

β -Cell Transcription Factors and Diabetes

No Evidence for Diabetes-Associated Mutations in the Gene Encoding the Basic Helix-Loop-Helix Transcription Factor Neurogenic Differentiation 4 (*NEUROD4*) in Japanese Patients With MODY

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The basic helix-loop-helix (bHLH) family of transcription factors plays an important role in the normal development and function of the endocrine pancreas. Heterozygous mutations in the gene encoding one member of this family, *NeuroD1/BETA2*, are associated with a monogenic form of diabetes that resembles maturity-onset diabetes of the young (MODY) in many respects. This result prompted us to screen the genes encoding related bHLH transcription factors that are also expressed in pancreatic islets for diabetes-associated mutations. We have screened 57 unrelated Japanese subjects with a clinical diagnosis of MODY for mutations in the *NeuroD4/Math-3/ATH-3* gene (*NEUROD4*). This analysis revealed seven frequent polymorphisms that were not associated with MODY, including five in the 5'-untranslated region (UTR) (-477G/A, -436delA, -324delT, -107insTTTT, and -104T/C [cDNA sequences]) and two in the 3'-UTR (1027C/T and 1076C/A). A missense mutation, K68T (203A/C), was found in a heterozygous state in one MODY subject and two nondiabetic subjects. The results of our study suggest that genetic variation in *NEUROD4* is not a common cause of MODY in Japanese. *Diabetes* 49:1955-1957, 2000

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BETA2, β -cell E-box transactivator 2; bHLH, basic helix-loop-helix; HNF, hepatocyte nuclear factor; IPF, insulin promoter factor; MODY, maturity-onset diabetes of the young; *NeuroD1*, neurogenic differentiation 1; RT-PCR, reverse transcriptase-polymerase chain reaction; UTR, untranslated region.

The development and normal function of the endocrine pancreas is dependent on a dynamic network of basic helix-loop-helix (bHLH) proteins that affect gene expression in both a positive and a negative manner (1-4). The balance between negatively and positively acting bHLH proteins appears central to the control of islet development with some proteins, such as *Hes-1*, acting in a negative manner and with other proteins, such as *NeuroD1/BETA2* and *neurogenin 3*, acting as positive regulators of gene expression. The bHLH transcription factor neurogenic differentiation 1 (*NeuroD1*) or β -cell E-box transactivator 2 (*BETA2*) plays a particularly prominent role in pancreatic β -cell function. It is required for both normal islet development and regulation of insulin gene transcription (3). Homozygous *NeuroD1/BETA2*-null mice show a reduction in the number of insulin-producing β -cells and fail to develop mature islets. Islet morphogenesis appears to be arrested between E14.5 and E17.5, a period characterized by a major expansion of the β -cell population. In addition, heterozygous mutations in the human *NeuroD1/BETA2* gene (*NEUROD1*) are associated with a monogenic form of diabetes that resembles maturity-onset diabetes of the young (MODY) in many respects except perhaps for a slightly later age at onset (5). A search for other bHLH proteins expressed in the developing pancreas and mature islet cells revealed the presence of several other members of this family, including the *NeuroD1*-related protein *NeuroD4/Math-3/Ath-3* (M.S.G., unpublished observations). *NeuroD4* is widely expressed in the developing nervous system and, after birth, it is expressed at its highest levels in the retina (6,7). It is also found in developing pancreas and mature rodent islet cells, and we have confirmed its expression in adult human islets by a reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay (data not shown). Because *NeuroD4* was expressed in islets and was structurally related to the diabetes-associated bHLH transcription factor *NeuroD1/BETA2*, mutations in *NeuroD4* might also be a cause of diabetes. We tested this hypothesis by screening the *NeuroD4*

TABLE 1
Polymorphisms in *NEUROD4*

Location	Nucleotide*	Nucleotide change†	Designation	Amino acid change	Designation	Frequency of major allele	
						MODY	Nondiabetic
Exon 1							
5'-UTR	-477	G/A	-477G/A	—	—	G, 0.78	0.76
5'-UTR	-436	delA	-436delA	—	—	A, 0.78	0.76
5'-UTR	-324	delT	-324delT	—	—	T, 0.77	0.76
5'-UTR	-107	insTTTT	-107insTTTT	—	—	—, 0.79	0.76
5'-UTR	-104	T/C	-104T/C	—	—	T, 0.79	0.76
Exon 2							
Codon 68	203	A/C	203A/C	Lys (AAG)>Thr (ACG)	K68T	A, 0.99	0.98
3'-UTR	1,027	C/T	1,027C/T	—	—	C, 0.65	0.57
3'-UTR	1,076	C/A	1,076C/A	—	—	C, 0.65	0.57

The nucleotide location within an intron is numbered relative to the splice donor (+) or acceptor (-) site. The frequency of each substitution was determined in 57 unrelated MODY patients and 50 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 markers -477G/A, -436delA, -324delT, -107insTTTT, -104T/C, 1,027C/T, and 1,076C/A are in Hardy-Weinberg equilibrium. There is highly significant linkage disequilibrium among the markers -477G/A, -436delA, -324delT, -107insTTTT, and -104T/C and between 1,027C/T and 1,076C/A. There is also significant linkage disequilibrium between these two clusters of markers. However, 203A/C is not in linkage disequilibrium with either cluster. UTR, untranslated region. *The A of the ATG of the initiator Met codon is denoted nucleotide +1; †the reference sequence is the cDNA sequence.

gene (*NEUROD4*) for mutations in a group of 57 unrelated Japanese subjects with MODY.

The exon-intron organization and partial sequence of *NEUROD4* was determined by analysis of the zp12c07 and the bacterial artificial chromosome clone 272N2 (Research Genetics, Huntsville, AL). Physical mapping using the G3 Radiation Hybrid Mapping Panel (Research Genetics) localized *NEUROD4* near *D12S1632* (logarithm of odds = 6.84) in chromosome band 12q13 (70.6–73.5 cM from pter), and this polymorphism can be used as a marker for *NEUROD4* in linkage studies. The *NEUROD4* consists of two exons spanning ~10 kb, the partial sequence of which has been determined including both exons and flanking intron regions as well as 0.4 kb of the promoter. This sequence has been deposited in the GenBank database with accession numbers AF203900 and AF203901 (this sequence is also available in an online appendix [Fig. A1] at www.diabetes.org/diabetes/appendix.htm). Exon 1 encodes the majority of the 5'-untranslated region (UTR) and exon 2 encodes nine nucleotides of the 5'-UTR, amino acids 1–331, and the 3'-UTR. The predicted amino acid sequence of human NeuroD4 has 88.5% identity with the mouse ortholog, and the two proteins have 100% identity within the bHLH region.

The minimal promoter region, 5'-UTR, coding region, a part of the 3'-UTR, and the flanking introns were screened for mutations in 57 unrelated Japanese subjects with MODY. This analysis revealed seven common polymorphisms (Table 1), none of which were associated with MODY, including five in the 5'-UTR (-477G/A, -436delA, -324delT, -107insTTTT, and -104T/C [cDNA sequences]) and two in the 3'-UTR (1027C/T and 1076C/A). One subject was heterozygous for the missense mutation K68T (203A>C), which affects a conserved amino acid located 19 amino acids NH₂-terminal to the basic region. (This residue is conserved and is also Lys in mouse, chicken, and frog [*Xenopus*] NeuroD4 [Fig. A2 in the online appendix])

The K68T mutation was found in a male subject who was diagnosed with diabetes at age 18 years. He was obese and had a BMI of 32.3 kg/m². He was treated with oral hypoglycemic agents, and his HbA_{1c} value was 8.2%. His urine C-peptide level was 47.9 µg/day, and he had no diabetic complications (including retinopathy). His father and paternal grandfather were also diabetic but were not available for study. The K68T mutation was also found in 2 of the 50 nondiabetic Japanese control subjects. They were previously confirmed to be nondiabetic by oral glucose tolerance testing but were not available for follow-up studies. Therefore, it is inconclusive whether this mutation was the cause of diabetes in this subject. Further studies are required to determine what role, if any, it may have on normal β-cell function and the development of diabetes.

Transcription factors play a key role in the morphogenesis, maturation, and normal function of pancreatic β-cells (8). Heterozygous mutations in the transcription factors insulin promoter factor (IPF)-1 and hepatocyte nuclear factor (HNF)-1α, -1β, and -4α are associated with β-cell dysfunction and MODY (9–12) and heterozygous mutations in NeuroD1/BETA2, a transcriptional activator of the IPF-1 and insulin genes with a monogenic form of diabetes that appears to have a later age of onset than MODY (5). The results presented here suggest that mutations in *NEUROD4* are not a common cause of MODY in Japanese. However, mutations in this gene may be contributory in other populations. The information presented here will facilitate the search for mutations in *NEUROD4* in other populations and studies of the role of NeuroD4 in determining normal β-cell function.

RESEARCH DESIGN AND METHODS

Study population. The study population consisted of 57 unrelated Japanese subjects with a diagnosis of MODY, the clinical features of which have been described previously (13). These subjects have previously been screened for mutations in the HNF-1α, -1β, and -4α and the IPF-1, NeuroD1/BETA2, Nkx2.2, and HNF-3β genes. Mutations in the HNF-1α gene have been excluded as the

TABLE 2
Sequences of primers for amplification and sequencing of *NEUROD4*

Region	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)
Promoter/Exon 1			
	F1 GACGTGCTGAAAAGGTGGGAG *CTTTACTCCTTAACCTCAGC	R1 TGATTGTGAGTGTCTATGTG *CCCCAGCCGAAAAGACACGCC *CCTTACCCAGAAGAGACTTTCC	1,104
Exon 2			
	F2 CAGGCACTAACACTGCAATG	R2 *GATGAAGGAGATGTGTTTCC *AGAGTAGCATGGCATGACTCG	902
	F3 *TCCTCCTGGAGAAGCACGAGG *AGCCATCTGCCTGACTGCAG	R3 *TTGGCACAGATTATGCAATG	757

The minimal promoter and exons 1 and 2 were amplified using primer pairs F1 and R1, F2 and R2, and F3 and R3 shown above, and the PCR products were then sequenced using the primers denoted with an asterisk. The PCR mixture contained, in addition to the standard reagents, 1.0 mmol/l MgCl₂. The annealing temperature for the PCR was 60°C.

cause of MODY in each of these subjects. However, this group does include one subject with a nonsense mutation in the HNF-1 β gene (10) and two subjects with putative diabetes-associated mutations in the HNF-4 α gene (14).

RT-PCR analysis of NeuroD4 mRNA. RNA was prepared from 5×10^4 adult human pancreatic islets obtained from the Islet Isolation Core Facility of Washington University School of Medicine. Two micrograms of total RNA was reverse transcribed using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD). The presence of NeuroD4 mRNA was demonstrated using a nested PCR strategy. The first PCR was carried out using the forward primer 5'-CATGGCTGTCACTGTGAAG-3' and the reverse primer 5'-ACCACCCTATGTTGGAGAGC-3'. An aliquot of the PCR product was then amplified with the inner primers 5'-CTCTCTCCAGCTCACAGTTGC-3' and 5'-AAGATCTCTGCAAGGCTAC-3'. The PCR mixture contained, in addition to the standard reagents, 1.8 mmol/l MgCl₂. The annealing temperature for the PCR was 60°C.

Screening for mutations in *NEUROD4*. The minimal promoter region, 5'-UTR, coding region, a part of the 3'-UTR, and the flanking introns were screened for mutations by amplifying specific regions using the primers shown in Table 2 and then by directly sequencing the PCR products using an ABI Prism dRhodamine terminator cycle-sequencing ready-reaction kit (PE Applied Biosystems, Foster City, CA).

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