

Lack of Association of the Ala⁴⁵Thr Polymorphism and Other Common Variants of the NeuroD Gene With Type 1 Diabetes

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Variation in genes necessary for normal functioning and development of β -cells, e.g., *NEUROD1*, which encodes a transcription factor for the insulin gene and is important in β -cell development, causes maturity-onset diabetes of the young. Some studies have reported an association between a nonsynonymous Ala⁴⁵Thr (+182G→A) single nucleotide polymorphism (SNP) in *NEUROD1* and type 1 diabetes, but this result has not been consistently found. To clarify this, we genotyped Ala⁴⁵Thr in 2,434 type 1 diabetic families of European descent and Caucasian ethnicity from five different countries. Taking the allele frequency of 36% for Thr⁴⁵ and an odds ratio (OR) of 1.2, this sample provided >99% power to detect an association ($P < 0.05$). We could not confirm the association ($P = 0.77$). No evidence of population heterogeneity in the lack of association of Thr⁴⁵ with type 1 diabetes was observed. To evaluate the possibility that another *NEUROD1* variant was associated with type 1 diabetes, we resequenced the gene in 32 U.K. affected individuals and identified and genotyped all common SNPs (minor allele frequency >10%; $n = 5$) in 786 families. We report no evidence of association of these common variants in *NEUROD1* and type 1 diabetes in these samples. *Diabetes* 53: 1158–1161, 2004

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MODY, maturity-onset diabetes of the young; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test.

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The pathogenesis of type 1 diabetes may involve intrinsic functional defects in the insulin-producing β -cells of the pancreas in addition to the extrinsic destruction of these cells by the immune system. Hence, genes that affect β -cell development, function, or apoptosis might conceivably influence susceptibility to type 1 diabetes.

Maturity-onset diabetes of the young (MODY) encompasses a group of single-gene disorders characterized clinically by nonimmune diabetes that develops in the young and is generally inherited in an autosomal-dominant fashion (1). Mutations in genes such as hepatocyte nuclear factor-4 α , glucokinase, and hepatocyte nuclear factor-1 α , which are necessary for the normal functioning of β -cells, have previously been associated with various subsets of MODY (MODY-1, -2, and -3, respectively). NeuroD is a helix-loop-helix protein that acts as a transcription factor for the insulin gene and plays a pivotal role in the development of pancreatic β -cells (2). Mutations in this gene have been associated with a form of MODY found in Iceland (3). Mice deficient in *Neurod1* develop severe diabetes and die in the perinatal period with severe hyperglycemia due to the absence of pancreatic β -cells (4). Sequence changes that lead to synthesis of a transcriptionally inactive form of NeuroD have been demonstrated in two families with premature onset of non-immune-mediated diabetes, although not all individuals with these changes developed diabetes (5).

The level of expression of NeuroD influences apoptosis in β -cells; overexpression induces apoptosis in transfected cells (6). β -Cell apoptosis results in the release of numerous autoantigens that may be relevant to the induction of self-tolerance and the prevention of autoimmunity. Genetic variation that increases thymic insulin gene expression correlates with protection against type 1 diabetes, perhaps due to increased tolerance to the insulin autoantigen (7,8). It is therefore possible that polymorphisms in *NEUROD1* may affect the development of self-tolerance and hence the genetic predisposition to type 1 diabetes, as well as influence basic β -cell development.

TABLE 1
Transmission analysis of the Ala⁴⁵ Thr polymorphism of *NEUROD1* in 2,434 families (sets 1 and 2)

DIL reference number	Parental minor allele frequency (%)	TDT			GTRR
		T	U	P_{TDT}	
2402 (Ala ⁴⁵ Thr)	36.6	1510	1494	0.77	0.62

Values for the Thr⁴⁵ allele from heterozygous parents to affected children with correction for multiple affected siblings. The minor allele frequency is an average from all parents. DIL, Diabetes and Inflammation Laboratory; GTRR, genotype relative risk; T, transmitted; U, untransmitted.

Interestingly, the gene encoding for this protein has been mapped to chromosome 2q31-q35, where three putative loci have been previously linked to type 1 diabetes (*IDDM7*, *IDDM12*, and *IDDM13*) (9). Mutation scanning in Caucasians followed by case-control and family association studies failed to demonstrate an association between type 1 diabetes and variation in several candidate genes including *HOXD8*, *NEUROD1*, and *IGFBP5* (10). These investigators described a variant in *NEUROD1* consisting of a G→A transition at the first position of codon 45 in exon 2, resulting in an amino acid substitution (Ala⁴⁵Thr). The minor (A) allele frequency was 35%. Studies in the Japanese (11) and French (12) populations have failed to show evidence for association of this variant with MODY or type 2 diabetes.

However, Iwata et al. (13) reported an association between this polymorphism and type 1 diabetes in a case-control study in a Japanese population (60 subjects with type 1 diabetes and 174 unaffected control subjects). The relative risk of type 1 diabetes for the variant versus wild homozygote was estimated to be 3.1. A subsequent case-control study performed in a French population with 80% power to detect an effect similar to that reported by Iwata et al. failed to detect an association between the variant and type 1 diabetes (as well as BMI and age at onset of diabetes) (14), although, again, the sample size was small (87 case and 114 control subjects). In another case-control study, Hansen et al. (15) failed to detect an association between the Ala⁴⁵Thr variant and type 2 diabetes. However, this group reported a positive association with type 1 diabetes in 138 Danish sibpair families. Of note, in this study, in contrast to the report by Iwata et al. (13), the Ala⁴⁵ NeuroD variant conferred susceptibility to type 1 diabetes, suggesting the possible presence of another (unidentified) functional variant in the gene in linkage disequilibrium with the Ala⁴⁵Thr polymorphism.

TABLE 2
Common polymorphisms identified by resequencing of *NEUROD1* in 32 randomly selected probands

DIL reference number	Position relative to initiation codon	IUB code	Minor allele frequency (%)	Within coding sequence?	Sequence context
4565	36	r	33	5' UTR	GCAGGAGGC[A/G]CGGCGTCC
2402	1799	r	34	Ala to Thr	ACCTCGAA[A/G]CCATGAAC
4566	4571	r	8	No	TGTCACAG[A/G]AAAATTTA
4567	5033	w	8	No	ACATTGGA[A/T]GACTACCC
4568	7124	s	21	No	CTGGCCAG[C/G]CATTATGT

DIL, Diabetes and Inflammation Laboratory; IUB, International Union of Biochemistry; UTR, untranslated region.

More recently, Cinek et al. (16) in a small case-control study of 285 Czech children with type 1 diabetes and 289 control children reported that the Thr⁴⁵ NeuroD variant increased susceptibility to type 1 diabetes.

Despite the putative association of this polymorphism with type 1 diabetes, the functional consequences of this polymorphism are unclear, as activation of the human insulin promoter by Thr⁴⁵ NeuroD does not seem to be different from Ala⁴⁵ NeuroD (15). This is in marked contrast to the effects of the rare Arg111Leu and His206finsC variants of *NEUROD1* that have been reported to be associated with type 2 diabetes. These variants reduce the activity of the rat insulin-2 promoter in vivo, suggesting interference with the transcription of insulin and other β -cell-specific genes in subjects carrying these variations (5).

Given this prior evidence for a primary role of NeuroD in type 1 diabetes, we genotyped the Ala⁴⁵Thr polymorphism in 2,434 type 1 diabetic families of European descent from five different countries. Assuming a minor allele frequency of 36% and an OR of 1.2, this would provide >99% power to detect an effect of this variant on risk of type 1 diabetes at $P < 0.05$.

Parental genotype frequencies were found to be consistent with Hardy-Weinberg equilibrium. Transmission analysis was performed, and we were unable to confirm the reported association of NeuroD Ala⁴⁵Thr with type 1 diabetes (Table 1). It is possible that Thr⁴⁵ is associated with type 1 diabetes in certain populations, perhaps via interaction with alleles of unlinked loci that vary significantly in frequency between populations. Therefore, analysis by population was undertaken, but no evidence of population heterogeneity in the lack of association of Thr⁴⁵ with type 1 diabetes was observed (online supplementary data, Appendix 1.4 [available at <http://diabetes.diabetesjournals.org>]).

It is also possible that previous reports of association with type 1 diabetes are indicative of a true effect, but caused by another polymorphism in linkage disequilibrium with the Ala⁴⁵Thr variant.

We, therefore, resequenced polymerase chain reaction (PCR) products from 32 individuals with type 1 diabetes encompassing, exon 1, intron 1, and exon 2 of *NEUROD1*. In addition, we resequenced 3 kb upstream of exon 1 and 3 kb downstream of exon 2 so as to identify all common polymorphisms in the regions most likely to harbor promoter/regulator sequences. The individuals were probands selected at random from the Diabetes U.K. Warren 1 affected sibpair family collection (17). Five additional single nucleotide polymorphisms (SNPs) were identified (Table 2) and subsequently genotyped in our collection of

TABLE 3
Transmission analysis of other common alleles identified in *NEUROD1* in 786 families (set 1)

DIL reference number	Parental minor allele frequency (%)	TDT			GTRR
		T	U	P_{TDT}	P_{GTRR}
4565	36.4	525	548	0.52	0.7
4566*	15.6	312	268	0.09	0.16
4567	13.0	257	287	0.20	0.45
4568	37.6	473	450	0.48	0.75

*Transmission analysis of DIL4566 in sets 1 and 2 (T 728, U 690: $P_{TDT} = 0.31$; $P_{GTRR} = 0.32$). DIL, Diabetes and Inflammation Laboratory, GTRR, genotype relative risk; T, transmitted; U, untransmitted.

type 1 diabetic families. By using a SNP discovery-sequencing panel of 32 affected individuals, we had 88% probability of detecting SNPs with minor allele frequencies of 3.3%, 96% probability for 5% frequency, and 99.8% for 10% frequency.

One variant (DIL4564) in the region 5' to the gene was extremely rare (minor allele frequency <0.001%); association testing was not performed for this SNP. In addition to the Ala⁴⁵Thr (DIL2402), of the SNPs identified by resequencing, one SNP (DIL4565) was located in the 5' untranslated region, while the remainder were in the region 3' to the gene (Table 2). D' and r^2 values for the common polymorphisms were calculated (online supplementary data, Appendixes 1.1 and 1.2).

To evaluate the possibility of other variation in *NEUROD1* contributing to the pathogenesis of type 1 diabetes, all other common variants were genotyped using a two-stage approach. This strategy utilizes two subsets of families with type 1 diabetes. One subset is initially genotyped (set 1: 786 multiplex families), and if the results of a transmission disequilibrium test (TDT) are below a threshold of $P < 0.2$ when testing by single-locus TDT, genotyping is continued in the second subset (set 2) and analyzed together with the set 1 families (total 2,434 families) (18). Our set 1 families provided ~90% power to detect a causal allele with a frequency of 10% and an OR of 1.3. One marker (DIL4566, $P = 0.09$ in stage 1), in order to further investigate its possible association with type 1 diabetes, was subsequently genotyped in the rest of the family collection. No evidence of association of DIL4566 with type 1 diabetes was found ($P = 0.31$) (Table 3). There was no evidence of population heterogeneity in the lack of association of DIL4566 with type 1 diabetes; our sample size provided adequate power (>60%) to detect an association for OR at 1.5 and $P < 0.05$ in three of the five populations studied (online supplementary data, Appendix 1.4).

We have performed a large genetic study of *NEUROD1* describing all common variants in the gene for the U.K. type 1 diabetes population and subsequently performing adequately powered association studies with type 1 diabetes. Given the negative results obtained in our study, it is unlikely that the common variants (>10% minor allele frequency) in *NEUROD1* studied here influence susceptibility to type 1 diabetes in any major way, at least in the populations we studied.

RESEARCH DESIGN AND METHODS

All families were Caucasian and of European descent and were composed of two parents and at least one affected child. The population studied consisted of 458 multiplex families from the Diabetes U.K. Warren 1 collection (17), 328 multiplex families from Human Biological Data Interchange (U.S.) (19), 80 simplex families from Yorkshire (U.K.) (20), 250 multiplex/simplex families from Belfast (21), 159 Norwegian simplex families (22), 233 Romanian simplex families (23), and 926 Finnish multiplex/simplex families (24). All DNA samples were collected after informed consent was obtained.

PCR and sequencing. DNA samples from 32 individuals were amplified using specifically designed forward and reverse primers. PCR was performed in 96-well polypropylene microtitre plates (ABgene; Epsom, Surrey, U.K.) in a 25- μ l final reaction volume. Stock (5 μ l) of genomic DNA (4 ng/ μ l) was dispensed into each well of the plate together with 20 μ l of PCR mix containing 0.5 mmol/l dNTP, 2 mmol/l MgCl₂, 15 ng/ μ l forward and reverse primer, and 0.5 units TaqGold (Perkin Elmer Applied Biosystems, Foster City, CA) and the plate sealed with an Adhesive Sealing Sheet (ABgene). Direct sequencing of PCR products was performed using an Applied Biosystems 3700 capillary sequencing instrument (Applied Biosystems).

Genotyping. DIL4565 and DIL2402 were genotyped by biplex Invader (Third Wave Technologies, Madison, WI). For DIL2402, a 343-bp fragment of DNA was amplified by means of forward and reverse primers (5'-GTTTGCCTCTC CCTGTGTA-3' and 5'-TCTCAATTTAAACGCTCCAG-3', respectively). In the case of DIL4565, an 864-bp fragment of DNA was amplified by means of forward and reverse primers (5'-TAATCTCTCTGCGGGTAAAAA-3' and 5'-CTCCCAATGTGCGTAAAAACA-3', respectively). The SNPs were then genotyped according to the manufacturer's instructions using probe sets designed and synthesized by the manufacturer (Third Wave Technologies). The SNPs DIL4566, DIL4567, and DIL4568 were genotyped by a TaqMan 5' nuclease assay (Applied Biosystems, Warrington, U.K.). TaqMan probes and primers were designed by the suppliers (Applied Biosystems). All genotyping data were double scored by a second researcher to minimize error.

Statistical analysis. All statistical analyses were performed with STATA (<http://www.stata.com>) making use of the Genassoc package (<http://www-gene.cimr.cam.ac.uk/clayton/software/>). To test for Hardy-Weinberg equilibrium, a modified test was used that allows for allelic frequencies to differ between known population groups. Allelic association was tested using the TDT. Genotype relative risk, a 2-degree-of-freedom test was determined by conditional logistic regression using case and pseudocontrol subjects as previously described (25). Robust variance estimates were used for the calculation of P values and 95% CI in order to correct for nonindependence of transmissions within families with more than one affected sibling. The power calculations assumed a multiplicative model and that all 2,434 families were trios (two parents and one affected child); as such, the values obtained were slight underestimations, since 804 families had more than one affected child.

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