

***NeuroD/BETA2* Gene Variability and Diabetes**

No Associations to Late-Onset Type 2 Diabetes but an A45 Allele May Represent a Susceptibility Marker for Type 1 Diabetes Among Danes

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Mutations in the *NeuroD/BETA2* gene have been shown to associate with type 2 diabetes. In the present study, we examined mutations in the *NeuroD/BETA2* gene for association with either type 1 or 2 diabetes. Three variants were identified in patients with type 2 diabetes: Ala45Thr (allelic frequency 0.36, 95% CI 0.31–0.41), Pro197His (0.01), and Ser259Ser (0.01). Ala45Thr and Pro197His were not associated with type 2 diabetes, but the transmission disequilibrium test showed unequal transmission of the A45 allele to offspring with type 1 diabetes ($\chi^2 = 5.90$, $P < 0.02$, odds ratio 1.55, 95% CI 0.91–2.63). This association could not be explained by linkage disequilibrium between the Ala45 allele and *IDDM7* (*D2S152*), which is also located on chromosome 2q32. When tested in vitro, the biological activity of Thr45 ($117 \pm 36\%$ vs. Ala45) and His197 ($90 \pm 28\%$ vs. Pro197) on the regulation of the human insulin gene promoter appeared normal. In conclusion, mutations in the *NeuroD/BETA2* gene are not a common cause of late-onset type 2 diabetes among Danes. However, in the type 1 diabetic Danish population, the Ala45Thr variant of *NeuroD/BETA2* may represent a susceptibility marker independent of *IDDM7* on chromosome 2q32. *Diabetes* 49:876–878, 2000

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IPF, insulin promoter factor; MODY, maturity-onset diabetes of the young; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; TDT, transmission disequilibrium test.

After the discovery that mutations in members of the hepatocyte nuclear factor transcription factor family are responsible for types 1, 3, and 5 of maturity-onset diabetes of the young (MODY) and that insulin promoter factor (IPF)-1 is the cause of MODY 4 (1–4), mutations in genes encoding transcription factors essential to the normal development and functioning of pancreatic β -cells have become an important paradigm in the study of the genetics of type 2 diabetes. *NeuroD/BETA2*, an important regulator of insulin gene transcription (5) that is essential to normal pancreatic islet development (6), has been considered a plausible candidate gene for MODY (7) or late-onset type 2 diabetes (8,9). Moreover, in 2 families, an Arg111Leu and a His206finsC variant of *NeuroD/BETA2* has been shown to associate with type 2 diabetes (9). *NeuroD/BETA2* has also been considered a positional candidate gene for type 1 diabetes because of its close relation to the *IDDM7* locus (*D2S152*) at chromosome 2q32–33. Mutation scanning in U.S. Caucasians followed by case-control and family association studies could not show an association between type 1 diabetes and the Ala45Thr variant of *NeuroD/BETA2* (10); however, a study in the Japanese population did find a positive association between the Ala45Thr variant and type 1 diabetes (11).

The purposes of the present study were 1) to perform mutational analysis of the *NeuroD/BETA2* gene (GenBank accession number U50822) in 82 patients with type 2 diabetes, 2) to perform association studies of identified genetic variabilities in 246 type 2 diabetic patients and 236 age- and sex-matched glucose-tolerant control subjects, 3) to perform association studies of the A45T variant in 138 unrelated type 1 diabetic Danish Caucasian sib-pair families, and 4) to test the biological activity of identified variants in vitro.

Other than the previously reported A45T, P197H, and an S259S variant, we did not identify any new missense variants in *NeuroD/BETA2*. The A45T polymorphism was investigated in an association study, and the allelic frequency (0.36, 95% CI 0.31–0.41, vs. 0.37, 0.32–0.42) and carrier distribution (data not

TABLE 1

Transmission of the A45 (wild-type) and T45 (mutant) alleles of *NeuroD* from heterozygous parents to offspring with type 1 diabetes (affected) and without type 1 diabetes (unaffected)

	Transmitted allele		χ^2_{tdt}	P_{tdt}
	A45	T45		
Affected offspring	115 (59)	81 (41)	5.90	0.02
Unaffected offspring	38 (48)	42 (52)	0.20	0.66

Data are n (%), unless indicated otherwise. TDT was performed as described by Spielman et al. (19) with the ETDT Version 1.0 program (www.gene.ucl.ac.uk/pub/packages/curtis) (20). Because of the low number of transmissions observed to unaffected offspring, no significant difference in transmission pattern was demonstrated between unaffected and affected offspring ($\chi^2 = 2.6$, $P = 0.11$). $P < 0.05$ was chosen as level of significance.

shown) were similar and in Hardy-Weinberg equilibrium in both the type 2 diabetic patients and the control subjects. The allelic frequencies of the P197H variant and the S259S variant were 0.01. The H197 variant did not segregate convincingly with type 2 diabetes in 2 families carrying this variant (data not shown). The S259S variant was not further investigated.

In the families with type 1 diabetes, the wild-type allele A45 (allelic frequency 0.59, 95% CI 0.53–0.64) was transmitted to 59% of affected offspring, and the T45 allele (0.41, 0.35–0.47) was transmitted to 41% (χ^2 transmission disequilibrium test [χ^2_{tdt}] = 5.90, $P_{\text{tdt}} < 0.02$, odds ratio 1.55, 95% CI 0.91–2.63) (Table 1). The A45 allele was transmitted to 48% of the unaffected offspring, and the T45 allele was transmitted to 52% ($\chi^2_{\text{tdt}} = 0.2$, $P_{\text{tdt}} = 0.7$), thus suggesting that the T45 allele might be protective against the development of type 1 diabetes. Because the A45 and T45 alleles are separated by only 2 cM from the *IDDM7* locus (*D2S152*) on chromosome 2q32 (10), the association between these 2 alleles could potentially be explained by linkage disequilibrium between the A45T variant of *NeuroD/BETA2* and *IDDM7*, a locus that is also associated with type 1 diabetes in the examined families (12). However, the association of A45 with type 1 diabetes could not be explained by linkage disequilibrium between the A45T variant of *NeuroD/BETA2* and *IDDM7* ($P = 0.78$) (data not shown).

Another susceptibility locus for type 1 diabetes (*IDDM2*) has been shown to be caused by a functionally active variable number of tandem repeats (classes I–III) of the minisatellite upstream of the insulin gene on chromosome 11p15 (13). The protective class III alleles have been shown to increase the transcription of the insulin gene in vitro (14) and to be associated with higher levels of insulin mRNA in the thymus (15). Increased expression of insulin in the thymus has been suggested to protect against the development of type 1 diabetes by inducing tolerance to this important β -cell-specific autoantigen (14,15). Likewise, Arg111Leu and a His206fsC variant of *NeuroD/BETA2* that associate with type 2 diabetes have been shown to reduce the activity of the rat *insulin2* promoter, suggesting possible interference with the normal transcription of β -cell-specific genes in subjects carrying these mutations (9).

Therefore, to test for potential pathogenic impact of the other variants of the *NeuroD/BETA2* proteins on regulation of the insulin gene, we investigated their biological function by comparing the transactivational efficiency of all 3 variants of

TABLE 2

Transcriptional activity on the human insulin promoter of the T45 and H197 variants of *NeuroD* compared with the wild-type (A45) clone transiently transfected into NIH 3t3 cells and estimated by luciferase activity in whole-cell lysates

Clone	pCMV4	T45	H197	A45
Luciferase activity (arbitrary units)	24 \pm 6%	117 \pm 36%	90 \pm 28%	100%

Data are means \pm SD of 7 individual experiments performed in duplicate (see text). Each experiment was normalized to 100% for the wild-type A45 clone of *NeuroD*.

human *NeuroD/BETA2* (A45/P197, T45/P197, and A45/H197) on a luciferase construct harboring nucleotide –333 to +114 of the human insulin promoter. Each of the 3 *NeuroD/BETA2* variants transfected into NIH 3t3 mouse fibroblasts together with its co-activators: IPF-1 and E47 resulted in a comparable activation of the insulin promoter, as estimated by luciferase activities (Table 2). Based on these functional studies, we conclude that in terms of *insulin* gene transcription, both the T45 and the H197 mutations are likely to represent functionally normal *NeuroD* variants with no direct role in the pathogenesis of either type 1 or type 2 diabetes.

Recent evidence, however, suggests that *NeuroD/BETA2* is an important regulator of cell differentiation in enteroendocrine cells, and overexpression of *NeuroD/BETA2* induces apoptosis in transfected cells (16). Because the A45 is the wild-type variant of *NeuroD/BETA2*, it could be expected to be more active than the T45 variant and to potentially induce a higher level of apoptosis in the β -cells. Hypothetically, just a small increase in β -cell apoptosis conferred by the A45 allele would release a sufficient amount of autoantigens to provoke an autoimmune response in those individuals who carry also other susceptibility genes for type 1 diabetes (*IDDM1*, *IDDM2*, *IDDM3*, etc.). Such a polygenic model could also help to explain why the A45 variant associates with type 1 diabetes but not with type 2 diabetes negative for anti-GAD antibodies. Polygenic interaction was further suggested when we stratified the affected offspring according to HLA haplotypes (*IDDM1*) (83 DR3/4 heterozygotes [high-risk] and 114 non-DR3/4 [low-risk]). In those subjects carrying the high-risk HLA haplotype (DR3/4), the A45 allele was transmitted more often than expected (52 transmissions of A45 and 31 transmissions of T45; $\chi^2_{\text{tdt}} = 5.83$, $P_{\text{tdt}} = 0.02$).

However, in the Japanese population, the T45 allele associates with type 1 diabetes (11), whereas in the Danish population it appears to be the A45 allele that does so. These data indicate that it is not the A45T variant of *NeuroD/BETA2* that confers susceptibility; rather, the A45/T45 alleles in the Danish and Japanese populations, as a result of different genetic recombinations, could be in linkage disequilibrium with the same yet unidentified functional mutation in either *NeuroD/BETA2* regulatory elements or in a different but closely linked gene at the *NeuroD/BETA2* locus. However, until more detailed maps of chromosome 2q32 and studies of the linkage disequilibrium across the *NeuroD/BETA2* locus are available, we cannot conclude on the specific status of the A45T variant. Nevertheless, the studies from the Japanese and Danish populations suggest that the *NeuroD* gene may be

regarded as an independent susceptibility locus for type 1 diabetes on chromosome 2q32 that is close to, but genetically distinct from, the *IDDM7* locus.

RESEARCH DESIGN AND METHODS

Subjects. The primary mutation analysis comprised 82 type 2 diabetic patients (45 men and 37 women) who were negative for anti-GAD antibodies, had a mean age of 67 years (range 43–87), a mean BMI of 30.6 kg/m² (22.3–48.8), and a mean reported disease duration of 55.7 years (27–75). Of these patients, 11% were treated with diet, 83% were treated with diet and/or oral hypoglycemic agents, and 6% were treated with insulin. Each patient was a member of a 2-generation family, and ~80% had at least 1 first-degree relative with either impaired glucose tolerance or type 2 diabetes. The patients were recruited either from the Danish family resource bank at the Department of Human Genetics, University of Copenhagen, or from the outpatient clinic at Steno Diabetes Center. Association with type 2 diabetes was performed on a sample of 246 type 2 diabetic patients who tested negative for anti-GAD antibodies and were recruited from the outpatient clinic at Steno Diabetes Center and in 236 age- and sex-matched glucose-tolerant control subjects who were traced through the Central Personal Register in the County of Copenhagen (17). For association studies with type 1 diabetes, 138 unrelated type 1 diabetic Danish Caucasian sib-pair families with 289 affected and 121 nonaffected offspring, including both parents in 99 families and 1 parent in 39 families, were available for genotyping (12). All study participants were Danish Caucasians by self-report. Before participation, the purpose and risks of the study were carefully explained both vocally and in writing, and informed consent was obtained. The protocol was approved by the committee of Ethics in Copenhagen County and was in accordance with the Helsinki Declaration.

Genetic analyses. Polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) and heteroduplex analyses were performed at 2 different experimental settings (18) in a primary gene scanning of *NeuroD/BETA2* on genomic DNA from 82 patients with type 2 diabetes. Information on the primers and cycling conditions used in the amplification and sequencing of the human *NeuroD* gene is described in Table A1, which can be found in an online appendix on the *Diabetes* website (www.diabetes.org/diabetes/appendix.asp). SSCP variants were sequenced as described previously (18). The prevalent A45T amino acid polymorphism in *NeuroD/BETA2* was identified using PCR-restriction fragment length polymorphism with restriction enzyme *MwoI* and primers 2a/b. The reactions were analyzed on 3% agarose gels.

Transactivation assays. The human *NeuroD/BETA2* gene was cloned with primers (5'-cggaattcatgacaaatcgtagcgca-3' [forward] and 5'-gcgaattcctaa tcagaaatggcat-3' [reverse]) in a PCR reaction with *pfu* polymerase on 2 µg DNA from wild-type, heterozygous, and homozygous carriers of P197H and A45T. PCR products were *EcoRI* digested and cloned into pCSA eucaryotic expression plasmid using standard procedures. Cloned *NeuroD/BETA2* (10 ng) was transfected into 30,000 NIH 3T3 mouse fibroblasts with the following co-activators: human *IPF-1* (10 ng) and *E47* (5 ng), *renilla* as the internal standard (6 ng), human *insulin* promoter construct (200 ng), pCMV4 as the negative control (25 ng), and pBluescript as the carrier (250 ng). Lipofectamine (Gibco, Grand Island, NY) was used according to the manufacturer's recommendations. Each series of transfections was done in duplicate, and luciferase/*renilla* activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

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