Analysis of Trinucleotide-Repeat Combination Polymorphism at the *rad* Gene in Patients With Type 2 Diabetes Mellitus

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A combined (GTT)n (ATT)n trinucleotide-repeat polymorphism designated as *RAD1* has been identified at intron 2 of the *rad* gene on chromosome 16q. An association between the total length of the *RAD1* locus and type 2 diabetes has been shown in white American subjects, but not in Finns. We genotyped 115 Japanese patients with type 2 diabetes and 114 nondiabetic control subjects at the *RAD1* locus by the direct sequencing method, and found 16 *RAD1* alleles composed of various combinations of GTTs and ATTs. Allele 14 consisting of four GTTs and seven ATTs accounted for the majority in both control subjects and diabetic patients, suggesting that *RAD1* polymorphism is not a major genetic component for susceptibility to common forms of diabetes in the Japanese. There was no significant association between total repeat length and diabetes. However, the frequency of minor alleles containing five GTTs or three GTTs was significantly higher in diabetic patients versus nondiabetic subjects (4.8% *v* 0.9%, $P = .012$). Thus, genetic variability at the *rad* gene in linkage disequilibrium with *RAD1* could be associated with a predisposition to type 2 diabetes in the Japanese population.

THE RAS-RELATED guanosine triphosphatase (GTPase), Rad (Ras associated with diabetes), was initially identified by subtraction cloning from skeletal muscle of humans with type 2 (non–insulin-dependent) diabetes.[1] Rad mRNA is expressed in the skeletal muscle, heart, lung, and placenta, but not in the brain, liver, kidney, or pancreas. Insulin resistance of skeletal muscle is a common feature of type 2 diabetes mellitus. Northern blot analysis has shown that Rad mRNA expression is markedly increased in the muscle of diabetic patients compared with normal controls, suggesting the possible involvement of Rad in insulin resistance.

Overexpression of Rad molecules by transfection of human Rad cDNA into myocytes and adipocytes results in a reduction of insulin-stimulated glucose uptake,[2] although the association between augmented Rad expression in skeletal muscle and insulin resistance in type 2 diabetes is still controversial.[3] Overexpression of Rad in diabetic muscle could be related to chronic hyperinsulinemia, since insulin induces an increase in Rad mRNA levels.[4] Molecules belonging to the Ras superfamily regulate a diversity of cellular functions, including the budding and fusion of vesicles, cytoskeletal organization, and gene expression.[5,6] Ras GTPases occupy a key position in a signal transduction pathway that links cell-surface receptors via a protein kinase cascade to changes in gene expression and cell morphology. Rad may be involved in skeletal muscle motor function and cytoskeletal organization, because Rad interacts with muscle β-tropomyosin and the cytoskeleton in a guanine nucleotide–dependent manner.[7]

A combined (GTT)n (ATT)n trinucleotide-repeat polymorphism designated as *RAD1* has been identified at intron 2 of the *rad* gene on chromosome 16q.[8] The frequency distribution of *RAD1* alleles was different between white American patients with type 2 diabetes and control subjects. When *RAD1* alleles were grouped into four classes based on the length of the trinucleotide repeat, class III accounted for greater than 80% of the alleles in both diabetic and normal subjects. An excess of minor alleles belonging to class I, II, or IV was found in patients with type 2 diabetes. However, an association of the *RAD1* class and type 2 diabetes was not observed in Finns.[9] In an attempt to verify these findings, we determined *RAD1* allele frequency with a direct sequencing method in 115 patients with type 2 diabetes and 114 nondiabetic control subjects.

SUBJECTS AND METHODS

The subjects of the study were 115 patients with type 2 diabetes aged 57.3 ± 13.2 years and 114 control subjects aged 56.9 ± 6.0 years with normal glucose tolerance according to World Health Organization criteria (Table 1). Of the diabetic patients, 32.2% were treated with insulin or insulin plus oral hypoglycemic agents and 41.7% with oral hypoglycemic agents alone. After informed consent was obtained, genomic DNA was extracted from whole blood by the phenol/chloroform method following incubation with proteinase K (Sigma, St Louis, MO). A polymerase chain reaction (PCR) of the *RAD1* locus was performed in a total volume of 10 μL containing 0.45 U Amplitaq Gold Taq DNA polymerase (Perkin Elmer, Foster City, CA), 10 mmol/L Tris hydrochloride, pH 8.3, 50 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 100 μmol/L dNTPs, and the following primers: 5′-GCTAGGACTAGGGGCTGAGA-3′ (forward) and 5′-AAGGGATTCTCCTGCCTCAG-3′ (reverse). After 35 cycles of 1 minute at 94°C, 1 minute at 63°C, and 1 minute at 72°C, aliquots (2 μL) of PCR products were analyzed on 2% agarose gels to confirm proper amplification. Free nucleotide and primers in PCR products were removed by centrifuge-driven dialysis (Ultrafree-MC, Millipore, Bedford, MA). Cycle sequencing was performed using both sense and antisense primers in different reactions with AmpliTaq DNA polymerase and dichlororhodamine dye terminators (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. After removal of excess dye terminators with CentriSep spin columns (Princeton Separations, Adelphia, NJ), the reactions were analyzed on an ABI Prism 310 automated sequencer (Applied Biosystems).

Statistical Analysis

The results are presented as the mean ± SD. Differences between group means were estimated by the unpaired *t* test. The χ^2 test and

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Table 1. Subject Characteristics

Group	Sex (F/M)	Age (yr)	Age at Diagnosis (yr)	BMI (kg/m²)	HbA_{1c} (%)
Diabetes (n = 115)	58/57	57.3 ± 13.2	46.2 ± 14.3	24.1 ± 4.4	8.0 ± 1.9
Control (n = 114)	42/72	56.9 ± 6.0		23.5 ± 2.5	

Abbreviations: F, female; M, male; BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}.

Fisher's exact test were used to compare frequencies. A P value less than .05 was considered statistically significant. The relative risk of type 2 diabetes according to genotype was estimated by the odds ratio and 95% confidence interval.

RESULTS

A total of 229 subjects were genotyped at the *RAD1* locus using the direct sequencing method. The polymorphic site consisted of various combinations of GTT- and ATT-repeat sequences. In the Japanese population, we found 16 *RAD1* alleles (Table 2). Allele 14 consisting of four GTT triplets and seven ATT triplets accounted for the majority (77% in both control subjects and diabetic patients). There was no significant association between diabetes and the total length of the *RAD1* locus (GTTs plus ATTs) or the number of ATT triplets. However, the number of GTT triplets was significantly associated with diabetes (P = .034). Alleles with four GTT triplets accounted for the majority in both diabetic patients (95.2%) and control subjects (98.2%), whereas minor alleles containing five GTT repeats or three GTT repeats (alleles 3 to 7 and allele 16) were more common in patients with diabetes versus normal controls (4.8% *v* 0.9%, P = .012 by χ^2 test and P = .021 by Fisher's exact test). No significant differences were observed between patients with alleles containing five or three GTT repeats and those without the alleles in terms of the basal C-peptide level, body mass index, age at diagnosis, or family history of diabetes in first-degree relatives. No other mutation was detected in the examined region of the *rad* gene.

Table 2. Distribution of *RAD1* Alleles in Patients With Type 2 Diabetes and Nondiabetic Control Subjects

	No. of Triplets		No of Alleles	
RAD1 Allele	GTT	ATT	Control	Type 2 Diabetes
1	7	12	1	0
2	7	10	1	0
3	5	13	0	1
4	5	12	1	1
5	5	11	1	4
6	5	7	0	1
7	5	6	0	1
8	4	14	4	5
9	4	13	19	21
10	4	12	16	8
11	4	11	5	5
12	4	10	2	1
13	4	8	2	0
14	4	7	176	178
15	4	5	0	1
16	3	5	0	3
Total			228	230

DISCUSSION

The trinucleotide-repeat polymorphism at the *rad* locus (*RAD1*) has been described in association with type 2 diabetes in white Americans.[8] When the alleles were grouped into four classes based on the length of the trinucleotide repeat, class III accounted for greater than 80% of the alleles of both diabetic and normal subjects. An excess of minor alleles belonging to class I, II, or IV was found in patients with type 2 diabetes. However, it was also previously reported that the *RAD1* locus was not associated with diabetes in Finns.[9] These controversial reports prompted us to examine the polymorphism in Japanese subjects.

We directly sequenced the *RAD1* region considering the possibility that each allele may consist of different combinations of GTT and ATT repeats when alleles are classified only by the total length. In studies on caucasian populations,[8,9] 10 alleles have been identified on the basis of the total number of GTT and ATT repeats. Although we also found 10 alleles with a different total length. the distribution was not the same as for caucasians. The major allele contained 11 triplet repeats, whereas the allele with 12 repeats is predominant in caucasians. Furthermore, five consisted of two or three alleles with different combinations of GTT and ATT repeats. Thus, 16 alleles were identified at the *RAD1* locus in Japanese subjects. The allele with four GTT triplets and seven ATT triplets comprised 77% of the alleles in both diabetic patients and control subjects, suggesting that the polymorphism may not be associated with type 2 diabetes in the Japanese. It is difficult to determine the role of either very rare or very common alleles by an association study. When the alleles were classified based on total repeat length according to the method of Doria et al,[8] we did not observe any significant association. However, these data can be analyzed in multiple different ways. When the alleles were grouped according to the number of GTTs, the frequency of minor alleles containing five GTTs or three GTTs was significantly higher in diabetic patients versus nondiabetic subjects.

Although *RAD1* polymorphism is unlikely a major genetic component of the susceptibility to common forms of diabetes in the Japanese population, genetic variability at the *rad* gene in linkage disequilibrium with *RAD1* could be involved as one of the polygenes in the predisposition to type 2 diabetes.

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