

A Hepatocyte Nuclear Factor-4 α Gene (*HNF4A*) P2 Promoter Haplotype Linked With Late-Onset Diabetes Studies of *HNF4A* Variants in the Norwegian MODY Registry

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Variants in hepatocyte nuclear factor (HNF)-4 α cause maturity-onset diabetes of the young, type 1 (MODY1) and may also be risk factors for type 2 diabetes. We sequenced the *HNF4A* gene of 95 MODY3-negative probands from the Norwegian MODY Registry. We found three novel coding variants in exon 8 of *HNF4A*: G326R, T339I, and W340X. In intron 7, we noted a single nucleotide polymorphism in the binding site of a previously published primer pair, which in some cases caused allelic drop out when amplifying exon 8. We also detected two novel sequence variants of the P2 promoter region, of which P2 -192C>G showed linkage with diabetes in two families (maximal logarithm of odds score of 3.1 and 0.8, respectively). This variant and a surrounding haplotype restricted by 3.7 Mb was also found in two Danish MODY pedigrees. The age of onset was higher in the P2 -192C>G carriers (median 45 years) compared with that reported for other MODY1 individuals. We could not support a biological role of the P2 promoter variant by in vitro transfection assays. In conclusion, we have identified three novel *HNF4A* mutations and a 3.7-Mb haplotype, including the *HNF4A* P2 promoter, which was linked with diabetes. *Diabetes* 55:1899–1903, 2006

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HNF, hepatocyte nuclear factor; LOD, logarithm of odds; MODY, maturity-onset diabetes of the young; SNP, single nucleotide polymorphism.

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Maturity-onset diabetes of the young (MODY) can result from disease-causing sequence variants in any of at least six different genes that encode the glycolytic enzyme glucokinase (GCK) and five transcription factors: hepatocyte nuclear factor (HNF)-1 α , -1 β , and -4 α , insulin promoter factor-1, and NEUROD1 (1). Recently, genetic variants in the β -cell-specific P2 promoter region of the *HNF4A* (MODY1) gene have gained substantial attention in MODY (2–5) and in type 2 diabetes (6–12).

We have investigated the prevalence of sequence variants of the *HNF4A* gene in probands recruited from the Norwegian MODY Registry (13) after first excluding the presence of mutations in *HNF1A* (MODY3). In clinically relevant cases, we had also excluded mutations in *GCK* (MODY2). Here, we present a summary of sequence variants detected during the screening and discuss their role in the development of monogenic diabetes.

RESEARCH DESIGN AND METHODS

Since 1997, physicians have referred subjects to the Norwegian MODY Registry based on at least two of the following criteria: 1) first-degree relative with diabetes, 2) onset of diabetes before age 25 years in at least one family member, 3) low dose of insulin requirement (<0.5 units \cdot kg⁻¹ \cdot day⁻¹), 4) early-onset diabetes defined as diabetes diagnosed between age 25 and 40 years, and 5) subjects with an unusual type 1 diabetes (low-dose insulin requirement, no antibodies, or atypical history). Obviously, the conventional criteria of MODY (1) are not met in all cases. Nevertheless, inclusion of subjects based only on the conventional criteria will exclude some true MODY patients, e.g., those with de novo mutations, age at diagnosis after age 25 years, or limited clinical data on the family history of diabetes.

We sequenced *HNF4A* in 95 probands recruited from the Norwegian MODY Registry. Twenty-three probands fulfilled conventional MODY criteria (1), whereas the remaining 72 subjects were categorized as having suspected MODY. All probands were negative for mutations in *HNF1A* (13). Moreover, *GCK* mutations were excluded in 42 probands who had a MODY2-like phenotype. Standard oral glucose tolerance tests were performed (75 g glucose), and World Health Organization criteria for diabetes were applied. The study was approved by the regional committee for research ethics. We obtained written informed consent from the patients or their parents.

Molecular genetics. *HNF4A* was sequenced using previously reported (14) or newly designed (online appendix Table 1 [available at <http://diabetes.diabetesjournals.org>]) primers. In particular, a new primer pair for exon 8 had to be used in order to avoid allelic drop out observed when using the published strategy (14). For the determination of allele frequencies of the novel variants and the IVS7 nt-88C>T single nucleotide polymorphism (SNP)

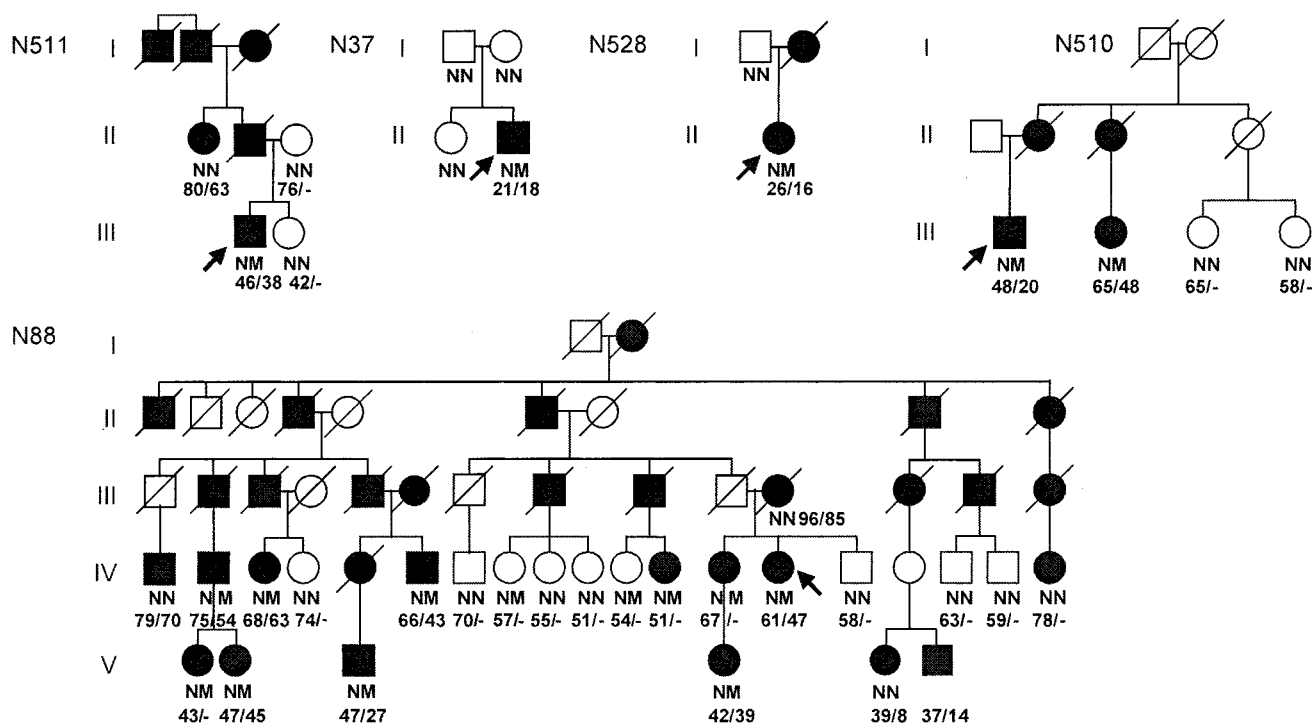


FIG. 1. Pedigrees of Norwegian MODY families with *HNF4A* gene variants. Circles denote female family members, squares denote male family members, open symbols denote unaffected family members, and symbols with a slash denote deceased family members. Solid symbols indicate diabetes or impaired glucose tolerance. Individual mutation status is given where tested: N, normal allele; M, mutated allele. Numbers below the subject symbols denote age at study/age at diagnosis of diabetes. Arrowheads indicate the probands. In the text, the subjects are referred to with Roman numerals for the generation number and Latin numerals counting consecutively from the left. The proband in family N88, IV-14, was 47 years old at onset of diabetes but was referred to the MODY Registry based on her gestational diabetes at the ages of 34 and 37 years and the aggregation of diabetes in the family. In family N88, the following subjects were coded as unknown in the linkage analysis because before the genotyping we considered them to have diabetes from a different cause than the rest of the family: subjects V-5 (elevated GAD65 auto antibodies and high-risk haplotypes HLA-DR3-DQ2/DR4-DQ8) and V-6 (by history and not available for blood sampling) were considered to have type 1 diabetes. The spouses III-6 and III-11 were considered to have common type 2 diabetes. Subjects IV-1 and IV-19 are phenocopies but were coded as affected in the linkage analysis.

causing allelic drop out, we sequenced the relevant exon and flanking introns directly in 100 blood donors. The P2 -192C>G SNP was genotyped in 1,006 healthy Norwegian subjects using Sequenom's MassARRAY system. The allele frequencies of the polymorphisms were estimated in 95 MODY probands.

Linkage analysis. Power calculation using 1,000 replicates and SLINK/MSIM software yielded maximal average logarithm of odds (LOD) scores of 4.4 and 0.3 in families N88 and N510, respectively. To assess the significance of the P2 variant cosegregating with MODY, we used a previously described parametric LOD score analysis with age-related penetrances (15) and the MLINK/VITESSE (version 1.0) program. To investigate a possibly shared haplotype, we genotyped family members with the microsatellite markers *D20S855*, *D20S96*, *D20S43*, *D20S89*, *D20S119*, and *D20S1151*. Estimation of individual haplotypes in families N88 and N510 were done using the SIMWALK (version 2.89) software, and haplotype frequencies among unrelated controls were calculated with PHASE (version 2.1.1).

Plasmid constructs. A luciferase reporter gene construct for the P2 promoter studies was kindly provided by Dr. Gerhard Ryffel, Essen, Germany (5). We used specifically designed mutagenesis primers to create P2 promoter constructs containing the -192C>G and -50G>C sequence variants (Quick-

Change XL kit; Stratagene, La Jolla, CA). Sequences of the constructs were confirmed by DNA sequencing.

Cell culture and transfection studies. Rat insulinoma INS-1 cells were transfected and assessed as previously described for HeLa cells (13). Each experiment was performed in triplicate on each of 5 consecutive experimental days. Transcriptional activation was measured as luciferase activity generated from reporter constructs transfected into INS-1 cells. We performed transfection of the cells using 4 μl FuGene6 (Roche Molecular Biochemicals, Indianapolis, IN) and a total amount of 2 μg DNA, including 0.025 μg pRLSV40 (Promega, Madison, WI) for normalization of transfection efficiencies and various amounts of wild-type or mutant constructs. The total quantity of plasmids was maintained constant by adding suitable amounts of empty pGL3-Basic (Promega) reporter vector. The cells were harvested 24 h post-transfection, and total cell extracts were prepared. The transcriptional activity was measured using the dual-luciferase reporter assay system (Promega).

Statistical analysis. We used linear regression to estimate any difference in the luciferase activity for the wild-type and mutant constructs. We chose a significance level of 5% and used Stata (version 7.0; Stata, College Station, TX).

TABLE 1
HNF4A gene mutations observed in Norwegian MODY families

Family	MODY categorization	<i>HNF4A</i> gene location	Designation at DNA level*	Designation at protein level
N88	Suspected MODY	Promoter P2	P2 -192C>G	—
N510	Conventional MODY	Promoter P2	P2 -192C>G	—
N511	Suspected MODY	Exon 8	c.976G>A	G326R
N37	Suspected MODY	Exon 8	c.1016C>T	T339I
N528	Conventional MODY	Exon 8	c.1020G>A	W340X

*c.1 is the first nucleotide of the ATG initiation codon in the cDNA.

A

Family	N510	N88	N88	N88	DK-657	DK-515
Subject	III-1	V-2	IV-3	IV-6	657-1	515-12
Affected	Yes	Yes	Yes	Yes	Yes	Yes
No of carriers in family	2	10	10	10	4	2

Marker	Physical Location (kb)	N510	N88	N88	N88	DK-657	DK-515
<i>D20S855</i>	39082	113	113	113	109/111	107	109
<i>D20S96</i>	41529	116	116	116	116	116	116
<i>D20S43</i>	41633	196	196	196	196	196	196
<i>P2-192</i>	42428	G	G	G	G	G	G
<i>D20S89</i>	42774	181	181	147/173	181	181	181
<i>D20S119</i>	43082	112	112	110/112	112	112	112
<i>D20S1151</i>	43320	250	250	243/246	250	250	239

B

SNP	Physical Location (bp)	Families		Estimated haplotypes in blood donors			
		N510	N88	A 41 %	B 28 %	C 14 %	D 7.2 %
rs1884613	-4030	C	C	C	C	C	G
P2 -192C>G	0	G	G	C	C	C	C
rs2144908	1272	G	G	G	G	G	A
rs1028583	66316	G	G	G	T	T	G
rs3818247	73035	G	G	G	T	G	G

FIG. 2. A: Haplotypes of recombinant chromosomes for individuals in the Norwegian (N88 and N510) and Danish (DK-515 and DK-657) families based on microsatellite markers. Critical recombinant chromosomes in affected family members are presented. B: Deduced SNP-based mutation-carrying haplotypes in families N88 and N510 based on SNPs in the *HNF4A* region with positions given relative to P2 -192C>G. All mutation carriers in both families carried the identical mutation-carrying haplotype. Haplotypes in 50 blood donors were estimated with PHASE (version 2.1.1) based on the SNP genotypes, and the haplotypes with an estimated frequency >5% are shown.

RESULTS

We found four previously undescribed mutations in *HNF4A* (Fig. 1 and Table 1), three in exon 8 (G326R, T339I, W340X), and one in the P2 promoter (P2 -192C>G). It was not possible to extend the pedigrees N511 and N528 for an evaluation of cosegregation for the G326R and W340X variants, respectively. T339I was a spontaneous mutation, as the healthy parents did not harbor the mutation and the family relationships were confirmed by microsatellite analysis. In addition, we observed the previously reported exon 7 variant V255M (16) in two families in which there was no cosegregation with diabetes (N91 and N557, data not shown). None of the putative pathogenic variants were identified in 200 control chromosomes. We also observed several polymorphisms and intron variants (online appendix Table 2).

During sequencing of exon 8, we observed an allelic drop out caused by an SNP (IVS7 nt-88C>T) in the binding site of the originally published forward primer (14). The frequency of the C allele of the IVS7 nt-88C>T SNP was 59% in 95 MODY probands and 57% in unrelated blood donors.

We observed two novel variants of the P2 promoter, P2 -192C>G (families N88 and N510; Fig. 1) and P2 -50G>C (families N515; data not shown), of which only the former

showed a pattern of cosegregation with disease. The P2 -192C>G variant was not found among 1,006 control individuals. We genotyped additional family members from families N88 and N510 for the P2 -192C>G variant. By linkage analysis, we found a maximum two-point LOD score of 3.1 and 0.80, respectively, in the two families (online appendix Table 3). Haplotype determination using microsatellite markers surrounding the *HNF4A* locus showed that the N88 and N510 families shared a minimal haplotype of <3.7 Mb flanked by the markers *D20S855* and *D20S89* (Fig. 2A). Furthermore, this haplotype was also shared with two Danish families harboring the same P2 -192C>G variant (families DK-657 and DK-515; Fig. 2A) (17). The same microsatellite marker haplotype was not present among 20 Norwegian blood donors. In contrast, on constructing a haplotype of closely spaced SNPs spanning *HNF4A* and the P2 promoter region, we found that the P2 -192C>G variant resides on the most common *HNF4A* SNP haplotype in Norway (Fig. 2B).

An elevated age at onset of diabetes was the most distinguishing clinical feature of subjects with the P2 -192C>G variant (median onset of 45 years) compared with subjects with coding variants. In addition, the subjects were generally lean (Table 2). Since sulfonylurea has been reported to be effective in MODY1 patients (18), we

TABLE 2
Clinical features of Norwegian MODY1 families

Characteristics	Coding mutations	P2 -192C>G
Number of families	3	2
Number of subjects (male)	3 (2)	14 (4)
Current age (years)	26 (21–46)	56 (42–75)
Age at diagnosis (years)	18 (16–38)	45 (20–63)
Normal glucose tolerance/impaired glucose tolerance/diabetes	0/0/3	2/3/9
Number of subjects diagnosed with diabetes before age 25 years	2	1
Late diabetes complications (yes/no)	2/1	0/7
Treatment in diabetic subjects (oral hypoglycemic agents/insulin)	1/1	5/2
Insulin requirement (units/kg)	0.97	0.91 (0.40–1.41)
BMI (kg/m ²)	25.4 (22.1–30.4)	22.6 (18.5–30.1)
A1C (%)	9.2 (8.0–9.3)	7.1 (5.4–8.3)
Glucose (mmol/l)	7.4 (7.3–7.4)	7.9 (5.2–12.3)
Insulin C-peptide (nmol/l)	0.9 (0.9–0.9)	0.5 (0.0–1.2)
Triglycerides (mmol/l)	1.28 (1.22–1.33)	0.75 (0.31–2.30)
Total cholesterol (mmol/l)	4.9 (4.4–5.3)	5.7 (4.0–6.8)
HDL cholesterol (mmol/l)	1.2 (1.1–1.3)	1.6 (0.8–2.5)
LDL cholesterol (mmol/l)	2.9 (2.6–3.2)	3.4 (2.6–4.6)
Apolipoprotein AI (mg/dl)*	120	185 (149–236)
Apolipoprotein AII (mg/dl)*	28	31 (30–36)
Apolipoprotein B (mg/dl)*	118	109 (87–113)
Apolipoprotein CII (mg/dl)*	4.7	4 (3–7)

Data are median (range), unless otherwise indicated. All biochemical blood values except A1C were measured in serum. Glucose, insulin, C-peptide, and lipids were sampled in fasting patients. *Calculations were based on one subject with a coding variant (the proband in family N511) or on six subjects with the P2 -192C>G variant.

noted that among the subjects with the P2 promoter variant P2 -192C>G, five subjects were treated with sulfonylurea and used on average a dose of 43% of the maximum licensed dose (with a mean level of HbA_{1c} [A1C] 7.7%). Among the subjects with coding variants, one subject was using sulfonylurea, requiring 8% of the maximum licensed dose (A1C 9.2%). We found no significant difference in transcriptional activity between the wild-type and either of the two P2 promoter variants when they were coupled to a reporter gene and tested in INS-1 cells (online appendix figure). We found that disease-associated variants in the coding and promoter regions of *HNF4A* account for 9% (2 of 23) of MODY3-negative probands meeting conventional criteria and 4% (3 of 72) among the probands with suspected MODY, based on the putative causal effects of P2 -192C>G, G326R, T339I, and W340X (Table 1).

DISCUSSION

Mutation screening of *HNF4A* in 95 probands recruited from the Norwegian MODY Registry revealed three coding mutations in exon 8 (G326R, T339I, and W340X) not previously reported, as well as a previously undescribed disease-associated variant of the P2 promoter. Cosegregation was not possible to evaluate for G326R and W340X. The T339I was a de novo mutation and hence the second

spontaneous mutation to be reported in MODY1 (18). G326R, T339I, and W340X were all located in a genomic region encoding the transactivating domain of the protein, and they affected residues strictly conserved in the human, mouse, rat, and *Drosophila* HNF-4 α homologues. Moreover, they were not found in 200 control chromosomes. Thus, these variants are likely to cause monogenic diabetes. In our material, variants in the coding and promoter regions of *HNF4A* causing monogenic diabetes account for 9% of MODY3-negative probands meeting conventional criteria. We therefore consider, in Norway, MODY1 to be much less common than MODY3 and consider MODY2 to be the third most prevalent type (13; unpublished data).

Due to a highly frequent SNP in a primer binding site, we observed an allelic drop out (19) when sequencing exon 8 with the originally suggested primer pairs (14). Resequencing should therefore be considered in patients tested with the previously published primers. Notably, all the three novel coding variants that we observed occurred in exon 8.

We found two single nucleotide substitutions in the P2 promoter region. The P2 -192C>G variant cosegregated with diabetes in two Norwegian families (maximal LOD score 3.1 and 0.80, respectively). The P2 -192C>G variant is located 10 bases upstream of the HNF-1 binding site and is conserved in the corresponding position in mouse. Interestingly, the same variant was also found in five Danish pedigrees (17) but not in 1,006 control subjects. We tested two of the Danish pedigrees and found a shared haplotype block of 3.7 Mb. Other haplotype-specific variants in *HNF4A* were not found. We believe that the observed maximum two-point LOD score (3.1) for the -192C>G variant at a recombination fraction of 0.10 (and not 0.00) in family N88 results from a slight misspecification of the model that underestimates the true phenocopy rate (subjects IV-1 and IV-19 are phenocopies). Based on the genetic data, we have established that the P2 variant or another variant within the shared 3.7-Mb haplotype is linked to diabetes in the two Norwegian and in two of the Danish pedigrees. We could not support a functional role of the P2 promoter variant by an in vitro transfection assay. This result does not, however, exclude that the P2 promoter variant is biologically relevant. Interestingly, Ek et al. (17) provide in vitro data showing that P2 -192C>G disrupts the binding of an unidentified sequence-specific DNA-binding complex present in human islet extracts.

Most of the subjects with the P2 -192C>G variant were lean and had a later onset of diabetes (median onset age of 45 years) compared with other recently described *HNF4A* mutation carriers (mean onset age of 23 years) (18), suggesting that it defines a particular subgroup of diabetic subjects. Late-onset monogenic diabetes has also been noted for the coding *HNF4A* variant V393I (20).

Several studies in various ethnic populations have suggested that haplotypes spanning the regulatory regions of *HNF4A* associate with type 2 diabetes (7–10,12), while other studies have failed to show this relationship (11), a problem generally noted in association studies (21). One reason for the discrepancy between the studies might be allelic heterogeneity (22). Rare risk variants, like the described P2 -192C>G variant, with modest attributable risk in the population but with major impact in a family, will most likely not be detected by conventional multi-population association studies (23,24). In such cases, exhaustive sequencing in extended families showing linkage to the region might be an alternative approach.

In conclusion, variants in the coding and promoter regions of *HNF4A* leading to monogenic diabetes account for ~9% of MODY3-negative probands meeting conventional criteria, making MODY1 the third most prevalent MODY form in Norway. An *HNF4A* P2 promoter haplotype was linked with late-onset diabetes, further supporting the role of the *HNF4A* region in type 2 diabetes.

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