Brief Genetics Report

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

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Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes characterized by autosomal dominant inheritance, onset usually before 25 years of age, and β-cell dysfunction (1,2). Recent studies have shown that MODY can result from mutations in four different transcription factors (hepatocyte nuclear factor [HNF]-1α [3], HNF-1β [4], HNF-4α [5], and insulin promoter factor [IPF]-1 [6]), as well as the glycolytic enzyme glucokinase (7). Because genetic studies have suggested that MODY may be primarily a disorder of abnormal gene expression in the pancreatic β-cell, we have begun to examine other transcription factors, especially those that play a key role in β-cell differentiation and maturation, for mutations in patients with MODY.

Molecular biological studies have identified a diverse assemblage of transcription factors that are expressed in islets and insulinoma cells (8,9), several of which appear to play important roles in islet and β-cell differentiation and maturation. These include IPF-1, mutations of which are associated with MODY (6), BETA2/NeuroD1 (10,11), Nkx2.2 (8), Isl-1 (12), and Pax-4 (13) and -6 (14,15). BETA2/NeuroD1, a member of the basic helix-loop-helix family of transcription factors, was isolated as an important regulator of transcription of the insulin gene (10). Mice homozygous for a targeted disruption of the NeuroD1 gene exhibit abnormal islet morphology with reduced numbers of β-cells, develop neonatal diabetes, and die 3–5 days postpartum (11). Nkx2.2 is a member of a family of proteins whose homeodomains are homologous to that of the Drosophila NK-2 protein (16). Within the pancreas, Nkx2.2 is expressed in insulin-producing β-cells and glucagon-producing α-cells and pancreatic polypeptide (PP)-secreting cells of the islet, but not in somatostatin-containing δ-cells (23). Mice homozygous for a targeted disruption in the Nkx2.2 gene die within a few days of birth with severe hyperglycemia (23). Immunohistochemical studies show that these animals lack mature β-cells and that there is a reduction in the number of α- and PP cells. While there are no insulin-expressing cells, there is a large population of cells expressing a subset of β-cell proteins such as amylin and IPF-1, suggesting a block in maturation of β-cells in animals lacking Nkx2.2. These studies indicate that NeuroD1 and Nkx2.2 play a central role in β-cell development and that deficiency of either protein is associated with diabetes. These results led us to screen the NeuroD1 and Nkx2.2 genes for mutations in a group of patients with MODY.

The study population consisted of 57 unrelated Japanese subjects with a clinical diagnosis of MODY based on presentation of non–type 1 diabetes before 25 years of age and/or being a member of a family in which type 2 diabetes was present in three or more generations. Of these subjects, 32 met strict criteria for a diagnosis of MODY (i.e., diabetes in at least three generations with autosomal dominant transmission and diagnosis before 25 years of age in at least one affected subject), and 7 were members of a family in which at least 2 subjects were diagnosed with non–type 1 diabetes before 25 years of age. The clinical features of this study population have been summarized previously (17). Mutations in the HNF-1α gene have been excluded as the cause of MODY in each of these subjects. However, this group does include one subject with a nonsense mutation in the HNF-1β gene (4) and two subjects with possible mutations in the HNF-4α gene (17).

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FISH, fluorescence in situ hybridization; HNF, hepatocyte nuclear factor; IPF, insulin promoter factor; MODY, maturity-onset diabetes of the young; PAC, P1-derived artificial chromosome; PCR, polymerase chain reaction; PP, pancreatic polypeptide; YAC, yeast artificial chromosome.

TABLE 1
Sequences of primers for amplification and sequencing of *NEUROD1* and *NKX2B*

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (base pairs)
NEUROD1			
Exon 2–1	ACTTTTCGCAAGCATTTGTACAGG	CGCGTTCAGTCCGTGCATGC	483
Exon 2–2	ATGACTAAGGCTCGCCTGGA	GGAGGCTTAACGTGGAAGAC	564
Exon 2–3	AGTCCGCCTTACGGTACCATG	GACAGTCACTGTAAGCACAG	447
NKX2B			
Exon 1	ACGAATTGACCAAGTGAAGCTAC	AACCCGGGCTGCGGCTGCAGGAAT	380
Exon 2–1	ATCCAGGGTGCTCCGAGTCTGGTGCA	CGCGCTTCATCTTGTAGCGGT	377
Exon 2–2	GAGCGCGAACACCTGGCCAGCCTC	GTGGAGCCGAGAGTCAACTCGACT	494

The PCR mixture contained, in addition to the standard reagents, 1.0 mmol/l $MgCl_2$ and 5% dimethylsulfoxide. The annealing temperatures for the PCR for *NEUROD1* and *NKX2B* were 60 and 65°C, respectively.

The human NeuroD1 gene (*NEUROD1*) contains two exons and has been mapped to human chromosome band 2q32 (18). Exon 1 encodes part of the 5′-untranslated region of NeuroD1 mRNA and exon 2 codes for 11 nucleotides of the 5′-untranslated region and the 356 amino acid NeuroD1 protein. The entire protein coding region and splice-acceptor site of intron 1 of *NEUROD1* were screened for mutations: intron 1, 91 nucleotides adjacent to and including the splice acceptor site; and exon 2, 11 nucleotides of 5′-untranslated region, entire coding region, and 38 nucleotides of the 3′-untranslated region. This region was amplified using the primers shown in Table 1, and the polymerase chain reaction (PCR) products were sequenced directly on both strands using an AmpliTaq FS dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and the ABI PRISM 377 DNA sequencer (Foster City). The sequences of this region of *NEUROD1* in the 57 unrelated Japanese MODY subjects were identical to one another and to the *NEUROD1* sequence listed under GenBank accession number AF045152 (this sequence is also available in an on-line appendix [Fig. 1] at www.diabetes.org/diabetes/appendix.htm) except for a previously described G-to-A replacement in codon 45 (GCC to ACC) that results in an Ala-to-Thr substitution (A45T) (19). Of 57 MODY subjects, 6 (10.5%) were heterozygous for this polymorphism, as were 7 of 53 (13.2%) nondiabetic Japanese control subjects. Thus, the A45T polymorphism does not appear to be associated with MODY.

The exon-intron organization and partial sequence of the human Nkx2.2 gene (*NKX2B*) was determined by analysis of P1-derived artificial chromosome (PAC) clone 310N11 (Genome Systems, St. Louis, MO), which was identified by PCR screening of PAC DNA pools using the primers Nkx2.2-P1 (5′-ACGAATTGACCAAGTGAAGCTAC-3′) and Nkx2.2-P2 (5′-CGTTGGTGTCCGGCAGGTCTAAG-3′). The chromosomal localization of *NKX2B* was determined by fluorescence in situ hybridization (FISH) to normal human metaphase chromosomes (20), and its localization in the integrated genetic and physical map, by determining its yeast artificial chromosome (YAC) address (21). FISH analysis showed that biotin-labeled PAC 310N11 DNA specifically hybridized only to human chromosome 20 (data not shown). Specific labeling of 20p11-p12 was observed on four (18 cells), three (6 cells), or two (1 cell) chromatids of the chromosome 20 homologues in 25 cells examined. Of 92 signals observed (92 of 100 20p chromatids were labeled), all 92 (100%) were located at 20p11-p12. Of these, 84 (91%) were located at 20p11, 4 (4.5%) were located at the junction of 20p11-p12, and 4 signals (4.5%) were located at 20p12. No background signals were observed at other chromosomal sites. These results localized the human Nkx2.2 gene to chromosome 20, band p11. Physical mapping studies confirmed this localization and placed *NKX2B* in the overlapping YACs 770D4 and 855E7. These two YAC clones include the polymorphic simple sequence repeat polymorphisms D20S868 and D20S180, which can be used as markers for *NKX2B* in linkage studies.

The human *NKX2B* consists of two exons that span a region of ~3.5 kilobase pairs of human chromosome 20, band p11. The partial sequence of *NKX2B* has been deposited in the GenBank database with accession numbers AF019414 and AF019415 (this sequence is also available in an on-line appendix [Fig. 2] at www.diabetes.org/diabetes/appendix.htm). The single intron interrupts codon 87. Exon 1 includes the 5′-untranslated region and codons 1–86 and the first nucleotide of codon 87, and exon 2 includes the second and third nucleotides of codon 87, codons 88–273, and the 3′-untranslated region. The amino acid sequence of Nkx2.2 is highly conserved, and there is 98% identity between the human and mouse orthologs (22). There is 93% nucleotide sequence identity between the protein coding regions of the human and mouse Nkx2.2 genes.

The protein coding region and splice-donor and acceptor sites of *NKX2B* were screened for mutations by amplifying regions of exons 1 and 2 and adjacent regions of intron 1 using the primers shown in Table 1. The sense strands of the PCR products were sequenced directly using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems). Sequences that were ambiguous were repeated and determined on the antisense strand. The region of *NKX2B* screened for mutations included: exon 1, 33 nucleotides of the 5′-untranslated region and the coding region; intron 1, 41 and 54 nucleotides in the region of the splice donor and acceptor sites, respectively; and exon 2, the coding region and 116 nucleotides of the 3′-untranslated region. The sequence of the coding region and flanking intron region of *NKX2B* was identical in the 57 unrelated Japanese subjects with MODY to that from PAC 310N11, except for a C-to-A substitution located in the 3′-untranslated region 13 nucleotides downstream of the translation termination codon, TGA. Of 57 MODY subjects, 3 (5.3%) were heterozygous for this polymorphism. We did not determine the frequency of

this polymorphism in nondiabetic Japanese subjects. In addition to the C-to-A substitution in the 3′-untranslated region described above, we observed a G-to-A substitution in codon 42 (GCC to ACC) resulting in an Ala-to-Thr substitution (A42T) in a 45-year-old nondiabetic white woman with no family history of diabetes. The A42T substitution was not seen in any of the Japanese MODY patients, and we did not screen other white subjects or subjects of other racial or ethnic backgrounds for the presence of this sequence change.

Transcription factors play a key role in the development and maturation of the cells of the islets of Langerhans. Deficiency of *IPF1* is associated with absence of the pancreas, and heterozygous mutations in this gene are associated with MODY (6). The demonstration that deficiency of the transcription factors NeuroD1 and Nkx2.2 in mice were associated with defects in islet formation and diabetes led us to screen these genes for mutations in patients with MODY. Our results indicate that mutations in the coding regions of these genes are not a common cause of MODY in Japanese. However, they do not exclude the possibility of promoter variants that lead to abnormal expression of these genes and thereby contribute to diabetes. While there is no evidence that these genes are associated with MODY in Japanese, they may be involved in the development of MODY in other populations or the cause of other disorders, such as congenital diabetes. The information presented in this report will facilitate genetic studies of the role of *NEUROD1* and *NKX2B* in diabetes and other disorders.

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