Brief Genetics Report

Lack of Association of the Ala^{45}Thr Polymorphism and Other Common Variants of the NeuroD Gene With Type 1 Diabetes

Adrian Vella,[1] Joanna M.M. Howson,[1] Bryan J. Barratt,[1] Rebecca C.J. Twells,[1] Helen E. Rance,[1] Sarah Nutland,[1] Eva Tuomilehto-Wolf,[2] Jaakko Tuomilehto,[2,3] Dag E. Undlien,[4] Kjersti S. Rønningen,[5] Cristian Guja,[6] Constantin Ionescu-Tîrgovişte,[6] David A. Savage,[7] and John A. Todd[1]

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 Ala^{45}Thr (+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 Ala^{45}Thr in 2,434 type 1 diabetic families of European descent and Caucasian ethnicity from five different countries. Taking the allele frequency of 36% for Thr^{45} 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 Thr^{45} 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

From the [1]Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, U.K.; the [2]Diabetes and Genetic Epidemiology Unit, National Public Health Institute, University of Helsinki, Helsinki, Finland; the [3]Department of Public Health, University of Helsinki, Helsinki, Finland; the [4]Institute of Medical Genetics, Ulleval University Hospital, University of Oslo, Oslo, Norway; the [5]Laboratory of Molecular Epidemiology, Division of Epidemiology, Norwegian Institute of Public Health, Oslo, Norway; the [6]Clinic of Diabetes, Institute of Diabetes, Nutrition and Metabolic Diseases 'N. Paulescu,' Bucharest, Romania; and the [7]Department of Medical Genetics, Queen's University Belfast, Belfast City Hospital, Belfast, Northern Ireland.

Address correspondence and reprint requests to Professor John A. Todd, JDRF/WT Diabetes and Inflammation Laboratory, University of Cambridge, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, U.K. E-mail: john.todd@cimr.cam.ac.uk.

Received for publication 18 December 2003 and accepted in revised form 5 January 2004.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

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

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-4α, glucokinase, and hepatocyte nuclear factor-1α, 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}	P_{GTRR}
2402 (Ala45Thr)	36.6	1510	1494	0.77	0.62

Values for the Thr^{45} 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 ($Ala^{45}Thr$). 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 $Ala^{45}Thr$ 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 Ala^{45} 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 $Ala^{45}Thr$ polymorphism. 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 Thr^{45} 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 Thr^{45} NeuroD does not seem to be different from Ala^{45} 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 $Ala^{45}Thr$ 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 $Ala^{45}Thr$ with type 1 diabetes (Table 1). It is possible that Thr^{45} 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 Thr^{45} 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 $Ala^{45}Thr$ 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 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	TGTCCCAG[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.

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 Ala[45]Thr (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-μl final reaction volume. Stock (5 μl) of genomic DNA (4 ng/μl) was dispensed into each well of the plate together with 20 μl of PCR mix containing 0.5 mmol/l dNTP, 2 mmol/l $MgCl_2$, 15 ng/μl 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 CCTTGTTGA-3′ and 5′-TCTCAATTTAAAACGCTCCAG-3′, respectively). In the case of DIL4565, an 864-bp fragment of DNA was amplified by means of forward and reverse primers (5′- TAATCTCTCCTGCGGGTAAAAA-3′ and 5′-CTCCCAATGTGCGTAAAATACA-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.

REFERENCES

1. Fajans SS, Bell GI, Polonsky KS: Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *N Engl J Med* 345:971–980, 2001
2. Madsen OD, Jensen J, Petersen HV, Pedersen EE, Oster A, Andersen FG, Jorgensen MC, Jensen PB, Larsson LI, Serup P: Transcription factors contributing to the pancreatic beta-cell phenotype. *Horm Metab Res* 29:265–270, 1997
3. Kristinsson SY, Thorolfsdottir ET, Talseth B, Steingrimsson E, Thorsson AV, Helgason T, Hreidarsson AB, Arngrimsson R: MODY in Iceland is associated with mutations in HNF-1alpha and a novel mutation in NeuroD1. *Diabetologia* 44:2098–2103, 2001
4. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ: Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* 11:2323–2334, 1997
5. Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T, Saad M, Warram JH, Montminy M, Krolewski AS: Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet* 23:323–328, 1999
6. Mutoh H, Naya FJ, Tsai MJ, Leiter AB: The basic helix-loop-helix protein BETA2 interacts with p300 to coordinate differentiation of secretin-expressing enteroendocrine cells. *Genes Dev* 12:820–830, 1998
7. Kennedy GC, German MS, Rutter WJ: The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription. *Nat Genet* 9:293–298, 1995
8. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C: Insulin expression in human

thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat Genet* 15:289–292, 1997
9. Copeman JB, Cucca F, Hearne CM, Cornall RJ, Reed PW, Ronningen KS, Undlien DE, Nistico L, Buzzetti R, Tosi R, et al.: Linkage disequilibrium mapping of a type 1 diabetes susceptibility gene (IDDM7) to chromosome 2q31-q33. *Nat Genet* 9:80–85, 1995
10. Owerbach D, Naya FJ, Tsai MJ, Allander SV, Powell DR, Gabbay KH: Analysis of candidate genes for susceptibility to type 1 diabetes: a case-control and family-association study of genes on chromosome 2q31-35. *Diabetes* 46:1069–1074, 1997
11. 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
12. Dupont S, Vionnet N, Chevre JC, Gallina S, Dina C, Seino Y, Yamada Y, Froguel P: No evidence of linkage or diabetes-associated mutations in the transcription factors BETA2/NEUROD1 and PAX4 in type II diabetes in France. *Diabetologia* 42:480–484, 1999
13. Iwata I, Nagafuchi S, Nakashima H, Kondo S, Koga T, Yokogawa Y, Akashi T, Shibuya T, Umeno Y, Okeda T, Shibata S, Kono S, Yasunami M, Ohkubo H, Niho Y: Association of polymorphism in the NeuroD/BETA2 gene with type 1 diabetes in the Japanese. *Diabetes* 48:416–419, 1999
14. Dupont S, Dina C, Hani EH, Froguel P: Absence of replication in the French population of the association between beta 2/NEUROD-A45T polymorphism and type 1 diabetes. *Diabetes Metab* 25:516–517, 1999
15. Hansen L, Jensen JN, Urioste S, Petersen HV, Pociot F, Eiberg H, Kristiansen OP, Hansen T, Serup P, Nerup J, Pedersen O: 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: Danish Study Group of Diabetes in Childhood, and the Danish IDDM Epidemiology and Genetics Group. *Diabetes* 49:876–878, 2000
16. Cinek O, Drevinek P, Sumnik Z, Bendlova B, Sedlakova P, Kolouskova S, Snajderova M, Vavrinec J: NEUROD polymorphism Ala^{45}Thr is associated with type 1 diabetes mellitus in Czech children. *Diabetes Res Clin Pract* 60:49–56, 2003
17. Bain SC, Todd JA, Barnett AH: The British Diabetic Association: Warren repository. *Autoimmunity* 7:83–85, 1990
18. Lowe CE, Cooper JD, Chapman JM, Barratt BJ, Twells RCJ, Green EA, Savage DA, Guja C, Ionescu-Tirgoviţe C, Tuomilehto-Wolf E, Tuomilehto J, Todd JA, Clayton DG: Cost-effective analysis of candidate genes using htSNPs. *Genes Immun.* In press
19. Lernmark A: Human cell lines from families available for diabetes research (Letter). *Diabetologia* 34:61, 1991
20. Feltbower RG, McKinney PA, Parslow RC, Stephenson CR, Bodansky HJ: Type 1 diabetes in Yorkshire, UK: time trends in 0–14 and 15–29-year-olds, age at onset and age-period-cohort modelling. *Diabet Med* 20:437–441, 2003
21. Patterson CC, Carson DJ, Hadden DR, Waugh NR, Cole SK: A case-control investigation of perinatal risk factors for childhood IDDM in Northern Ireland and Scotland. *Diabetes Care* 17:376–381, 1994
22. Undlien DE, Akselsen HE, Joner G, Dahl-Jorgensen K, Aagenaes O, Sovik O, Thorsby E, Ronningen KS: No difference in the parental origin of susceptibility HLA class II haplotypes among Norwegian patients with insulin-dependent diabetes mellitus. *Am J Hum Genet* 57:1511–1514, 1995
23. Ionescu-Tirgoviste C, Guja C, Herr M, Cucca E, Welsh K, Bunce M, Marshall S, Todd JA: Low frequency of HLA DRB1*03-DQB1*02 and DQB1*0302 haplotypes in Romania is consistent with the country's low incidence of type I diabetes. *Diabetologia* 44 (Suppl. 3):B60–B66, 2001
24. Tuomilehto J, Lounamaa R, Tuomilehto-Wolf E, Reunanen A, Virtala E, Kaprio EA, Akerblom HK: Epidemiology of childhood diabetes mellitus in Finland: background of a nationwide study of type 1 (insulin-dependent) diabetes mellitus: the Childhood Diabetes in Finland (DiMe) Study Group. *Diabetologia* 35:70–76, 1992
25. Cordell HJ, Clayton DG: A unified stepwise regression procedure for evaluating the relative effects of polymorphisms within a gene using case/control or family data: application to HLA in type 1 diabetes. *Am J Hum Genet* 70:124–141, 2002