Brief Genetics Report

Systematic Search for Single Nucleotide Polymorphisms in the Resistin Gene

The Absence of Evidence for the Association of Three Identified Single Nucleotide Polymorphisms With Japanese Type 2 Diabetes

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Resistin is a novel polypeptide specifically secreted from adipocytes, and its serum levels are increased in obese diabetic mice. Resistin antagonizes insulin and could account for insulin resistance. To determine whether there are single nucleotide polymorphisms (SNPs) in the resistin gene associated with type 2 diabetes, sequences for 24 Japanese type 2 diabetic patients were initially analyzed using PCR direct sequencing. Three SNPs were found in the introns, but none were present in the coding regions. The allele frequencies of genomic −167C>T, +157C>T, and +299G>A in 99 Japanese control subjects were determined to be 3.5, 6.6, and 39.4%, respectively. In each pair of these SNPs, linkage disequilibria were found between either −167C>T and +299G>A or +157C>T and +299G>A. A linkage disequilibrium was also detected among −167C>T, +157C>T, and +299G>A, and only four of the eight possible haplotypes defined by these SNPs were found. A comparison of the frequencies of these SNPs and haplotypes between 99 type 2 diabetes and 99 control subjects revealed no evidence for any association. These identified SNPs, which were in linkage disequilibrium, represent potentially useful tools for searching for their association with specific phenotypes of diabetes. *Diabetes* 51:863–866, 2002

Type 2 diabetes is characterized by insulin resistance in insulin target tissues and an impaired insulin secretion from pancreatic β-cells (1). Gene mutations, which are associated with this disease, have been identified in molecules such as insulin and insulin receptor (2). These mutations account for specific types of diabetes as single genetic factors and constitute only a small proportion of all type 2 diabetic cases. Common type 2 diabetes is thought to be a polygenic disease, and its major genetic factors remain to be elucidated. Most recently, it has been reported that single nucleotide polymorphisms (SNPs) in calpain-10 and peroxisome proliferator–activated receptor-γ (PPAR-γ) are associated with type 2 diabetes (3,4).

Resistin (resistance to insulin) was recently discovered as a polypeptide that is specifically secreted from adipocytes (5). Serum levels of resistin are increased in both genetic (*ob*/*ob* and *db*/*db*) and diet-induced obese diabetic mice. Treatment with the PPAR-γ ligand rosiglitazone reduces serum resistin levels in mice. Interestingly, rosiglitazone also reduces resistin gene expression in 3T3-L1 adipocytes.

Resistin antagonizes the effect of insulin both in vitro and in vivo (5). An anti-resistin antibody improves blood glucose levels and insulin sensitivity in diet-induced obese mice, whereas recombinant resistin impairs glucose tolerance and the action of insulin in normal mice. In 3T3-L1 adipocytes, insulin-stimulated glucose uptake is enhanced by the immunoneutralization of resistin and is also blunted by recombinant resistin. Therefore, resistin represents a potential candidate gene for the insulin resistance observed in type 2 diabetes.

In view of this, we initiated a systematic search for SNPs in the resistin gene of Japanese subjects. The exon-intron boundaries were determined based on the human draft sequence for the FiZZ 3 (found in inflammatory zone 3) gene on chromosome 19 (GenBank accession no. NT_011145) and the human resistin cDNA sequence (no. AF323081). We initially examined the three exons, as well as the introns of the resistin gene, in 24 type 2 diabetic patients using PCR direct sequencing (Table 1 lists the primers used). The sequencing of both strands revealed the presence of three intronic SNPs, namely, genomic (g.) −167C>T, +157C>T, and +299G>A. These SNPs did not involve splice/donor sites.

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PPAR-γ, peroxisome proliferator–activated receptor-γ; RELM, resistin-like molecule; SNP, single nucleotide polymorphism.

TABLE 1
Primers used for PCR and sequencing of the resistin gene

Mainly amplified region	Primers (5′-3′)	5′ Position of each primer
Exon 1	1F: AGCCCTTACTGTCTGCTCA	−379
	1R: CCATGGAGGGAGTAGGATC	−73
Exon 2	2F: AGTGACAGCTGCTCCTGCG	−202
	2R: ATGAGATTTGGTGAGCGCT	+223
Exon 3	3F: ATGCCCACAGGGACCTAGC	+391
	3R: GTCAGGACCAAGATCCTAG	+690
Exon 4	4F: CCAGCTCAGAGTCCACGCT	+855
	4R: GGGCCTACTAAAGAAACCA	+1177
Intron 2	5F: GAGAGGATCCAGGAGGTCG	+88
	5R: GTGAGACCAAACGGTCCCT	+460

The 5′ positions are shown by defining the translation start site as +1. The putative exon and intron numbers shown are based on the comparison between the human draft sequence for the FiZZ 3 (found in inflammatory zone 3) gene on chromosome 19 (GenBank accession no. NT_011145) and the human resistin cDNA sequence (no. AF323081). It should be noted that exact exon-intron boundaries of the resistin gene remain to be experimentally determined. F, forward; R, reverse.

We then further sequenced the regions with these three SNPs in 99 control subjects (Table 2 lists clinical data). The allele frequencies of g. −167C>T, +157C>T, +299G>A were 3.5, 6.6, and 39.4%, respectively (Table 3). We then examined the linkage disequilibrium between any two pairs of these three SNPs. Linkage disequilibrium was found between two of the three pairs. The first was detected between −167C>T and +299G>A (Table 4*A*). The estimated haplotype frequencies were f (C–G) = 0.60, f (C–A) = 0.36, f (T–G) = 0.00, and f (T–A) = 0.04. The other was found between +157C>T and +299G>A. The estimated haplotype frequencies were f (C–G) = 0.61, f (C–A) = 0.33, f (T–G) = 0.00, and f (T–A) = 0.06. The linkage disequilibrium was also found among these three SNPs, and only four of the eight possible haplotypes defined by these SNPs were detected (Table 4*B*). The estimated haplotype frequencies were f (C–C–G) = 0.60, f (C–C–A) = 0.29, f (C–T–A) = 0.07, and f (T–C–A) = 0.04.

We next sequenced the regions with these three SNPs in 75 additional type 2 diabetic patients, and we compared the allele frequencies of these SNPs between 99 type 2 diabetic patients and 99 nondiabetic control subjects (Table 2 lists clinical data). No significant differences in the allele frequencies of either g. −167C>T, +157C>T, or +299G>A were detected between type 2 diabetes and the control subjects (Table 3). The estimated haplotype frequencies were then determined for type 2 diabetes. In these 99 type 2 diabetic subjects, the same linkage disequilibria as for the normal control subjects were found, and the estimated haplotype frequencies were also similar to those in normal control subjects. The estimated haplotype frequencies between −167C>T and +299G>A were f (C–G) = 0.64, f (C–A) = 0.34, f (T–G) = 0.00, and f (T–A) = 0.02. The estimated haplotype frequencies between +157C>T and + 299G>A were f (C–G) = 0.64, f (C–A) = 0.30, f (T–G) = 0.00, and f (T–A) = 0.06. The estimated haplotype frequencies between these three SNPs were f (C–C–G) = 0.64, f (C–C–A) = 0.28, f (C–T–A) = 0.06, and f (T–C–A) = 0.02. To examine whether other SNPs exist, the entire resistin gene was also sequenced in 25 of the 75 type 2 diabetic patients, in addition to the initial screening, which involved 24 patients. No other SNPs were detected in these patients, suggesting that most of all the SNPs, at least those with relatively high frequencies, were identified in this study.

TABLE 2
Clinical features of the control and type 2 diabetic subjects

	Control	Type 2 diabetes
n (M/F)	99 (53/46)	99 (45/54)
Age (years)	49.1 ± 6.7	59.9 ± 10.5
Age of onset (years)	—	47.0 ± 11.5
Duration of diabetes (years)	—	13.2 ± 9.3
Height (cm)	162.5 ± 8.7	157.7 ± 8.3
BW (kg)	60.5 ± 9.6	57.9 ± 10.4
Max BW (kg)	—	68.5 ± 13.1
ΔBW (kg)	—	10.6 ± 6.7
Age of max BW (years)	—	43.5 ± 13.7
BMI (kg/m2)	22.8 ± 2.6	23.2 ± 3.2
Max BMI (kg/m2)	—	27.4 ± 4.2
ΔBMI(kg/m2)	—	4.2 ± 2.6
HbA_{1c} (%)	4.8 ± 0.3	7.8 ± 1.7
Treatment (diet/OHA/insulin)	—	(19/57/23)

Data are means ± SD or *n*. BW, body weight; Max BW, maximum body weight; ΔBW=Max BW − BW; ΔBMI=Max BMI − BMI; OHA, oral hypoglycemic agent.

Our findings show that Japanese subjects had two major haplotypes determined by −167C>T and +299G>A, namely C–G and C–A. Similarly, Japanese subjects had two major haplotypes determined by +157C>T and +299G>A, namely C–G and C–A. Among the haplotypes defined by the three SNPs, two major haplotypes (C–C–G and C–C–A) were detected in Japanese subjects. Because most Japanese subjects had C at both −167 and +157, the +299G>A represents a simple potential marker for roughly classifying these two major haplotypes. The +299G>A SNP would be useful in searching for an association with specific phenotypes in Japanese type 2 diabetes, especially in cases where a large number of samples is involved.

Our findings demonstrate the lack of evidence for a major effect of resistin gene polymorphisms on susceptibility to type 2 diabetes. It should be noted that a small effect of these polymorphisms on susceptibility cannot be excluded, since the sample size was limited in the present study. It is known that Japanese subjects gain less weight than Caucasians as the result of lower insulin response (6,7). In fact, the mean BMI of type 2 diabetic patients examined was similar to that of normal control subjects. Because the serum resistin levels are increased in obese insulin-resistant rodents, the resistin gene may well be involved in the development of type 2 diabetes associated with severe obesity.

Another molecule might be present in the human as a functional homologue of the mouse resistin, since the homology between human and mouse resistin genes is low. Steppan et al. (8) reported on a family of resistin-like molecules (RELMs) in rodents and humans. RELMα is expressed most abundantly in adipose tissue, and RELMβ is expressed only in the gastrointestinal tract. Resistin and the RELMs share a cysteine composition and other signa-

TABLE 3
Allele frequency of SNPs in the resistin gene for control and type 2 diabetic subjects

SNP	Control (n = 99)	Type 2 diabetes (n = 99)	χ^2	P
g.−167C>T	7/198 (3.5)	4/198 (2.0)	0.842	0.543
+157C>T	13/198 (6.6)	11/198 (5.6)	0.177	0.834
+299G>A	78/198 (39.4)	71/198 (35.9)	0.527	0.534

Fisher's exact probability test was used for the statistical analysis. Allele frequencies represent minor alleles different from the reference sequence (GenBank accession no. NT_011145). The nucleotide number of each SNP is counted from A of the start codon as 1. Data are frequency (%).

ture features, suggesting that these molecules comprise a class of tissue-specific signaling molecules.

Further investigation will be required for the regulation of resistin gene expression in obese rodents and the effect of thiazolidinediones. Serum resistin levels are increased in obese insulin resistant *ob*/*ob* and *db*/*db* mice (5). Rosiglitazone reduces serum resistin levels in mice. Rosiglitazone also decreases resistin gene expression in 3T3-L1 adipocytes. Recently, Way et al. (9) showed that resistin gene expression is decreased in the white adipose tissue of obesity models, such as *ob*/*ob*, *db*/*db*, *tub*/*tub*, and KKAy mice. Several different classes of PPAR-γ agonists cause an increase in resistin expression in adipose tissue of both *ob*/*ob* mice and Zucker fatty rats.

In summary, we report here on a systematic search for SNPs in the resistin gene and the identification of three intronic SNPs. No association of these SNPs or haplotypes defined by these SNPs in linkage disequilibrium with type 2 diabetes was evident. The issue of how these SNPs affect resistin gene expression in adipocytes and its serum levels, and whether SNPs in resistin gene are associated with type 2 diabetes in other ethnic groups, remains unclear. Further study will be required to clarify these points.

RESEARCH DESIGN AND METHODS

This study involved an initial screening of 24 unrelated type 2 diabetic outpatients from the Ehime University Hospital and the Ehime Prefectural Hospital. The subjects were chosen based on the fact that they showed the typical characteristics of type 2 diabetes, namely, age of onset between 40 and 60 years, treatment with diet alone or oral hypoglycemic agents, and the presence of first-degree relatives with type 2 diabetes. Diabetes was diagnosed based on the American Diabetes Association criteria, reported in 1998 (10). The entire resistin gene was initially sequenced in these 24 diabetic patients. For the association study, the regions containing the SNPs identified were sequenced in 75 additional unrelated type 2 diabetic subjects and 99 nondiabetic subjects. To confirm that SNPs, at least those with relatively high frequencies, were identified in the initial screening, the entire resistin gene was also sequenced in 25 patients randomly chosen from these 75 type 2 diabetic subjects. The 99 nondiabetic control subjects were chosen based on the fact that they had no history of diabetes, an absence of diabetic patients among their first-degree relatives, and normal glucose tolerance, as evidenced by a 75-g oral glucose tolerance test. All patients and control subjects were informed of the purpose of the study, and their consent was obtained. The study was approved by the ethics committee of the Ehime University Hospital and Ehime Prefectural Hospital. The clinical characteristics of the 99 type 2 diabetic subjects and the 99 control subjects are summarized in Table 2. The age of the control subjects was comparable to the age of onset of the type 2 diabetic subjects.

PCR direct sequencing was performed as described previously (11,12). Genomic DNA was extracted from leukocytes using a DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim, Indianapolis, IN). The 5′ flanking region and three exons, as well as the introns of the resistin gene, were individually amplified using primers, as described in Table 1. The PCR

TABLE 4
Linkage disequilibrium between the identified SNPs in the resistin gene in Japanese subjects

Haplotype	Estimated HF	Expected HF	LD	RLD	χ^2	P
Linkage disequilibrium between two of the three SNPs						
−167 +299					11.2	$<10^{-3}$
C - G	0.606	0.585	+0.021	+1		
C - A	0.359	0.380	−0.021	−1		
T - G	0.000	0.021	−0.021	−1		
T - A	0.035	0.014	+0.021	+1		
+157 +299					21.4	$<10^{-5}$
C - G	0.606	0.566	+0.040	+1		
C - A	0.328	0.368	−0.040	−1		
T - G	0.000	0.040	−0.040	−1		
T - A	0.066	0.026	+0.040	+1		
Linkage disequilibrium among the three SNPs						
−167 +157 +299				—	26.5	$<10^{-4}$
C - C - G	0.606	0.546	0.060	—		
C - C - A	0.293	0.355	−0.062	—		
C - T - G	0.000	0.038	−0.038	—		
C - T - A	0.066	0.025	0.041	—		
T - C - G	0.000	0.020	−0.020	—		
T - C - A	0.035	0.013	0.022	—		
T - T - G	0.000	0.001	−0.001	—		
T - T - A	0.000	0.001	−0.001	—		

To examine a deviation from linkage equilibrium between two SNPs, a χ^2 test was performed as described in Imanishi et al. (15). For the test of linkage disequilibrium between three SNPs, a likelihood ratio test in the EH program (13) was performed. HF, haplotype frequency; LD, linkage disequilibrium parameter; RLD, relative linkage disequilibrium value.

between +691 and +854 were not feasible, probably because of the long region of repetitive C bases, although different sets of primers in this region were tested. We amplified 30 ng of genomic DNA in a 15 μl reaction mixture, which included 0.375 units of *Taq* DNA polymerase (Takara Shuzo Biomedical Group, Shiga, Japan), 3 pmoles of each primer, 3 nmoles of each dNTP, and 5% DMSO. After the first denaturing for 3 min at 94°C, PCR was carried out for 30 cycles, with denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension period of 3 min. The amplified PCR products were electrophoresed on a 1% agarose gel and then stained with Cyber Green I (BioWhittaker Molecular Application, Rockland, ME) to confirm the size of each molecule.

These PCR products were sequenced using forward and/or reverse primers, after purification using a Multiscreen PCR filter (Millipore, Bedford, MA). The sequencing reaction was carried out using *Taq* Dye Deoxy and ABI Prism terminator cycle sequencing kits (Applied Biosystems, Perkin-Elmer, Foster City, CA). The products were then purified using either an Autoseq G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ) or a Multiscreen HV plate (Millipore) loaded with Sephadex G-50 Superfine. These products were then electrophoresed on an ABI gene analyzer 3100 system (PE Applied Biosystems). Both strands of the entire resistin gene were sequenced for the initial screening of the 24 type 2 diabetic subjects to detect any unknown SNPs. A nested primer 5′-ATCATCATCATCTCCAGGT-3′ was used as a reverse primer for exon 4 when GAT repeats affected the sequencing. Sequences of a plus strand of intron 1 and a minus strand of intron 2, where the identified SNPs were located, were then checked for the association study because these strands allow the polymorphisms to be identified more precisely. The other strand was also sequenced, when required.

A χ^2 test was used for statistical analysis unless otherwise indicated. Haplotype frequencies between two or three SNPs were estimated based on the EH program (13). The relative linkage disequilibrium value for two SNPs was defined as the ratio of the linkage disequilibrium parameter to the possible maximum linkage disequilibrium parameter (14). To examine deviations from linkage equilibrium between the two SNPs, a χ^2 test was performed as described by Imanishi et al. (15). In the case of haplotypes consisting of three SNPs, linkage disequilibrium was defined as the difference between the estimated haplotype frequency and the product of each allele frequency. For the test of linkage disequilibrium between three SNPs, a likelihood ratio test in the EH program (13) was performed.

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