K.) with an interassay coefficient of variation of 8.9%. The incremental trapezoidal area during the first 10 min was called first-phase insulin response. Plasma C-peptide concentrations were measured by radioimmunoassay (Linco Research, St. Charles, MO), with an interassay coefficient of variation of 9%.

Plasmid constructs. In vitro mutagenesis was performed on full-length human *HNF1α* cDNA using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide used in mutagenesis was 5′-CCTTCATCTCCACCCAGA*A*GGCCTCTTCCTCC-3′, with the mutated nucleotide underlined corresponding to the M626K substitution. Wild-type and mutant *HNF1α* were subcloned into a pcDNA3.1 expression vector (Invitrogen, NV Leek, the Netherlands). Sequences of the created constructs were verified by DNA sequencing before the expression studies.

Transactivation assay. HeLa cells (1.5×10^5) or MIN6 cells (3×10^5) were transfected using LipofectAMINE Plus Reagent (Life Technologies, Rockville, MD), with indicated amounts of wild-type or mutant *HNF1α*-pcDNA3.1 together with 0.5 or 2 μg GLUT2/pGL3 basic luciferase vector (Promega, Madison, WI) and 25 or 100 ng pRL-TK internal control vector (Promega), respectively. The transcriptional activity was measured after 24 h (HeLa) or 48 h (MIN6) using the Dual Luciferase Assay System (Promega) on a Victor^2 Wallac 1420 Multilabel counter (PerkinElmer, Stockholm, Sweden). Each experiment was performed in triplicate and independently repeated three times. The calculation of transcriptional activity as a percentage was performed by subtracting the value of the empty vector.

Western blot analysis. HeLa and MIN6 cells were transfected with 5 μg wild-type or mutant *HNF1α*-pcDNA3.1. Blotting was performed as described (24) using anti-HNF1α (N-19) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase–conjugate anti-goat IgG (Santa Cruz Biotechnology).

Electrophoretic mobility shift assay. HeLa cells were transfected with 5 μg of wild-type or mutant *HNF1α*-pcDNA3.1. After 24 h, nuclear protein extracts were prepared as previously described (25), and the electrophoretic mobility shift assay was performed (24) using 4.0 μg of nuclear extract with ^{32}P-labeled, GLUT2 promoter, HNF1α binding-site sequence (CTCAGTAAAGAT TAACCAT) as the probe.

Immunolocalization studies. HeLa cells grown on Falcon Culture slides (Becton Dickinson Labware, Bedford, MA) were transfected with 1.0 μg wild-type *HNF1α* and M626K/*HNF1α* stained with anti-HNF1 (BD Biosciences, San Jose, CA) before confocal and immunofluorescence microscopy analysis as previously described (24).

Statistics. Data are presented as the mean ± SE or ±SD. A P value of <0.05 was considered statistically significant. Differences between group means were tested by the Mann-Whitney nonparametric test, and differences in dual luciferase assay results were calculated using the paired t test. Correlations were calculated using Spearman.

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