

ORIGINAL ARTICLE

Mutations in MODY Genes Are not Common Cause of Early-Onset Type 2 Diabetes in Mexican Families

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ABSTRACT

Context Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes mellitus characterized by autosomal dominant inheritance, early age of onset and a primary insulin secretion defect. Certain MODY gene sequence variants may be involved in polygenic forms of type 2 diabetes.

Objective We assessed the contribution of MODY genes to the etiology of type 2 early-onset diabetes in 23 Mexican families, including five with apparently autosomal dominant inheritance.

Patients Twenty-three unrelated Mexican families with early-onset type 2 diabetes previously screened for the presence of glucokinase mutations, were studied.

Design We screened MODY genes for sequence variants by PCR-SSCP analysis and automated sequencing. We performed a functional analysis of the HNF-1alpha P379H

recombinant protein *in vitro* in both HeLa and RINm5f beta-cell lines.

Main outcome measures MODY gene mutation screening and P379H mutant protein transactivation assay.

Results No mutations were detected in the HNF-4alpha, IPF-1, NEUROD1 or HNF-1beta genes in any of the families studied. A new mutation (P379H) of the HNF-1alpha gene was identified in one MODY family. RINm5f and HeLa cell transfection assays revealed decreased transactivation activity of the mutant protein on the human insulin promoter.

Conclusions All known MODY genes were screened for abnormalities in this cohort of early-onset diabetes families which included 5 MODY pedigrees. We identified a new HNF-1alpha MODY mutation (P379H) and demonstrated that it reduces the transactivation potential of the mutant protein on the human insulin promoter. No other

mutation was identified in this cohort indicating that abnormalities in MODY genes are generally not a common cause of early-onset diabetes and this includes MODY families in Mexico.

INTRODUCTION

Type 2 diabetes mellitus (T2DM), a chronic disease affecting glucose metabolism, is characterized by both insulin resistance and pancreatic beta-cell dysfunction. It is estimated that 150 million people are affected worldwide [1]. In Mexico, diabetes has become the first cause of death [2] and prevalence estimations are as high as 12% [3]. Characteristically, the age of onset in the Mexican population is earlier than expected, as almost 15% of the cases are diagnosed before age 40 (early-onset T2DM) [4]. The premature morbidity and mortality associated with early-onset T2DM has a much greater socio-economic impact than the late-onset form.

Maturity-onset diabetes of the young (MODY) is a genetically and clinically heterogeneous subtype of diabetes mellitus characterized by early-onset, autosomal dominant inheritance and beta cell dysfunction. Six genes have been identified as responsible for MODY: HNF-4alpha, glucokinase (GCK), HNF-1alpha, IPF-1, HNF-1beta and NEUROD1 [5, 6, 7, 8, 9, 10]. Additionally, in several populations other yet unknown genes are responsible for the disease in 16-45% of MODY families [11]. This

group is designated as MODY X.

MODY gene sequence variations have been associated with the more common polygenic forms of T2DM in several populations [12, 13, 14, 15]. The aim of this study was to establish whether mutations in genes encoding the transcription factors HNF-1alpha, HNF-4alpha, IPF-1, HNF-1beta or NEUROD1 are involved in the etiology of early-onset diabetes in a group of Mexican families previously screened for the presence of glucokinase mutations, including five families fulfilling the MODY criteria [16]. No mutations were identified in HNF-4alpha, IPF-1, HNF-1beta and NEUROD1 genes in any of the analyzed families. However, a new HNF-1alpha gene mutation (P379H) was found in a MODY family.

MATERIALS AND METHODS

Subjects

A total of 23 extended families were included in the analysis, recruited from the diabetes clinics of three different major referral centers in Mexico City. The families selected were those which included a proband with early onset diabetes initially not requiring insulin treatment, and at least one affected first degree relative diagnosed before the age of 40. Glucokinase gene mutations had been screened for and ruled out in this cohort in a previous study [16]. Five families showed a typical MODY inheritance pattern. The clinical characteristics of the probands from the 23 families studied are shown in Table 1;

Table 1. Clinical characteristics of the probands.

Type	Number of families	Age at diagnosis (years)	Insulin treatment	BMI ^b (kg/m ²)	Ketoacidosis	Glycemia at diagnosis (mg/dL)	Post-treatment glycemia (mg/dL)
MODY X ^a	4	14.2±1.9	1 (25%)	23.8±9.2	None	276±65	114±49
Early-onset diabetes	18	13.2±1.6	15 (83%)	25.2±5.3	4 (22%)	344±131	117±54
HNF-1alpha	1	12	None	27.3	None	400	85
P values ^c	-	0.286	0.046	0.680	0.554	0.331	0.920

Data are reported as frequencies and mean±SD

^a MODY type diabetes with no involvement of the six known MODY genes

^b BMI: body mass index

^c Comparison between MODY X and early-onset diabetes patients

the family structures and phenotypic profile of the probands were described elsewhere [16]. Islet cell antibodies were positive in 3 cases who fulfilled the inclusion criteria and had pedigrees compatible with autosomal dominant inheritance. Family history was positive for diabetes on both the paternal and maternal lines in 18 cases, while an autosomal dominant transmission pattern was evident through one of the parental lines in only 5 cases. Moreover, 60.9% of the probands were overweight (n=10; body mass index (BMI) ranging 25.6-28.5 kg/m²) or obese (n=4; BMI greater than 30 kg/m²), while the BMI was less than 25 kg/m² in the remaining 9 cases.

DNA Extraction and PCR Amplification

Genomic DNA was obtained from peripheral blood or lymphocyte cell lines using the proteinase-K, phenol-chloroform extraction method as described [16]. PCR primers used for amplification have been previously reported [5, 6, 7, 8, 9, 10]. The analysis included the exons and intron-exon boundaries of the HNF-4alpha, HNF-1alpha, IPF-1, HNF-1beta and NEUROD1 genes. Minimal promoter regions (500 bp) of the HNF-4alpha and HNF-1alpha genes [17] as well as the alternative promoter P2 of HNF-4alpha were also screened [18]. PCR amplifications were carried out using 50-100 ng of DNA, 10 pmoles of each primer, 0.1 µCi of alpha³²PdCTP (Amersham Biosciences, Buckinghamshire, England); specific activity: 3,000 Ci/mmol), 1 mM dNTPs, 1.5 mM MgCl₂.

PCR-SSCP and Automated Sequencing

SSCP analysis was performed as described [16]. Samples were run in 6% polyacrylamide gels in the presence or absence of 10% glycerol at room temperature at 8 watts for 12 h. Control samples were analyzed simultaneously. PCR fragments were sequenced using the 3100 ABI PRISM Genetic Sequence Analyzer following the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA).

Transactivation Assay

RINm5f and HeLa cell lines were grown in RPMI containing 10% fetal bovine serum, 200 mM L-glutamine and 1X non-essential aminoacids to approximately 80% confluency. 1.5 x 10⁵ cells were transfected using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) with 50 ng of HNF1alpha-cDNA3.1 wild type, mutant or the empty vector, together with 0.5 µg human insulin promoter/pGL3-basic luciferase vector and 10 ng of pRL-TK internal control vector (Promega, Madison, WI, USA). The transcriptional activity was measured 48 hours later using the Dual Luciferase Assay System (Promega, Madison, WI, USA) in a LumicountTM luminometer (Packard, Meriden, Connecticut, USA). Transactivation results were derived from two independent experiments, each carried out in sixuplicate. The results were normalized for transfection efficiency and by subtracting the value of the empty vector.

Preparation of Nuclear Extracts and Western Blot Analysis

RINm5f and HeLa cells were transfected with 300 ng of the wild type or mutant HNF1alpha-pcDNA 3.1 construct. After 48 h, the cells were lysed in a buffer containing 50 mM Tris-HCL (pH 8), 150 mmol/L NaCl, 1% Nonidet P-40 (NP-40), 0.5% deoxycolate, 1 mmol/L sodium orthovanadate, 15 µg/mL aprotinin and 1 mmol/L phenylmethylsulphonyl fluoride. Cell lysate protein concentrations were measured using a protein assay kit (BioRad, Hercules, CA, USA). Samples (30 µg protein) were run in 10% SDS-PAGE and transferred to a Hybond-PTM (Amersham Biosciences, Buckinghamshire, England) membrane. The membrane was blocked with a solution containing 5% milk powder, 0.05% Tween-20TM (BioRad, Hercules, CA, USA) in PBS and incubated in a 1:500 dilution of anti-HNF-1alpha (Geneka Biotechnology, Montreal, Quebec, Canada) for 1.5 h, followed by incubation in a 1:10,000 dilution of horseradish peroxidase

Table 2. HNF-4alpha, HNF-1alpha and IPF-1 gene sequence variations identified in early-onset type 2 diabetes Mexican families.

Gene	Exon/Intron	Nucleotide change	Amino acid and position
Synonymous			
HNF-1alpha	4	GGG>GGC	G288G
HNF-1alpha	6	CCA>CCG	P394P
HNF-1alpha	7	CTG>TTG	L459L
HNF-4alpha	2	GCC>GCT	A58A
IPF-1	1	CTG>CTA	L54L
Intronic			
HNF-1alpha	6	A>G	IVS6nt-2A>G
HNF-1alpha	6	C>T	IVS6nt+27C>T
HNF-1alpha	7	G>A	IVSnt+7G>A
HNF-4alpha	2	C>T	IVSnt-4C>T
Non-synonymous			
HNF-1alpha	7	AAC>AGC	S487N
HNF-1alpha	4	CTG>ATG	L254M

(HRP) goat anti-rabbit IgG as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was developed using the ECL quimioluminescence kit (Amersham Biosciences, Buckinghamshire, England) following the manufacturer's recommendations.

ETHICS

Informed consent was obtained from all participants through their attending physicians. The protocol was approved by the Ethics Committee in each of the referral Institutions.

STATISTICS

Data are expressed as mean±standard deviation and frequencies, and they are compared by means of the Student's t and the Fisher's exact tests. Two-tailed P values less than 0.05 are considered statistically significant.

RESULTS

No HNF-4alpha, IPF-1, NEUROD1 or HNF-1beta gene mutations were identified in any of the families analyzed. Sequence polymorphisms found in the HNF-4alpha, HNF-1alpha and IPF-1 genes are shown in Table 2.

A previously unreported mutation (P379H) in exon 6 of the HNF-1alpha gene was identified in one MODY family (Figure 1). The mutation was found in a heterozygous form in the proband, the affected father and a paternal great-aunt, but not in the unaffected siblings or mother. The proband was diagnosed with diabetes at age 13, and is currently on oral antihyperglycemic therapy (glibenclamide/metformin) and a diet/exercise regime. On diagnosis, her BMI was 27.3 kg/m², but is currently normal (22.9 kg/m²). She and the other affected relatives have not developed ketoacidosis. Her father was diagnosed at age 38; his weight is normal (BMI is 23.0 kg/m²) and he is on oral antihyperglycemic therapy. Both the proband and her father are free of chronic complications 5 and 12 years after

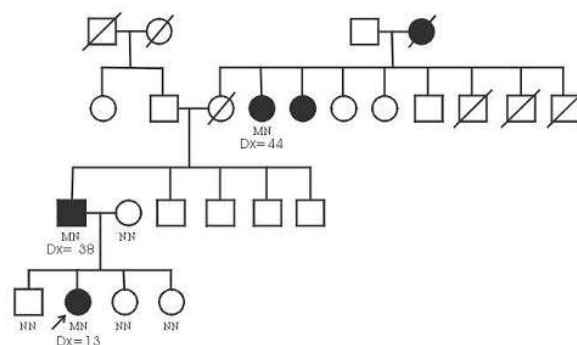


Figure 1. Family with the P379H mutation in the HNF-1alpha gene. Dark symbols indicate affected individuals. M, mutant allele; N, normal allele. Dx, age of diagnosis.

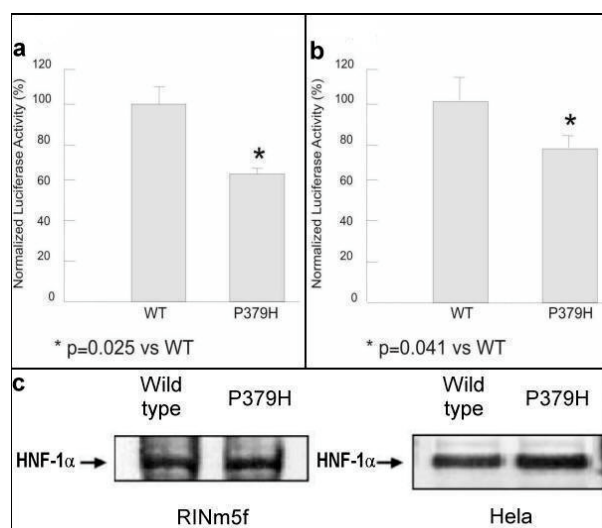


Figure 2. Transcriptional activation of wild-type (WT) and P379H HNF-1alpha on the human insulin promoter in RINm5f (a.) and HeLa cells (b.). Mean±standard deviation values from two independent experiments performed in sixuplicate are indicated by the bars. Western blot analysis of wild-type and mutant HNF-1alpha in RINm5f and HeLa cells are also shown (c.).

diagnosis, respectively, although the paternal great-aunt has developed hypertension. The proband was negative for anti-beta cell auto-antibodies.

Table 1 displays the clinical characteristics of the probands of MODY X, early-onset diabetes and the P379H mutation families. A higher proportion of early-onset diabetes patients required insulin treatment (P=0.046) and had suffered ketoacidosis (although not significant: P=0.554) as compared to the MODY families. Age at diagnosis, BMI and glycemia at diagnosis and after treatment were not significantly different between MODY X and early-onset diabetes patients.

RINm5f and HeLa cell transfection studies revealed that the transactivation activity of the HNF-1alpha P379H mutant protein on the human insulin promoter was lower than that of the wild-type protein (38 and 42% respectively) (Figure 2 A and B). This decrease was not related to the reduced expression of the mutant protein, as ascertained by Western blot analysis of nuclear extracts (Figure 2C).

Among the 11 polymorphisms identified (4 intronic, 5 synonymous and 2 non-synonymous), four are new: A58A in HNF-

4alpha, L54L in IPF-1, and P394P and IVS6nt+27C>T in HNF-1alpha. Polymorphisms L254M, G288G, L459L, S487N, IVS6nt-2A>G and IVS7nt+7G>A in HNF-1alpha, and IVS2nt-4C>T in HNF-4alpha were reported previously [19]. Interestingly, three unrelated patients showed the same 3 polymorphisms in exon and intron 7 of the HNF-1alpha gene (L459L, S487N and IVS7nt+7G>A). Genotype analysis of the available relatives showed that the haplotype was carried as a block in a single allele.

DISCUSSION

We evaluated the contribution of the MODY genes to the etiology of early-onset diabetes in twenty-three unrelated Mexican families (including at least 5 with autosomal dominant inheritance) recruited from three different major referral centers. The Mexican population can thus be included among the population groups assessed for the contribution of MODY genes in early-onset type 2 diabetes.

Mutations in known MODY genes were not found in 4 of the five MODY families studied. Only one with apparent autosomal dominant inheritance carried a new P379H HNF-1alpha gene mutation. This finding is consistent with the observed frequency of HNF-1alpha mutations in autosomal dominant early-onset T2DM families or single affected carriers from other ethnic groups [17, 19, 20, 21, 22, 23, 24, 25]. The mutation lies within exon 6, encoding the transactivation domain of the protein. Codon 379 is probably a mutational hotspot of the gene, since other mutations have been reported at this site in several families [19]. However, all previously reported mutations at this position were either deletions and/or insertions which resulted in truncated HNF-1alpha forms (i.e., P379fsdelCT).

In vitro expression studies demonstrated that the transactivation potential of the recombinant P379H mutant protein on the human insulin promoter is reduced both in HeLa and in the pancreatic RINm5f cell lines. Cosegregation of the mutant allele and

diabetes in this family is consistent with the results of the in vitro expression studies. Moreover, the clinical phenotype is similar to that reported for other HNF-1alpha mutation patients [20, 26].

The four putative MODY families in which no mutations were identified, are likely a valuable source in identifying additional MODY genes. In this regard, a recent whole genome scan for MODY X reported linkage to chromosomes 3, 5, 6 and 10 in European families [27] and to 8p23 and 2q37 in U. S. families [28], providing further evidence for the high genetic heterogeneity underlying MODY etiology.

An alternative but less likely explanation for the low mutation rate found is the low sensitivity of PCR-SSCP to detect sequence variations [29, 30, 31]. In this regard, even though intronic and 5' regulatory sequence changes may have been missed with the designed method, the number of both new and previously reported polymorphisms identified, particularly in the HNF-1alpha gene, is noteworthy.

Among the 11 MODY gene polymorphisms identified during the course of the study, of particular interest were L459L, S487N and IVS7nt+7G>A in exon and intron 7 of the HNF-1alpha gene since they segregated as a haplotype in three unrelated probands. Whether this haplotype is in fact associated with diabetes risk in our population remains to be determined.

In summary, this is the first report in which all known MODY genes were screened for sequence variations in an early-onset diabetes Latin American cohort. We identified a new HNF-1alpha mutation (P379H) in a MODY family, and proved that it alters the transactivation potential of the mutant protein on the human insulin promoter. Our results are consistent with findings in Brazilian MODY families who show a high proportion of relatives without HNF-4alpha, HNF-1alpha or glucokinase gene mutations [32], and with a recent study failing to find HNF-4alpha, HNF-1alpha or glucokinase gene mutations in Mexican-American early-onset diabetes families [33]. This suggests that there are still

unidentified genes in the majority of MODY families in Latin American populations. Moreover, the thorough analysis of all known MODY genes led to the identification of a putative HNF-1alpha risk haplotype in this cohort, while HNF-4alpha, glucokinase (GCK), IPF-1, HNF-1beta and NEUROD1 apparently fail to contribute to the etiology of early-onset diabetes in Mexican families, providing further support for the high heterogeneity of this disease.

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Keywords Genetics; Mexico; Polymorphism, Genetic

Abbreviations GCK: glucokinase; HRP: horseradish peroxidase; MODY: maturity-onset diabetes of the young; NP-40: Nonidet P-40; T2DM: type 2 diabetes mellitus

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