Association of Polymorphism in the NeuroD/BETA2 Gene With Type 1 Diabetes in the Japanese

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NeuroD/BETA2, a transcription factor of the insulin gene, also plays an important role in the development of pancreatic β-cells. Recently, the NeuroD/BETA2 gene has been mapped to the long arm of human chromosome 2 (2q32) where the *IDDM7* gene has previously been mapped, implying its involvement in diabetes. To identify mutations in the NeuroD/BETA2 gene that may predispose patients to develop diabetes, we studied the gene in 50 Japanese subjects with diabetes (4 with type 1 and 46 with type 2) by the polymerase chain reaction (PCR) followed by single-strand conformation polymorphism and sequencing analyses. Further analysis was performed in 392 Japanese subjects (60 with type 1 and 158 with type 2 diabetes and 174 healthy control subjects) by mismatch PCR restriction fragment length polymorphism. We found a DNA polymorphism of the NeuroD/BETA2 gene. A nucleotide G-to-A transition results in the substitution of alanine to threonine at codon 45 (Ala45Thr). The frequencies of heterozygotes for the Ala45Thr variant were 9.8% in the control subjects, 9.5% in the patients with type 2 diabetes, and 25.0% in the patients with type 1 diabetes, a significant difference (P = 0.006). Because the variant of the NeuroD/BETA2 gene (Ala45Thr) is associated with type 1 but not type 2 diabetes, it may be implicated in the loss of pancreatic β-cells in type 1 diabetes. *Diabetes* 48:416–419, 1999

Type 1 diabetes is manifested by ketosis-prone hyperglycemia associated with an almost complete loss of insulin-producing pancreatic β-cells (1). It has been shown that type 1 diabetes is a multifactorial autoimmune disease for which susceptibility is determined by both environmental and genetic factors (1–3). Although major histocompatibility complex genotype is an important genetic determinant, the concordance rate in identical twins is only 50% and several other contributing genes have also been identified (2). By analyzing both linkage and linkage disequilibrium at 21 microsatellite marker loci in 348 affected sib-pair families and 107 simplex families, Copeman et al. (4) reported that *IDDM7* mapped to human chromosome 2q31-q33 within 2 cM of D2S152. In addition, the NeuroD/BETA2 gene was recently mapped to chromosome 2q32 (5).

The NeuroD gene was identified as one of the differentiation factors for neurogenesis (6), along with Neurogenin (7), NeuroD-related factor (NDRF) (8), and NeuroM (9), but the expressions of those genes, except for NeuroD, are restricted to neural tissue. On the other hand, other groups have independently cloned the same gene as the BETA2 (β-cell E-box transactivator 2), a class B basic helix-loop-helix (bHLH) protein expressed in pancreatic endocrine cells, intestine, and brain (10–13). The heterodimer of the NeuroD/BETA2 and ubiquitous bHLH proteins (E12/E47) binds insulin E-box complex with a high affinity and also activates the transcription of the insulin gene in pancreatic β-cells (11). It was recently reported that NeuroD/BETA2-deficient mice developed severe diabetes and died perinatally because of the striking reduction in the number of insulin-producing β-cells, thus suggesting that NeuroD/BETA2 is essential for the morphogenesis or differentiation of pancreatic β-cells (14).

All previous reports have suggested the significance of the NeuroD/BETA2 in the development and physiology of pancreatic β-cells and its possible involvement in the pathogenesis of diabetes. In the present study, we evaluated Japanese patients with diabetes for the presence of mutations in the NeuroD/BETA2 gene.

RESEARCH DESIGN AND METHODS

Study subjects. The 392 subjects evaluated were 158 Japanese patients with type 2 diabetes diagnosed according to World Health Organization criteria, 60 Japanese patients with type 1 diabetes who represented the classical criteria "ketosis prone, abrupt onset," and 174 nondiabetic healthy Japanese control subjects. Serum C-peptide concentrations were <1.0 ng/ml in all subjects with type 1 diabetes.

Polymerase chain reaction, single-strand conformation polymorphism, and nucleotide sequence of the NeuroD/BETA2 gene. Total DNA was extracted from 2×10^7 peripheral mononuclear cells of individuals by the guanidine thiocyanate method (SepaGene kits; Sanko Junyaku, Tokyo). DNA (0.2 μg) was used as a polymerase chain reaction (PCR) template. Five pairs of oligonucleotide primers (Table 1) were designed to be ~400 bp apart to amplify five segments, which were named fragments 1, 2, 3, 4, and 5. The human NeuroD/BETA2 gene comprises two exons. Exon 1 is untranslated, and the protein coding region is located only on exon 2 (15). The segments covered the entire coding region (nucleotide [nt] 173 to 1243) of the human NeuroD/BETA2 gene (GenBank accession number U50822). Reaction was performed as the manufacturer recom-

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Ab, antibody; bHLH, basic helix-loop-helix; nt, nucleotide; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

TABLE 1
Sequence of oligonucleotides for PCR

Fragment	Primer sequence	Sequence region (nt)	Size of the amplified fragment (bp)
1	Forward: 5′-GTTTGCCTCTCCCTTGTTGA-3′	136–155	343
	Reverse: 5′-TCTCAATTTAAAACGCTCCAG-3′	458–478	
2	Forward: 5′-AGAGGAGGAGGACGAAGATGA-3′	343–363	414
	Reverse: 5′-AAAGTCCGAGGATTGAGTTGC-3′	736–756	
3	Forward: 5′-GCGCTTGGCCAAGAACTACAT-3′	601–621	366
	Reverse: 5′-GTGCAATCAGTCAGAGGGCTT-3′	946–966	
4	Forward: 5′-GCGCTTCCTTCCCTGTACACC-3′	804–824	323
	Reverse: 5′-GAAGATTGATCCGTGGCTTTG-3′	1106–1126	
5	Forward: 5′-GTCCGCCGAGTTTGAGAAAAA-3′	1039–1059	379
	Reverse: 5′-CTTTGATCCCCTGTTTCTTC-3′	1398–1417	

mended. Briefly, after an initial incubation at 95°C for 5 min, 35 cycles of 1 min at 95°C, 1 min at 56°C (fragment 1), 58°C (fragments 2, 3, and 4), or 54°C (fragment 5), and 1 min 30 s at 72°C were performed. Mixtures of 2 µl PCR products and 18 µl denaturing solution (95% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol) were heated for 5 min at 95°C, chilled on ice, and subjected to 12% PAGE (acrylamide: bis = 49:1, 1.0 mm × 8.0 cm × 9.0 cm). Electrophoresis was performed at a constant voltage of 80V for 16 h. After electrophoresis, the DNA bands were visualized by silver staining with a commercially available reagent kit (Daiichi Pure Chemicals, Tokyo) (16). DNA fragments showing variations on the single-strand conformation polymorphism (SSCP) analysis were cloned into pT7blue T-vector (Novagen, Madison, WI) and sequenced.

Detection of missense mutation of NeuroD/BETA2 by mismatch PCR–restriction fragment length polymorphism analysis. One polymorphism was detected in fragment 1. The sequence analysis revealed the nt variations to be at the first position of codon 45 (GCC to ACC). This polymorphism was confirmed by mismatch PCR–restriction fragment length polymorphism (RFLP) using *Eco57*I digestion (17). The sequence of the downstream primer is 5′-TCT CAA TTT AAA ACG CTC CAG-3′. The upstream primer is 5′-GAC AAG AAG GAG GAC GAC CCT GAA-3′ which contains a 2-bp mismatch just proximal to its 3′ end (underlined) such that the 198-bp amplified product incorporates a restriction site for the restriction enzyme *Eco57*I in the presence of guanine at nt 305 but not in the presence of adenine. The mismatch PCR was performed for 30 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 1 min 30 s at 72°C. The product (5 µl) was digested with 1 U *Eco57*I at 37°C for 10 h and size-separated by 4% agarose gel electrophoresis (MetaPhor agarose; FMC BioProducts, Rockland, ME). The more frequent allele (305G allele) produced two fragments of 157 and 41 bp (the 41-bp band could not be visualized clearly) when digested with *Eco57*I, while the 198-bp fragment containing the 305A allele failed to be cleaved by *Eco57*I. Heterozygous individuals were clearly identified by the presence of two bands (198 and 157 bp), which were then further confirmed by DNA sequencing.

Anti-glutamic acid decarboxylase antibody assay. Anti-glutamic acid decarboxylase antibody (anti-GAD Ab) was determined by a radioimmunoprecipitation assay using the Cosmic kit (Cosmic, Tokyo) (18,19).

Statisticalanalysis. Differences in the means and proportions were tested by Student's t test and the continuity-corrected χ^2 test, respectively. Odds ratios (ORs) and 95% CIs were calculated from regression coefficients and standard errors in the logistic regression analysis. The statistical computations were done by PC-SAS version 6.04 (SAS Institute, Cary, NC).

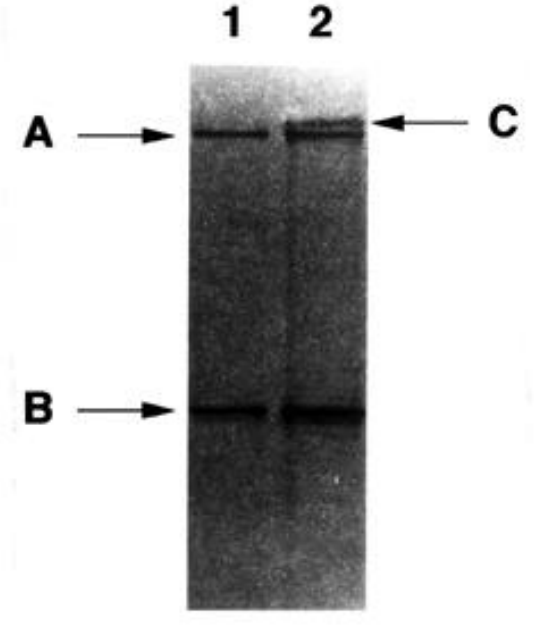

FIG. 1. PCR-SSCP analysis of NeuroD/BETA2 (fragment 1). Genomic DNA from the peripheral blood mononuclear cells was analyzed. Heat-denatured PCR products were electrophoresed in 12% polyacrylamide gels under the conditions described in METHODS. Two bands (A and B) in *lane 1* were detected in the wild homozygotes in fragment 1. An extra band (C) was detected in *lane 2*. DNA sequencing procedures revealed the subject in *lane 2* to be the Ala45Thr (GCC to ACC) heterozygote.

RESULTS

Identification of a DNA polymorphism in the human NeuroD/BETA2 gene. To identify any mutations in the NeuroD/BETA2 gene that predispose individuals to develop diabetes, 50 Japanese patients with diabetes (4 with type 1 and 46 with type 2) were initially screened using PCR-SSCP. One polymorphism was detected in fragment 1 in 6 subjects (2 of 4 with type 1 and 4 of 46 with type 2 diabetes) (Fig. 1). No polymorphism was detected in fragments 2, 3, 4, or 5. PCR-SSCP revealed one variant pattern in the coding region. The sequence analysis revealed a point-mutation with the replacement of guanine (G) with adenine (A) in the NeuroD/BETA2 gene at the first position of codon 45 (GCC to ACC) (data not shown), which resulted in the amino acid substitution Ala45Thr.

Frequencies of the Ala45Thr genotype in patients with type 1 and type 2 diabetes. We evaluated a normal population and populations with type 1 and type 2 diabetes to test whether the polymorphism has any association with the pathogenesis of diabetes. We performed a mismatch PCR-RFLP analysis on 174 nondiabetic control subjects and 218 patients with diabetes (60 with type 1 and 158 with type 2) (Fig. 2) and could not find any homozygotes of the Ala45Thr variant in the three populations. We compared the frequencies of heterozygotes for the Ala45Thr variant and determined them to be 25.0% in the subjects with type 1 diabetes, 9.5% in the subjects with type 2 diabetes, and 9.8% in the control subjects (Table 2). The heterozygotes of Ala45Thr variant were more

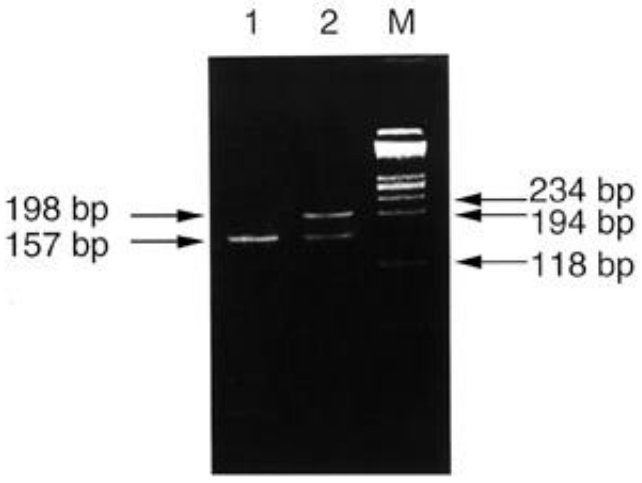

FIG. 2. Mismatch PCR-RFLP analysis: 4% agarose gel electrophoresis of mismatched PCR fragments after *Eco57*I digestion. *Lane 1*, wild homozygote; *lane 2*, Ala45Thr (GCC to ACC) heterozygote; *lane M*, size marker.

than twice as frequent in patients with type 1 diabetes as in the control subjects or patients with type 2 diabetes (P = 0.006 for both); the frequency of the variant in patients with type 2 diabetes was not significantly different from that of the controls (P = 1.00) (Table 2). The OR of type 1 diabetes for the heterozygous variant versus wild homozygote was estimated to be 3.1 (95% CI 1.6–6.2) based on comparison with the control subjects, and the OR of type 2 diabetes for the heterozygote versus wild homozygote was 1.0 (95% CI 0.5–2.0) compared with the control subjects (Table 2).

Ala45Thr genotype and other characteristics of type 1 diabetes. Type 1 diabetes is mainly caused by an autoimmune mechanism, and anti-GAD Ab is known to be a useful marker for the presence of anti-islet specific autoimmune process. To examine whether the polymorphism was associated with the presence of anti-GAD Ab, we compared the frequencies of anti-GAD Ab–positive type 1 diabetes patients with and without the variant (Table 3). There was no relation between anti-GAD Ab and the variant, nor was there any difference in HbA_{1c}, BMI, or age of onset between type 1 diabetes patients with and without the variant (Table 3).

TABLE 2
Subject characteristics and genotype frequencies of the NeuroD/BETA2 variant

	Type 1 diabetic subjects	Type 2 diabetic subjects	Control subjects
n	60	158	174
Sex (M/F)	28/32	85/73	85/89
Age (years)	36.7 ± 16.7	60.1 ± 12.8	48.4 ± 7.7
BMI (kg/m^2)	20.5 ± 2.5	22.8 ± 3.3	22.9 ± 2.7
HbA_{1c} (%)	8.5 ± 1.6	7.3 ± 1.5	4.7 ± 0.4
Ala45Thr heterozygotes	15 (25.0)*†	15 (9.5)‡	17 (9.8)
OR (95% CI) for the variant	3.1 (1.6–6.2)	1.0 (0.5–2.0)	1

Data are n, means ± SD, or n (%). *P = 0.006 vs. type 2 diabetes; †P = 0.006 vs. control subjects; ‡P = 1.00 vs. control subjects.

DISCUSSION

We found a variant (Ala45Thr) in the NeuroD/BETA2 gene and studied its association with diabetes. Recently, the same variant was reported in two other independent studies (20,21); in those studies, however, no association was found of the variant with maturity onset diabetes of the young in Japanese (21) or type 1 diabetes in American whites (20). In contrast, we found that in Japanese subjects, the frequency of this mutation in patients with type 1 diabetes was significantly higher than in patients with type 2 diabetes or control subjects. Because type 1 diabetes develops as a consequence of an extensive destruction of pancreatic β-cells, these results raise the possibility that the mutation of the NeuroD/BETA2 gene may be implicated in the loss of pancreatic β-cells. On the other hand, the mutation was also observed at a similar frequency in both healthy controls and patients with type 2 diabetes, indicating that the mutation alone is not sufficient to induce diabetes.

Our observation that the mutation of the NeuroD/BETA2 gene was associated with type 1 diabetes is also consistent with the previous finding in which *IDDM7* mapped to human chromosome 2q31-q33, where NeuroD/BETA2 gene was located (2q32) (4,5). Copeman et al. (4) suggested, however, that *IDDM7* might be the human homolog of mouse *idd5*, based on the conserved synteny between the regions of human chromosome 2q and mouse chromosome 1 where they are respectively localized. If this is the case, NeuroD/BETA2 may differ from the susceptibility gene represented by *IDDM7*, because mouse NeuroD/BETA2 gene was mapped to mouse chromosome 2 (4). To address this issue, the molecular nature of *IDDM7* and *idd5* have to be identified.

Since type 1 diabetes is an autoimmune disease directed against pancreatic β-cells and the presence of anti-GAD Ab is a reliable marker for the development of autoimmunity to pancreatic β-cells, we also evaluated the association of the NeuroD/BETA2 variant with the anti-GAD Ab in patients with type 1 diabetes. No frequency difference in the NeuroD/BETA2 variant was detected between individuals with and without the anti-GAD Ab or between young- and older-onset patients with type 1 diabetes. The polymorphism thus appears to pose an overall risk for the development of type 1 diabetes.

NeuroD/BETA2 can bind to the insulin E-box sequence as a heterodimer with the ubiquitous bHLH protein (E47/E12); the complex is called IEF-1 (insulin enhancer factor 1), which regulates the insulin gene transcription (11,13,22). The polymorphism was located in a 54–amino-acid cluster rich in glutamate and aspartate residues NH_2-terminal to the bHLH domain. The cluster exhibits a characteristic of some transcriptional activators (11,23). It might be possible that the mutated NeuroD/BETA2 protein could alter its functional properties through the phosphorylation of its replaced threonine residue in the putative activation domain.

A recent study on the targeted disruption of the NeuroD/BETA2 gene showed that homozygous NeuroD-null mice developed severe diabetes and died 3–5 days postpartum (14). In the histological examination of these mice, pancreatic islets failed to develop morphologically, and the number of specific islet populations, especially β-cells, was markedly decreased at the embryonic stage, suggesting NeuroD/BETA2 to be essential for the morphogenesis or differentiation of insulin-producing pancreatic β-cells. In addition, that study also indicated that apoptosis of the pancreatic β-cells was very likely to occur in the NeuroD/BETA2 knock-

TABLE 3
Genotype frequencies of the NeuroD/BETA2 variant in subjects with type 1 diabetes

	Ala45Thr heterozygotes	Wild homozygotes	*P* value
n	15	45	—
Sex (M/F)	7/8	21/24	1.00
BMI (kg/m^2)	20.2 ± 0.6	20.6 ± 0.4	0.63
HbA_{1c} (%)	8.5 ± 1.6	7.3 ± 1.5	0.45
Anti-GAD Ab–positive	6	19	1.00
Age of diabetes onset (years)	26.1 ± 3.9	27.2 ± 2.3	0.81
Onset before age 25 years	5	23	0.37

Data are *n*, means ± SE, or *P*.

out mice. These experimental data thus raise the possibility that the NeuroD/BETA2 variant plays some role in the development of apoptosis of pancreatic β-cells. It is possible that the altered form of NeuroD/BETA2 leads to the production of fewer islets in development. As a consequence, those with the variant might have a reduced "reserve" supply of islets when faced with an autoimmune assault, leading to greater susceptibility to develop disease. An alternative possibility exists in which the polymorphism is in disequilibrium with type 1 diabetes not because it is diabetogenic, but because it is in disequilibrium with another diabetogenic gene nearby. To test whether the polymorphism has any physiologic significance, studies on its metabolic and physiologic effect on β-cells are currently underway in our laboratory.

In summary, we identified a polymorphism of the NeuroD/BETA2 gene (Ala45Thr). Our study suggested that this variant may be associated with the risk for development of type 1 diabetes in the Japanese. Since the prevalence rate of type 1 diabetes in Japan is known to be lowest in the world (24), and the prevalence rate of type 1 diabetes varies greatly between different ethnic groups and countries, it is of great interest whether the mutation is also implicated in the development of type 1 diabetes in other ethnic groups and countries; a previous study suggested a lack of association of the variant with type 1 diabetes in American whites (20). In addition, further studies are required to clarify whether the NeuroD/BETA2 variant has a physiological effect or modulates the balance between survival and apoptotic signals in pancreatic β-cells. Such studies should provide us with further insight on the pathogenesis of type 1 diabetes.

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