

Brief Genetics Report

β -Cell Transcription Factors and Diabetes

No Evidence for Diabetes-Associated Mutations in the Hepatocyte Nuclear Factor-3 β Gene (HNF3B) in Japanese Patients With Maturity-Onset Diabetes of the Young

Yoshinori Hinokio, Yukio Horikawa, Hiroto Furuta, Nancy J. Cox, Naoko Iwasaki, Masashi Honda, Makiko Ogata, Yasuhiko Iwamoto, and Graeme I. Bell

Mutations in the transcription factors hepatocyte nuclear factor (HNF)-4 α and -1 α , insulin promoter factor-1, and HNF-1 β are the causes of four forms of maturity-onset diabetes of the young (MODY1 and 3–5, respectively). The winged-helix transcription factor HNF-3 β has been implicated in the regulation of expression of each of these MODY genes, suggesting that mutations in the HNF-3 β gene (HNF3B) may also cause MODY. We have tested this hypothesis by screening a panel of 57 unrelated Japanese subjects with a clinical diagnosis of MODY for mutations in HNF3B. This analysis revealed four frequent polymorphisms that were not associated with MODY, including one in the promoter region (-213A/G), two silent mutations in the codons for Ala 97 (291C/T) and Gly 279 (837A/G), and one in the 3'-untranslated region (1424C/T). Two rare substitutions in the 5'-untranslated region, -156C/T and -67A/C, were found in a heterozygous state in two subjects, and two subjects were heterozygous for putative missense mutations, S109N (326G > A) and A328V (983C > T). The two missense mutations were not found in 106 normal chromosomes from nondiabetic subjects. It was not possible to test for co-segregation of these mutations with diabetes and thus, it is unclear whether or not these mutations can cause MODY. The results of our study suggest that mutations in HNF3B are not a common cause of MODY in Japanese subjects. *Diabetes* 49:302–305, 2000

From the Howard Hughes Medical Institute (Y.Hi., Y.Ho, G.I.B.), and the Departments of Biochemistry and Molecular Biology (Y.Hi., Y.Ho, H.F., G.I.B.), Medicine (N.J.C., G.I.B.), and Human Genetics (N.J.C., G.I.B.), University of Chicago, Chicago, Illinois; the Diabetes Center (N.I., M.O., Y.I.), Tokyo Women's Medical University; and the Shiseikai Daini Hospital (M.H.), Tokyo, Japan.

Address correspondence and reprint requests to Dr. Graeme I. Bell, Howard Hughes Medical Institute, University of Chicago, 5841 S. Maryland Ave., MC 1028, Chicago, IL 60637. E-mail: g-bell@uchicago.edu.

Received for publication 27 May 1999 and accepted in revised form 15 October 1999.

Additional information can be found in an online appendix at www.diabetes.org/diabetes/appendix.asp.

HNF, hepatocyte nuclear factor; IPF, insulin promoter factor; MODY, maturity-onset diabetes of the young; PCR, polymerase chain reaction.

Recent studies have shown that heterozygous mutations in the transcription factors hepatocyte nuclear factor (HNF)-1 α (1), -1 β (2), -4 α (3), and insulin promoter factor-1 (IPF-1) (4) can cause maturity-onset diabetes of the young (MODY), a form of diabetes characterized by the following: 1) autosomal dominant inheritance; 2) onset usually before 25 years of age; and 3) pancreatic β -cell dysfunction (5,6). In addition to mutations in these four transcription factors, mutations in the gene encoding the glycolytic enzyme glucokinase and downstream target gene of IPF-1 have been associated with MODY (7,8). These findings highlight the importance of transcription factors expressed in the pancreatic β -cell and altered target gene expression in the development of diabetes and prompted us to examine other transcription factors expressed in the β -cell for mutations in patients with MODY.

The HNF-3 proteins were originally identified as factors mediating liver-specific transcription of the transthyretin gene (9). Subsequent studies have shown that they are involved in the regulation of expression of the MODY-associated genes HNF-1 α , -1 β , -4 α , and IPF-1 (10–13). There are three structurally related HNF-3 proteins in mammals (α , β , and γ) and they bind to DNA as monomers through a 100 amino acid winged-helix DNA binding domain, a motif found in the product of fork head, a gene required for terminal segment differentiation including gut invagination in *Drosophila* (9). HNF-3 α , -3 β , and -3 γ are expressed in islets and insulinoma cells (14). Each of these genes has been knocked out in mice. Knockout of the HNF-3 α gene results in decreased glucagon expression and hypoglycemia (15). Inactivation of the HNF-3 γ gene has no apparent effect on glucose homeostasis, possibly because of compensation due to increased expression of HNF-3 α and -3 β (16). The HNF-3 β knockout is an embryonic lethal (17). These findings suggest that the HNF-3 proteins, at least HNF-3 α and HNF-3 β , may be necessary for normal islet development and function. Moreover, they suggest that mutations in these genes may be associated

with MODY or another form of diabetes. To test this hypothesis, we screened a group of subjects with MODY for mutations in the HNF-3 β gene, HNF3B.

The exon-intron organization and partial sequence of HNF3B was determined by analysis of P1-derived artificial chromosome clones 34P11, 56E3, 90P17, and 231N19 (Genome Systems, St. Louis, MO). Physical mapping using the G3 Radiation Hybrid Mapping Panel (Research Genetics, Huntsville, AL) localized HNF3B near D20S184 in chromosome band 20p11, and this polymorphism can be used as a marker for HNF3B in linkage studies. The HNF3B consists of three exons spanning ~4.5 kb, the complete sequence of which has been determined as well as 0.6 kb of the promoter region. This sequence has been deposited in the GenBank database with accession number AF147787 (this sequence is available also in an online appendix [Fig. A1] at www.diabetes.org/diabetes/appendix.asp). Exon 1 encodes the majority of the 5'-untranslated region, exon 2 encodes one nucleotide of the 5'-untranslated region and amino acids 1–23 with intron 2 following codon 23, and exon 3 encodes amino acids 24–457 and the 3'-untranslated region. The predicted amino acid sequence of human HNF-3 β is highly conserved, having 96.7 and 97.8% identity with the mouse and rat orthologues, respectively (Fig. A2 in the online appendix at www.diabetes.org/diabetes/appendix.asp). The promoter region of HNF3B has binding sites for several transcription factors, including Nkx-2, cyclic AMP response element binding protein, HNF-6, and HNF-3.

The three exons, flanking introns, and minimal promoter region were screened for mutations in 57 unrelated Japanese subjects with MODY. This analysis revealed four frequent polymorphisms (Table 1) that were not associated with MODY, including one in the promoter (-213A/G), two silent mutations in the codons for Ala 97 (291C/T) and Gly 279

(837A/G), and one in the 3'-untranslated region (1424C/T). Two rare substitutions in the 5'-untranslated region, -156C/T and -67A/C, which do not disrupt any known transcription factor binding sites, were found in a heterozygous state in two subjects, and two subjects were heterozygous for putative missense mutations, S109N (326G > A) and A328V (983C > T). The first mutation, S109N, affects a conserved amino acid located in the linker region between transactivation domain region V and the winged-helix DNA binding domain; this residue is Ser in the human, mouse, rat, and *Xenopus* (frog) sequences and Thr in the zebrafish (Fig. A2 in the online appendix at www.diabetes.org/diabetes/appendix.asp). The second putative mutation, A328V, is located between the DNA binding domain and transactivation domain region II; this residue is Ala in the human sequence, Ser in the mouse and rat, and Gln in *Xenopus* and zebrafish. Neither mutation was found in 106 normal chromosomes from nondiabetic Japanese subjects.

The S109N mutation was found in a male subject, J2-96, diagnosed with diabetes at the age of 14 years. He has had diabetes for 17 years and is currently treated with insulin. This subject was heterozygous for a rare variant in the promoter region of the HNF-4 α gene (19). His mother and maternal grandmother also were diabetic, but they were not available for study, and he had no siblings. The proband with the A328V mutation, J2-12, was diagnosed with diabetes at 15 years of age. She has had diabetes for 18 years and started insulin treatment 10 years after diagnosis. She currently requires 20 U insulin/day. Her HbA_{1c} is 6.5% and BMI is 21.6. In 1997, her fasting serum C-peptide level was 0.9 ng/ml. She has proliferative retinopathy and diabetic nephropathy with increased serum creatinine (1.55 mg/dl). Her mother and father were both diabetic (ages at diagnosis, 40 and 54 years, respectively) as was her maternal grandmother (age at diagnosis, 50 years). The

TABLE 1
Mutations and polymorphisms in HNF3B

Location	Nucleotide* change	Nucleotide change	Designation	Amino acid change	Designation	Frequency of major allele	
						MODY	Nondiabetic
Promoter	-213 (-4)	A/G	c.-213A/G			A, 0.81	0.79
Exon 1							
5'-UTR	-156	C/T	c.-156C/T			C, 0.99	1.00
	-67	A/C	c.-67A/C			A, 0.99	1.00
Exon 3							
Codon 97	291	C/T	c.291C/T	Ala (GCC or GCT)		C, 0.79	0.77
Codon 109	326	G > A	c.326G > A	Ser (AGC) > Asn (AAC)	S109N	G, 0.99	1.00
Codon 279	837	A/G	c.837A/G	Gly (GGA or GGG)		A, 0.79	0.77
Codon 328	983	C > T	c.983C > T	Ala (GCG) > Val (GTG)	A328V	C, 0.99	1.00
3'-UTR	1424	C/T	c.1424C/T			C, 0.81	0.82

The polymorphism in the promoter region is located 4 bp upstream of the putative start of transcription. The frequency of each substitution was determined in 57 unrelated MODY patients and 53 unrelated nondiabetic (by oral glucose tolerance testing) subjects. Both the patients and control subjects were ascertained through the Diabetes Center, Tokyo Women's Medical University. The polymorphisms -213A/G, 291C/T, 837A/G, and 1424C/T are in the Hardy-Weinberg equilibrium. There is highly significant linkage disequilibrium among these polymorphisms with pairwise linkage disequilibrium coefficient D' values in control subjects of -213A/G and 291C/T, 0.93; -213A/G and 837A/G, 0.93; -213A/G and 1424C/T, 0.85; 291C/T and 837A/G, 0.87; 291C/T and 1424C/T, 0.84; and 837A/G and 1424C/T, 1.00. Similar D' values were observed in patients. *Nucleotide numbering: the A of the ATG of the initiator Met codon of the cDNA sequence is denoted nucleotide +1, and the lowercase c for cDNA in front of the nucleotide number indicates that the reference sequence is the cDNA sequence (if the reference sequence was the genomic sequence, lowercase g for genomic would precede the nucleotide number). The recommendations for a nomenclature system for human gene mutations suggest that these mutations and polymorphisms should be described as HNF3B-c.[nucleotide number][nucleotide change], e.g., HNF3B-c.-213A/G. UTR, untranslated region.

mother (BMI, 19.8) is currently treated with oral hypoglycemic agents: her HbA_{1c} is 9.2%. The mutant allele, V328, was inherited from her mother (the grandmother was deceased and no maternal aunts or uncles were available for study). The genetic studies are inconclusive with regard to whether or not these mutations are the cause of diabetes in these two families.

The functional properties of the two mutant forms of HNF-3 β were compared with those of the wild-type protein. The wild-type HNF-3 β protein stimulated transcription of HNF-1 α and HNF-4 α promoter-reporter gene constructs by 6.4- and 1.7-fold, respectively, in HeLa cells (there was no significant difference in the activity of proteins with or without the NH₂-terminal Xpress epitope tag). The wild-type and mutant proteins had very similar activities, although HNF-3 β -S109N consistently showed ~10% lower activity on both promoters compared with wild-type protein. The physiological significance of such a modest reduction in activity is unknown.

Transcription factors play a key role in the morphogenesis, maturation, and normal function of pancreatic β -cells (20,21). Heterozygous mutations in the transcription factors HNF-1 α , -1 β , and -4 α , and IPF-1 are associated with β -cell dysfunction and early-onset diabetes (1–4), and HNF-3 β plays a role in the regulation of expression of each of these genes (10–13). As an upstream regulator of four genes associated with MODY, HNF-3 β is a candidate diabetes gene itself, and this led us to screen it for mutations in patients with MODY. Our results revealed four common polymorphisms and two uncommon variants in this gene. We also identified two putative missense mutations that generate proteins with activities similar to (A328V) or slightly less than (S109N) wild-type HNF-3 β . The present genetic and molecular biological analyses do not allow us to ascribe a pathogenic role for either mutation, and further studies are required to determine what role they may have, if any, on normal β -cell function and the development of diabetes. Although there is no evidence that mutations in HNF-3 β are a common cause of MODY in Japanese, the mutations may be contributory in other populations. The information presented here will facilitate the search for mutations in other populations and studies of the role of HNF-3 β in determining normal β -cell function.

RESEARCH DESIGN AND METHODS

Study population. The study population consisted of 57 unrelated Japanese subjects with a clinical diagnosis of MODY, the clinical features of which have been described previously (18). These subjects were previously screened for mutations in the HNF-1 α , -1 β , and -4 α , IPF-1, NeuroD1/BETA2, and Nkx2.2 genes (18). **Screening for mutations in HNF3B.** The three exons, flanking introns, and minimal promoter region were screened for mutations by amplifying specific regions using the primers shown in Table 2 and then directly sequencing the polymerase chain reaction (PCR) products using an ABI PRISM dRhodamine terminator

cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA). Functional studies of wild-type and mutant forms of human HNF-3 β . The human HNF-3 β cDNA was generated by PCR and cloned into the mammalian expression vectors pcDNA3.1 and pcDNA3.1/HisB (Invitrogen, Carlsbad, CA). The S109N and A328V mutations were introduced by site-directed mutagenesis (QuikChange mutagenesis kit; Stratagene, La Jolla, CA) to generate pcDNA3.1-HNF-3 β -S109N or -A328V. The reporter gene constructs pGL3-HNF-1 α and pGL3-HNF-4 α were prepared by cloning fragments of the promoter regions of the human HNF-1 α (nucleotides 149–673, GenBank accession no. U73499) and HNF-4 α genes (nucleotides 549–1124, GenBank accession no. U72959) into the firefly luciferase reporter gene vector pGL3-Basic (Promega, Madison, WI). The sequences of all constructs were confirmed. HeLa cells were transfected with FuGENE 6 (Boehringer Mannheim, Indianapolis, IN) with 500 ng of pGL3-HNF-1 α or -4 α , 250 ng of pcDNA3.1-HNF-3 β , -HNF-3 β -S109N or -A328V with and without NH₂-terminal Xpress-epitope tag, and 25 ng of pRL-SV40 to control for efficiency of transfection. pcDNA3.1 DNA was added to each transfection so that the final amount of DNA added was 2 μ g. After 24 h, the transactivation activity of the normal and mutant HNF-3 β proteins was measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized with the Renilla luciferase activity to correct for variations in transfection efficiency. Transfections were carried out in triplicate and were repeated three times with different plasmid preparations.

ACKNOWLEDGMENTS

This study was supported by the Howard Hughes Medical Institute; the Blum-Kovler Foundation; and grants from the U.S. Public Health Service (DK-20595 and DK-44840), the Japanese Ministry of Health and Welfare (for Research on Human Genome and Gene Therapy); the Japanese Ministry of Science, Culture and Sport (10671084); the Uehara Memorial Foundation; and the Naito Memorial Foundation.

REFERENCES

1. Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, Southam L, Cox RD, Lathrop GM, Boriraj VV, Chen X, Cox NJ, Oda Y, Yano H, Le Beau MM, Yamada S, Nishigori H, Takeda J, Fajans SS, Hattersley AT, Iwasaki N, Hansen T, Pedersen O, Polonsky KS, Turner RC, Velho G, Chèvre J-C, Froguel P, Bell GI: Mutations in the hepatocyte nuclear factor-1 α gene in maturity-onset diabetes of the young (MODY3). *Nature* 384:455–458, 1996
2. Horikawa Y, Iwasaki N, Hara M, Furuta H, Hinokio Y, Cockburn BN, Lindner T, Yamagata K, Ogata M, Tomonaga O, Kuroki H, Kasahara T, Iwamoto Y, Bell GI: Mutation in hepatocyte nuclear factor-1 β gene (TCF2) associated with MODY. *Nat Genet* 17:384–385, 1997
3. Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI: Mutations in the hepatocyte nuclear factor-4 α gene in maturity-onset diabetes of the young (MODY1). *Nature* 384:458–460, 1996
4. Stoffers DA, Ferrer J, Clarke WL, Habener JF: Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat Genet* 17:138–139, 1997
5. Fajans SS: Maturity-onset diabetes of the young (MODY). *Diabetes Metab Rev* 5:579–606, 1989
6. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 20:1183–1197, 1997
7. Vionnet N, Stoffel M, Takeda J, Yasuda K, Bell GI, Zouali H, Lesage S, Velho G, Iris F, Passa P, Froguel P, Cohen D: Nonsense mutation in the glucokinase

TABLE 2
Sequences of primers for amplification and sequencing of HNF3B

Region	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Promoter	GAACCAAGAAAGAGCCTCCACATC	CTGATATTCAGCCGGCGTGTTTC	362
Exon 1	GAAATGCTGTGGGCACCTCGGTTG	GCAGATCCCTATGAAAGCCGGATT	413
Exon 2	GGGTGATTGCTGGTCTGTTGTTGTG	GAGGCGCCGCAAGTCAATATTTGA	365
Exon 3	GAATGAAACAGACTCGGAGTCCGG AAGACCTACAGGCGCAGCTACACG TTCTGGACCCTGCACCCTGACT GGAACACCACTACGCCTCAACCA	ATCTCGCTCAGCGTCAGCATCTTG CCTCCTTCAGCGCCAGCTGCTTCT AGGCCTTGAGGTCCATTTTGTG CTTGCTCTCTACTTGTCTCGAT	646 352 520 342

- gene causes early-onset non-insulin dependent diabetes mellitus. *Nature* 356:721–722, 1992
8. Watada H, Kajimoto Y, Miyagawa J, Hanafusa T, Hamaguchi K, Matsuoka T, Yamamoto K, Matsuzawa Y, Kawamori R, Yamasaki Y: PDX-1 induces insulin and glucokinase gene expressions in α TC1 clone 6 cells in the presence of beta-cellulin. *Diabetes* 45:1826–1831, 1996
 9. Lai E, Clark KL, Burley SK, Darnell JE: Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biological function. *Proc Natl Acad Sci U S A* 90:10421–10423, 1993
 10. Cereghini S: Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J* 10:267–282, 1996
 11. Wu K-L, Gannon M, Peshavaria M, Offield MF, Henderson E, Ray M, Marks A, Gamer LW, Wright CVE, Stein R: Hepatocyte nuclear factor 3 β is involved in pancreatic β -cell-specific transcription of the *pdx-1* gene. *Mol Cell Biol* 17:6002–6013, 1997
 12. Levinson-Dushnik M, Benvenisty N: Involvement of hepatocyte nuclear factor 3 in endoderm differentiation of embryonic stem cells. *Mol Cell Biol* 17:3817–3822, 1997
 13. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M: Regulation of a transcription factor network required for differentiation and metabolism. *Science* 281:692–695, 1998
 14. Vaisse C, Kim J, Espinosa R, Le Beau MM, Stoffel M: Pancreatic islet expression studies and polymorphic DNA markers in the genes encoding hepatocyte nuclear factor-3 α , -3 β , -3 γ , -4 γ , and -6. *Diabetes* 46:1364–1367, 1997
 15. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schütz G: Inactivation of the winged helix transcription factor HNF3 α affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* 13:495–504, 1999
 16. Kaestner KH, Hiemisch H, Schütz G: Targeted disruption of the gene encoding hepatocyte nuclear factor 3 γ results in reduced transcription of hepatocyte-specific genes. *Mol Cell Biol* 18:4245–4251, 1998
 17. Dufort D, Schwartz L, Harpal K, Rossant J: The transcription factor HNF3 β is required in visceral endoderm for normal primitive streak morphogenesis. *Development* 125:3015–3025, 1998
 18. Furuta H, Horikawa Y, Iwasaki N, Hara M, Sussel L, Le Beau MM, Davis EM, Ogata M, Iwamoto Y, German MS, Bell GI: β -Cell transcription factors and diabetes: mutations in the coding region of the BETA2/NeuroD1 (NEUROD1) and Nkx2.2 (NKX2B) genes are not associated with maturity-onset diabetes of the young in Japanese. *Diabetes* 47:1356–1358, 1998
 19. Furuta H, Iwasaki N, Oda N, Hinokio Y, Horikawa Y, Yamagata K, Yano N, Sugahiro J, Ogata M, Ohgawara H, Omori Y, Iwamoto Y, Bell GI: Organization and partial sequence of the hepatocyte nuclear factor-4 α /MODY1 gene and identification of a missense mutation, R127W, in a Japanese family with MODY. *Diabetes* 46:1652–1657, 1997
 20. Sander M, German MS: The β cell transcription factors and development of the pancreas. *J Mol Med* 75:327–340, 1997
 21. Edlund H: Transcribing pancreas. *Diabetes* 47:1817–1823, 1998
 22. Antonarakis SE: Recommendations for a nomenclature system for human gene mutations: Nomenclature Working Group. *Hum Mutat* 11:1–3, 1998