PRELIMINARY REPORT

Triple Genetic Variation in the *HNF-4α* Gene Is Associated With Early-Onset Type 2 Diabetes Mellitus in a Philippino Family

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Maturity-onset diabetes of the young–type 1 (MODY1) is a form of monogenic type 2 diabetes mellitus (T2DM) with long-term complications due to mutations in the *HNF-4α* gene. The *HNF-4α* gene is involved in hepatic differentiation and expression of genes regulating glucose transport, glycolysis, and lipid metabolism. The abnormal glucose-stimulated insulin secretion in MODY1 subjects may be due to reduced glucose transport and glycolysis. To date, 14 mutations in the *HNF-4α* gene have been identified as a cause of either MODY1 or late-onset type 2 diabetes. So far, no screening has been performed in subjects from the Philippines. We recruited a Philippino family with autosomal dominant early-onset type 2 diabetes and screened the proband for mutations in the genes for HNF-1α, GCK, *HNF-4α*, IPF-1, HNF-6, and NGN3. We identified a new missense mutation in exon 5 (V199I) of the *HNF-4α* gene and 2 new single-nucleotide substitutions in intron 4, IVS4-nt4 (G→A) and IVS4-nt20 (C→T), all cosegregating with diabetes in the 3 affected available siblings. These variations were not present in 100 normal healthy subjects. Bioinformatic analysis suggests that these variations in the whole, and overall the IVS4-nt4 variation located at splicing site, may affect the splicing potential of intron 4. We have biochemically and clinically characterized the Philippine-1 family. We suggest that the V199I missense mutation located in the ligand binding/dimerization domain of *HNF-4α* contributes to type 2 diabetes in the Philippine-1 family. The intron variations may contribute susceptibility to diabetes.

BETWEEN 5% and 10% of type 2 diabetes mellitus (T2DM) is attributable to defined mutations in identified genes, so-called monogenic diabetes that are inherited in families with a high degree of penentrance. Monogenic forms of diabetes are comprised of a set of mutations in mitochondrial genes and somatic genes. The latter includes the set of identified maturity-onset diabetes of the young (MODY) genes, as well as an increasing number of other genes.[1-3] There are now 6 recognized MODY genes, 5 of which encode transcription factors that are critical for pancreas development. These transcription factors are HNF-4α (MODY1), HNF-1α (MODY3), Ipf-1 (Pdx-1) (MODY4), HNF-1β (MODY5), and Beta2/NeuroD (MODY6). MODY2 results from mutations in glucokinase2, a rate-limiting enzyme for the metabolism of glucose by the insulin-producing beta cells in the pancreas. MODY forms have autosomal dominant inheritance, and the proband has disease onset before 25 years of age, is not obese, and presents an insulin secretion defect with normal insulin sensitivity.[4] MODY is often underdiagnosed or misdiagnosed as type 1 diabetes mellitus and its occurrence is higher than reported. MODY1 is a severe form of monogenic type 2 diabetes (T2DM) due to an insufficiency of the transcription factor HNF-4α. HNF-4α is a member of the nuclear receptor superfamily. Endogenous fatty acids are the natural ligand of HNF-4α.[5] HNF-4α is involved in hepatic differentiation and in the expression of genes regulating glucose transport, glycolysis, lipid metabolism, coagulation, and thyroid hormone transport. HNF-4α binds DNA as homodimer; it contains a DNA-binding-domain, a ligand-binding/dimerization domain, and a transactivation domain.[6] MODY1 mutations are uncommon[7-10] and are treated with oral hypoglicemic agents (50%) or insulin (35%). About 30% of patients have retinopathy and many have nephropathy, neuropathy, and macrovascular disease. The abnormal glucose-stimulated insulin response in MODY1 subjects[11,12] may be due to reduced glucose transport and glycolysis due to diminished expression of genes implicated in these processes.[13] MODY1 nondiabetic subjects have a reduced insulin secretion in response to increasing levels of glycemia. Priming effect is lost in the prediabetic and diabetic phase, in contrast to MODY3, in which it is maintained in the prediabetic phase, and to MODY2 in which it is maintained in the phase of overt diabetes.[4] HNF-4α is an activator of HNF-1α, and is clinically linked to HNF-1α in the T2DM pathogenesis of the MODY3/Italy-1 family due to a mutation in the HNF-4α DNA-binding site of the HNF-1α promoter.[14]

Several mutations have been identified in the *GCK* gene.[15] MODY2 is a mild form of diabetes, without long-term complications, and is treated either with diet or with diet and hypoglycemic oral agents. HNF-1α heterozygous mutations are a common cause of MODY.[14,16,17] MODY3 is severe: patients need insulin or oral hypoglycaemic agents and present complications.[14] *IPF-1* mutations are a rare cause of MODY. The only non-sense mutation was identified in homozygosity in a proband with pancreas agenesis, and in heterozygosity in other family members with diabetes. Patient carriers of the mutation are treated either with diet, or oral hypoglycemic agents or insulin.[18] *IPF-1* gene polymorphisms contributing to disease are rare in subjects with type 2 diabetes, constituting about 4% of the diabetic population in France.[19] The *HNF-1β* gene is implied in the pathogenesis of diabetes and in kidney develop-

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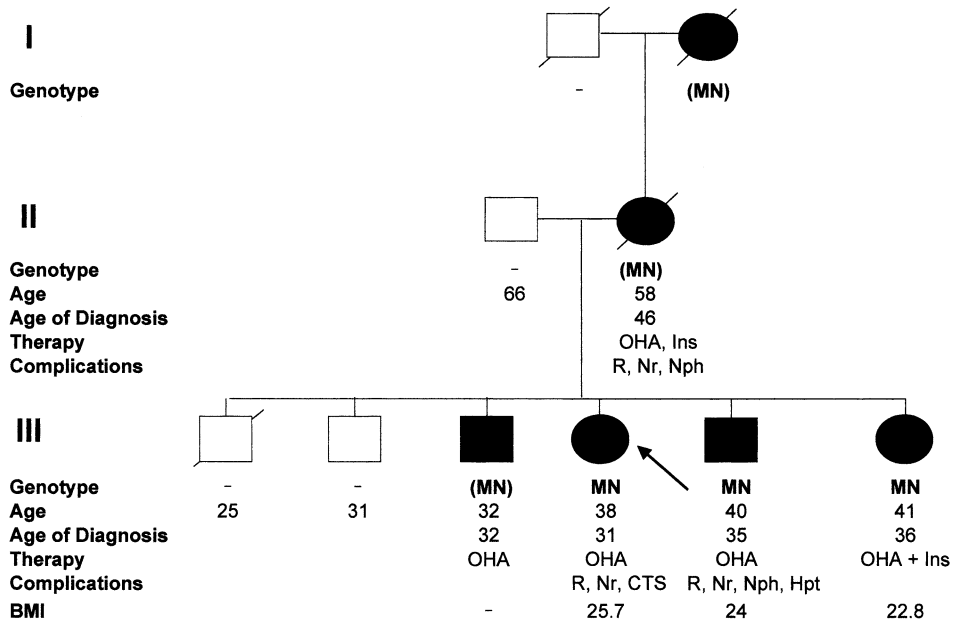

Fig 1. Pedigree and clinical data of Philippine-1 family. OHA, oral hypoglycemic agents; R, retinopathy; Nr, neuropathy; Nph, nephropathy; CTS, carpal tunnel syndrome; Hpt, hypertension; BMI, body mass index. (■) Type 2 diabetes; (□) unaffected; MN, inferred genotype; arrow indicates proband.

ment.[20] Heterozygous mutations in this gene are rare and the MODY5 form presents with various severity, precocious progressive nondiabetic renal dysfunction,[20] and occasionally with genital anomalies. Patients who are carriers of MODY5 mutations are treated either with diet, or with oral hypoglycemic agents or with insulin and present nephropathy. *NeuroD1* gene mutations are rarely a cause of MODY.[21]

We performed a genetic screening of β-cell transcription factors to identify the genetic variants contributing to autosomal dominant early-onset T2DM in a family from the Philippines.

MATERIALS AND METHODS

Subjects

We recruited 3 subjects from a family from the Philippines (Phillippine-1) with autosomal dominant early-onset T2DM. The subjects gave their consent after being informed about the nature and potential risks of the study. The Institutional Review Board for human subject research approved the study.

Genetic Screening

DNA was extracted from whole blood using the standard phenol/cloroform procedure.

The proband's DNA was screened for mutations by direct sequencing performed by ABI PRISM 3700 (Applied Biosystems, Foster City, CA) in exons 1a-10, flanking introns, and minimal promoter region of MODY1/HNF-4α and MODY2/glucokinase genes; in exons 1-10, flanking introns, and minimal promoter region of MODY3/*HNF-1*α gene; in exons 1-2 and flanking introns of MODY4/*Ipf-1* and *HNF-6* genes; and in exon, flanking introns, and promoter region of the neurogenin 3 gene.

Clinical Phenotyping

The Philippine-1 family was phenotyped for conditions and factors associated with diabetes and MODY1, including body mass index, metabolic profile (basal glucose, C-peptide and insulin, glycated hemoglobin, anti-insulin and anti-islets cell surface antibodies), lipid profile (total cholesterol, high-density lipoprotein [HDL], low-density lipoprotein [LDL], lipoprotein(a), triglycerides, apolipoprotein A, apolipoprotein B), liver function (AST, ALT, gamma glutamyl transpeptidase, albumin, total transferrin, total/direct bilirubin, ceruloplasmin, alpha-fetoprotein, somatomedin-C, thyroxine-binding globulin), coagulation function (factors VII, VIII, and IX, prothrombin time, Quick index, activated partial thromboplastin time, fibrinogen, antitrombin III), kidney function (azotemia, creatininemia, uricemia, erithropoietin, vitamin D_3), exocrine pancreatic function (serum amylase and lipase), and hypophyseal-thyroidal axis function (thyroglobulin, thyroid-stimulating hormone, triiodothyronine, thyroxine), and angiotensin-converting enzyme levels.

Software Analysis

Neural Network software was used for bioinformatic prediction of the splicing affinity of the wild-type and mutant sequences.

RESULTS

We did not identify any mutation/polymorphism in the above-mentioned genes except for 3 new genetic variations in the *HNF-4*α gene, including a GTC$\rightarrow$ATC base change resulting in a missense mutation in exon 5 (V199I), located in the endogenous ligand-binding/dimerization domain. The other 2 base changes are located in intron 4, IVS4-nt4 (G$\rightarrow$A) and IVS4-nt20 (C$\rightarrow$T). The variation IVS4-nt4 resides in the acceptor splicing site, and IVS4-nt20 is located 16 nucleotides upstream. These 3 variations were confirmed by sequencing of

Table 1. Philippine-1 Family

Parameter	III.4	III.5	III.6	Reference Values	
				Female	Male
Metabolic profile					
Basal glucose	249	216	207	65-110 mg/dL	
Basal C-peptide	2.1	1.8	0.8	1.1-5.0 ng/mL	
Basal insulin	14.6	7.6	13	< 20 μU/mL	
Glycated hemoglobin	8.1	9.6	8.2	3.8-5.5%	
Autoimmunity					
Anti-insulin antibody	6.2	4.6	2.4	< 10%	
Anti-pancreas antibody	Absent	Absent	Absent	Absent	
Lipid profile					
Total cholesterol	157	255	222	140-240 mg/dL	
HDL	54	63	54	> 45 mg/dL	
LDL	99	180	161	< 160 mg/dL	
Triglycerides	55	106	123	40-170 mg/dL	
Apolipoprotein A	147	161	146	115-220 mg/dL	115-190 mg/dL
Apolipoprotein B	66	132	117	55-125 mg/dL	55-140 mg/dL
Lipoprotein (a)	10	16	17	< 30 mg/dL	
Hepatosynthesis					
AST	16	40	22	10-37 U/L	
ALT	63	76	46	10-65 U/L	
γGT	53	72	47	< 50 U/L	< 70 U/L
Albumin	5	5.3	5.1	3.3-5.2 g/dL	
Transferrin	267	276	360	200-360 mg/dL	
Total bilirubin	0.35	0.61	0.3	0.2-1.0 mg/dL	
Direct bilirubin	0.07	0.15	0.11	< 0.25 mg/dL	
Ceruloplasmin	20	24	25	20-60 mg/dL	
Alpha-antitrypsin	93	100	110	90-200 mg/dL	
Alpha-fetoprotein (sieric)	1.5	4.2	3	< 10 ng/mL	
Somatomedin-C	226	213	213	96-424 ng/mL	119-494 ng/mL
Thyroxin binding globulin	1.4	1.4	1.6	1.0-4.2 mg/dL	
Coagulation function					
Factor VII	134	126	148	48-159 U/L	
Factor VIII	87	132	136	41-140 U/L	
Factor IX	93	102	100	63-150 U/L	
Quick index	100	100	100	70-120%	
APTT	28	29	27	27-35″	
Fibrinogen	232	250	246	150-400 mg/dL	
Antithrombin III	111	108	103	75-125%	
Kidney function					
BUN	23	22	33	10-50 mg/dL	
Creatinine	0.64	0.82	0.64	0.6-1.0 mg/dL	
Uric acid	2.3	6.6	5.7	2.4-5.7 mg/dL	3.4-7.6 mg/dL
Erythropoietin	9.4	6.3	30.3	2.6-34 mU/mL	
Vitamin D_3	30	12	13	9-38 ng/mL	
Microalbuminuria	30	136	30	< 50 mg/24 h	
Exocrine pancreas					
Pancreatic amylase	31	34	36	8-53 U/L	
Lipase	180	275	227	114-286 U/L	
Pituitary-thyroid axis					
Thyroglobulin	3	3	13	< 25 ng/mL	
TSH	1.7	1.6	2	0.3-5.5 U/mL	
T_3	3	3.4	2.7	2.0-4.2 pg/mL	
T_4	1.2	1.2	1.2	0.8-1.5 ng/dL	
ACE blood levels					
ACE	17	44	33	8-52 U/L	

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, gamma glutamyl transpeptidase; APTT, activated partial thromboplastin time; BUN, blood urea nitrogen; TSH, thyroid-stimulating hormone; T_3, triiodothyronine; T_4, thyroxine; ACE, angiotensin-converting enzyme.

both DNA strands and were shared by all 3 of the T2DM siblings tested. The variation IVS4-nt4 causes a higher affinity for the splicing in the acceptor site based on the Neural Network software prediction. The splicing score is 0.35 for the wild-type sequence, 0.50 for the sequence including IVS4-nt4, 0.63 when including IVS4-nt4 and IVS4-nt20, and 0.61 when including IVS4-nt4, IVS4-nt20 and V199I. These data predict that splicing affinity for intron-4 is higher in presence of these variations compared to the wild-type sequence and that the variation contributing to the altered splicing is IVS4-nt4. These variations were not found either in 100 normal chromosomes tested or in the screenings performed worldwide for MODY1 in individuals of multiple ethnic backgrounds/races. Therefore, we suggest that these mutations are not polymorphisms but rather causal or co-causal mutations associated with T2DM in the Philippine-1 family.

Pedigree and clinical data of the Philippine-1 family are described in Fig 1. All 3 affected siblings studied had poor metabolic control. Subject II.2 died of renal insufficiency. Subject III.1 died of unknown cardiac causes. Subjects III.2 and III.3 were not available for the study. Tested laboratory values of these patients are normal except for the following: fasting plasma glucose and hemoglobin A_{1c} in all 3 patients; AST, ALT, and γGT in subject III.5; γGT in subject III.4; and total cholesterol, LDL, and albuminemia in III.5 (see Table 1 for all data). These data are not significant, as the number of subjects is small, but it seems that the reported MODY1 mutation is not disrupting any clinical-biochemical function of the affected members, except for causing poorly controlled type 2 diabetes and potentially contributing to altered lipid profile and subtle biochemical liver alterations which are not due to any other hepatic dysfunction in subject III.5.

DISCUSSION

T2DM in this Philippino family may be caused by a conformational change in *HNF-4α* secondary structure and related functional alteration. Because V199I is in the endogenous ligand-binding/dimerization domain, it may alter endogenous ligand-binding affinity, thereby modifying the protein transcription function and homodimers dimerizing potential. As a consequence, disease may be caused by a gene dosage/haploinsufficiency mechanism. Furthermore, the variation IVS4-nt4, which increases splicing factors binding affinity, might cause preferential splicing of intron-4 variant compared to wild-type, potentially altering mRNA synthesis. The mRNA may be unstable and therefore prematurely degraded, or may translate to a dysfunctional protein. The second variation IVS-nt20 does not predict an effect on intron-4 splicing; however, the co-presence of IVS-nt4 variation may increase intron-4 splicing potential.

In the Philippine-1 family, the minimal alterations in hepatic function found may derive from altered liver *HNF-4α* transcription. So far few MODY1 families have been reported worldwide and a minority have been studied extensively clinically. The biochemical alterations caused by the MODY1 mutation in this family are consistent with those identified in the carriers of the *HNF-4α* R154X mutation in the German MODY1 family showing poor metabolic controls[8] and are not all in parallel with the biochemical alterations identified in the carriers of the *HNF-4α* Q268X mutation in the RW pedigree showing diminished apolipoprotein AII and tryglicerides values,[22] except for the lipoprotein(a) values specifically for the diabetic subject, which correlate with the average lipoprotein(a) values of our patient (14.3 mg/dL, n = 3). Although the triglyceride average value of our patients is in the lower range (94.6 mg/dL, n = 3), it is not consistent with the average value of the diabetic Q268X carriers (73.8 mg/dL). Also our data are not consistent with the data of the Swedish DS family in which carriers of the mutation K99fsdelAA had a low average of triglyceride levels (56.7 mg/dL), and a higher average for lipoprotein(a) values (30 mg/dL).[23] These differences may be explained by the diverse impact that each MODY1 gene mutation in each family may have at the functional level on the MODY1 target genes, and also by the number of subjects studied. In both cited cases the mutations were non-sense; therefore, in both cases we would expect higher biochemical derangement than in our Philippine-1 family, but only the study performed in the RW pedigree could exploit biochemical data deriving from 18 mutation carriers subjects, while the study conducted in the Dresden-11 pedigree was performed only in 5 mutation carriers. The study conducted in the Swiss family with the V121I *HNF-4α* mutation, even if limited, did non identify any lipid alterations.[24] These studies showed that retinopathy and neuropathy are the most common long-term complications associated with MODY1, as we have shown in our Philippine-1 family, where also for the first time a patient with carpal tunnel syndrome has been reported. Among the 3 variations described in our study, the missense V199I mutation appears to be a likely predisposing factor for the early-onset T2DM and for the long-term related complications in the Philippine-1 family. These patients may benefit by treatment with exogenous fatty acid agonist ligands of HNF-4α.[5]

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REFERENCES

1. Stride A, Hattersley AT: Different genes, different diabetes: Lessons from maturity-onset diabetes of the young. Ann Med 34:207-201, 2002

2. Mitchell SM, Frayling TM: The role of transcription factors in maturity-onset diabetes of the young. Mol Genet Metab 77:35-43, 2002

3. Iwasaki N: Diabetes mellitus (in Japanese). Rrinsho Byori 49: 161-164, 2001

4. Byrne MM, Sturis J, Menzel S, et al: Altered insulin secretory responses to glucose in diabetic and nondiabetic subjects with mutations in the diabetes susceptibility gene MODY3 on chromosome 12. Diabetes 45:1503-1510, 1996

5. Dhe-Paganon S, Duda K, Iwamoto M, et al: Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. J Biol Chem 277:37973-37976, 2002

6. Stoffel M, Duncan SA: The maturity-onset diabetes of the young (MODY1) transcription factor HNF4alpha regulates expression of

genes required for glucose transport and metabolism. Proc Natl Acad Sci USA 94:13209-13214, 1997

7. Yamagata K, Furuta H, Oda N, et al: Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). Nature 384:458-460, 1996

8. Lindner T, Gragnoli C, Furuta H, et al: Hepatic function in a family with a nonsense mutation (R154X) in the hepatocyte nuclear factor-4/MODY1 gene. J Clin Invest 100:1400-1405, 1997

9. Furuta H, Iwasaki N, Oda N, et al: Organization and partial sequence of the hepatocyte nuclear factor-4/MODY1 gene and identification of a missense mutation, R127W, in a Japanese family with MODY. Diabetes 46:1652-1657, 1997

10. Moller AM, Dalgaard LT, Ambur L, et al: A novel Phe75fsdelT mutation in the hepatocyte nuclear factor-4a gene in a Danish pedigree with maturity-onset diabetes of the young. J Clin Endocrinol Metab 84:367-369, 1999

11. Byrne MM, Sturis J, Fajan SS, et al: Altered insulin secretory responses to glucose in subjects with a mutation in the MODY1 gene on chromosome 20. Diabetes 44:699-704, 1995

12. Herman WH, Fajans SS, Ortiz FJ, et al: Abnormal insulin secretion, not insulin resistance, is the genetic or primary defect of MODY in the RW pedigree. Diabetes 43:40-46, 1994

13. Stoffel M, Duncan SA: The maturity-onset diabetes of the young (MODY1) transcription factor HNF4alpha regulates expression of genes required for glucose transport and metabolism. Proc Natl Acad Sci USA 94:13209-13214, 1997

14. Gragnoli C, Lindner T, Cockburn BN, et al: Maturity-onset diabetes of the young due to a mutation in the hepatocyte nuclear factor-4 alpha binding site in the promoter of the hepatocyte nuclear factor-1 alpha gene. Diabetes 46:1648-1651, 1997

15. Froguel P, Zouali H, Vionnet N, et al: Familial hyperglicemia due to mutations in glucokinase: Definition of a subtype of diabetes mellitus. N Engl J Med 328:697-702, 1993

16. Yamagata K, Oda N, Kaisaki PJ, et al: Mutations in the hepatocyte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3). Nature 384:455-458, 1996

17. Frayling TM, Bulamn MP, Ellard S, et al: Mutations in the hepatocyte nuclear factor-1a gene are a common cause of maturity-onset diabetes of the young in the United Kingdom. Diabetes 46:720-725, 1997

18. Stoffers DA, Zinkin NT, Stanojevic V, et al: Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet 15:106-110, 1997

19. Hani EH, Stoffers DA, Chevre JC, et al: Defective mutations in the insulin promoter factor-1 gene (IPF-1) in late-onset type 2 diabetes mellitus. J Clin Invest 104:R41-48, 1999

20. Lindner TH, Njolstad PR, Horikawa Y, et al: A novel syndrome of diabetes mellitus, renal dysfunction and genital malformation associated with a partial deletion of the pseudo-POU domain of hepatocyte nuclear factor-1beta. Hum Mol Genet 8:2001-2008, 1999

21. Kristinsson SY, Thorolfsdottir ET, Talseth B, et al: MODY in Iceland is associated with mutations in HNF-1alpha and a novel mutation in NeuroD1. Diabetologia 44:2098-2103, 2001

22. Shih DQ, Dansky HM, Fleisher M, et al: Genotype/phenotype relationships in *HNF-4α*/MODY1Haploinsufficiency is associated with reduced apolipoprotein (AII), apolipoprotein (CIII), lipoprotein(a), and triglyceride levels. Diabetes 49:832-837, 2000

23. Lehto M, Bitzen P-O, Isomaa B, et al: Mutation in the HNF-4a gene affects insulin secretion and triglyceride metabolism. Diabetes 48:423-425, 1999

24. Monney CT, Kaltenrieder V, Cousin P, et al: Large family with maturity-onset diabetes of the young and a novel V121I mutation in HNF4A. Hum Mutat 521:1-6, 2002