Brief Genetics Report

Association of Adiponectin Mutation With Type 2 Diabetes

A Candidate Gene for the Insulin Resistance Syndrome

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Adiponectin, also referred to as AdipoQ or ACRP30, is a plasma protein produced and secreted exclusively from adipose tissue. The protein contains a collagen-like domain and a C1q-like globular domain. A protease-generated globular segment enhances fatty acid oxidation in muscles, thereby modulating lipid and glucose metabolism. Plasma adiponectin levels are inversely correlated with the severity of insulin resistance. A recent genome-wide scan study mapped a susceptibility locus for type 2 diabetes and the metabolic syndrome to chromosome 3q27, where the adiponectin gene is located. Here, we screened Japanese patients with type 2 diabetes and age- and BMI-matched nondiabetic control subjects for mutations in adiponectin gene. We identified four missense mutations (R112C, I164T, R221S, and H241P) in the globular domain. Among these mutations, the frequency of I164T mutation was significantly higher in type 2 diabetic patients than in age- and BMI-matched control subjects ($P < 0.01$). Furthermore, plasma adiponectin concentrations of subjects carrying I164T mutation were lower than those of subjects without the mutation. All the subjects carrying I164T mutation showed some feature of metabolic syndrome, including hypertension, hyperlipidemia, diabetes, and atherosclerosis. Our findings suggest that I164T mutation is associated with low plasma adiponectin concentration and type 2 diabetes. *Diabetes* 51:2325–2328, 2002

Insulin resistance, which implies impairment of insulin signaling in the target tissues, is a common cause of type 2 diabetes. Adipose tissue plays an important role in insulin resistance syndrome through the dysregulated production and secretion of adipose-derived proteins, including tumor necrosis factor-α, plasminogen activator inhibitor-1, leptin, resistin, angiotensinogen, and adiponectin (1–7). The latter, also known as ACRP30, apM1, AdipoQ, and GBP28, is an adipocyte-derived protein present in circulating plasma with a range of 5–30 μg/ml in humans (8–13). Plasma adiponectin concentrations correlate inversely with the severity of insulin resistance (14–16). Recently, several groups have demonstrated that administration of ACRP30/adiponectin increased fatty acid oxidation in muscles and decreased hepatic glucose production, resulting in amelioration of insulin resistance and improvement of glucose metabolism in diabetic mice (17–20). Furthermore, two groups independently reported that one of the quantitative trait loci affecting insulin resistance/metabolic syndrome (21) and type 2 diabetes (22) was mapped on chromosome 3q27, where the adiponectin gene is located. These findings suggest that the reduced net function of adiponectin might be associated with the development of insulin resistance and type 2 diabetes.

In the present study, we screened 218 Japanese patients with type 2 diabetes for mutations in the adiponectin gene. Control studies were also conducted in 452 age- and BMI-matched nondiabetic control subjects. The entire coding regions of the adiponectin gene were amplified from the genomic DNA and directly sequenced. We have previously identified two different mutations of the adiponectin gene, including the R112C missense mutation and the G/T polymorphism at nucleotide 94 (23). In the present study, we identified three novel missense mutations in exon 3 of the adiponectin gene (Fig. 1*A*): *1*) a T-to-C substitution at nucleotide 517 led to amino acid substitution from isoleucine to threonine at position 164 (I164T); *2*) a C-to-A substitution at nucleotide 687 caused amino acid substitution from arginine to serine at position 221 (R221S); and *3*) an A-to-C substitution at nucleotide 748 led to amino acid substitution from histidine to proline at position 241 (H241P). Adiponectin possesses a signal sequence at the NH_2-terminal end and is composed of the collagen repeats domain and the COOH-terminal globular domain (10,14). All of the identified missense mutations resided in the globular domain. These missense mutations were found in heterozygous form.

No mutation was identified in 60, 23, and 33 bp of intron

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Received for publication 25 January 2002 and accepted in revised form 10 April 2002.

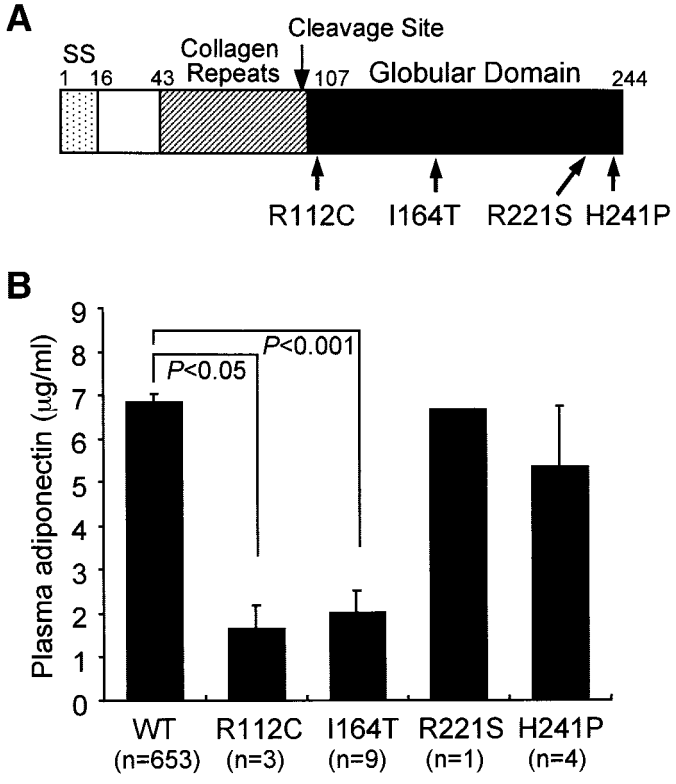

FIG. 1. Genetic mutations and plasma concentration of adiponectin. *A*: Domain structure and positions of mutations in adiponectin. *B*: Plasma adiponectin concentrations in control subjects with wild type (WT) and the subjects with various mutations in adiponectin gene. Data are means ± SE of the indicated sample numbers.

sequences of 3′-acceptor site of intron 1 or the 5′-donar and 3′-acceptor sites of intron 2, respectively. From these observations, we confirmed that there was no mutation to produce alternative splicing of adiponectin gene.

Table 1 shows the frequencies of mutations in type 2 diabetic and nondiabetic control subjects. The I164T mutation was found in 7 of 218 diabetic subjects and 2 of 452 nondiabetic subjects. Thus, the frequency of the I164T mutation was significantly higher in diabetic (3.2%) than in nondiabetic subjects (0.4%, $P = 0.007$). Furthermore, the two subjects carrying I164T mutations recognized as nondiabetic subsequently turned out to have impaired glucose tolerance by glucose tolerance test. Both the R112C and H241P mutations were identified in one subject in the diabetic group. No significant difference was observed in the frequency of these mutations between diabetic and nondiabetic subjects. R221S mutation was identified only in one nondiabetic subject.

TABLE 1
Frequency of mutations in adiponectin gene

	Nondiabetic control subjects	Type 2 diabetic subjects	*P*
n (M/F)	452 (312/140)	218 (137/81)	
I164T*	2 (0.4%)	7 (3.2%)	<0.01
R112C	2 (0.4%)	1 (0.5%)	NS
H241P	3 (0.7%)	1 (0.5%)	NS
R221S	1 (0.2%)	0 (0.0%)	NS

Mutation frequencies were compared by Fisher's exact probability test (for R112C, I164T, R221S, and H241P). *The two nondiabetic control subjects carrying I164T mutation exhibited impaired glucose tolerance by 75-g oral glucose tolerance test.

The subjects carrying I164T had markedly low plasma adiponectin concentrations compared with those without missense mutations (I164T mutation: 2.0 ± 0.5 μg/ml [means ± SE], no mutations: 6.9 ± 0.2 μg/ml; $P < 0.001$) (Fig. 1*B*). It has been demonstrated that the plasma adiponectin levels in men are significantly lower than in women (14). In the current study, plasma adiponectin levels in subjects with I164T mutation were lower than in the subjects without mutations for both sexes (men: 1.9 ± 1.1 vs. 6.0 ± 0.2 μg/ml and women: 2.2 ± 0.9 vs. 8.6 ± 0.4 μg/ml). The difference in plasma adiponectin level was still significant when plasma adiponectin levels of the subjects with I164T mutation were compared with those of 209 diabetic subjects without mutations (6.3 ± 0.3 μg/ml, $P < 0.001$), suggesting that the hypoadiponectinemia associated with I164T mutation was not the consequence of insulin resistance or type 2 diabetes. In our previous study, we reported that subjects with the R112C mutation had low plasma adiponectin concentrations. This was also confirmed in the present study in subjects with the R112C mutation (1.7 ± 0.5 μg/ml, $P < 0.05$). On the other hand, plasma adiponectin levels of the subjects with H241P and R221S were similar to those of wild-type subjects (Fig. 1*B*).

Table 2 shows the clinical profile of subjects with the mutations associated with low plasma adiponectin level (i.e., I164T and R112C). As described above, seven of the nine subjects carrying I164T mutation were type 2 diabetic patients, and the two subjects identified in the nondiabetic group exhibited impaired glucose tolerance by 75-g oral glucose tolerance test. All subjects carrying the I164T mutation had received medications for hypertension and/or had high blood pressure. Furthermore, the majority of subjects had lipid abnormalities and were on hypolipidemic agents. Six of nine subjects suffered from atherosclerotic vascular diseases. These results suggest that the I164T mutation of adiponectin gene in subjects with hypoadiponectinemia is strongly associated with the metabolic syndrome.

Two of three subjects with the R112C mutation had atherosclerotic vascular diseases, and one of them had type 2 diabetes. More subjects carrying R112C mutation need to be identified and evaluated for better assessment of the association between this mutation and metabolic syndrome.

Recent genome-wide scan studies have mapped diabetes susceptibility locus on chromosome 3q27, where the adiponectin gene is located. However, plasma adiponectin levels were not measured in these studies. In the present study, we measured plasma adiponectin levels by enzyme-linked immunosorbent assay and demonstrated that the I164T and R112C mutations were associated with markedly low plasma adiponectin levels and type 2 diabetes. More recently, Comuzzie et al. (24) identified two major and four potential loci for plasma variation in adiponectin by genome scan analysis. One of these was on chromosome 3. Taken together, genetic polymorphisms of adiponectin gene, which result in the lower production and/or secretion of adiponectin, may be underlying, at least in

TABLE 2
Clinical profile of the subjects with I164T and R112C mutation

Case subject	I164T mutation									R112C mutation			Control subjects*
	1	2	3	4	5	6	7	8	9	1	2	3	Mean (SE)
Age (years)	52	59	59	61	73	50	65	67	82	48	60	83	61.5 (0.4)
Sex	M	M	M	M	M	F	F	F	F	M	M	M	M/F = 437/216
Plasma adiponectin (μg/ml)	0.4	2.8	2.7	2.6	0.9	1.8	4.4	2.0	0.5	1.3	2.5	1.2	6.9 (0.2) M = 6.0 (0.2) F = 8.6 (0.4)
BMI (kg/m^2)	27.0	29.2	25.4	25.6	23.8	45.7	34.1	25.0	21.7	22.3	29.0	25.3	24.5 (0.2)
Systolic blood pressure (mmHg)	150	170	120	180	142	166	144	158	190	105	130	106	132 (0.7)
Diastolic blood pressure (mmHg)	110	116	80	100	80	110	78	92	100	55	80	88	75 (0.5)
Antihypertensive drug (yes/no)	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	no	
Total cholesterol (mg/dl)	268	208	213	236	184	316	284	247	274	243	203	226	200 (1.6)
Triglycerides (mg/dl)	387	372	126	79	134	333	235	267	302	145	331	140	157 (6.2)
HDL cholesterol (mg/dl)	23	37	54	73	34	32	47	47	34	78	34	54	49 (0.7)
Antihyperlipidemic drug (yes/no)	no	yes	yes	no	no	yes	yes	yes	no	no	no	no	
Fasting plasma glucose (mg/dl)	104	105	299	148	97	308	114	120	296	109	112	147	114 (1.7)
HbA_{1c} (%)	4.9	6.8	6.9	6.3	5.5	14.4	5.9	5.8	11.1	5.1	5.2	8.1	6.0 (0.1)
Antidiabetic drug (yes/no)	yes	no	yes	yes	no	yes	no	yes	yes	no	no	yes	
Diabetes or IGT	DM	IGT	DM	DM	IGT	DM	DM	DM	DM	—	—	DM	
Atherosclerotic diseases	AP	—	AP	CVA	MI	—	AP	AP	—	—	AP	AP	

*Subjects without mutations in adiponectin gene. AP, angina pectoris; CVA, cerebrovascular accident; DM, diabetes; IGT, impaired glucose tolerance; MI, myocardial infarction.

part, the pathophysiology of the insulin resistance syndrome.

In the current study, the subjects with I164T or R112C mutation showed markedly low plasma adiponectin concentrations, even in heterozygotes. Adiponectin/ACRP30 forms a high-ordered multimeric structure similar to complement C1q (25). The I164T and R112C mutations may disturb the normal assembly and/or secretion of the protein. The mutations may also produce less active protein for lipid and glucose metabolism. Furthermore, in vitro mutational and functional studies are necessary to clarify these possibilities.

It will also be important to elucidate the sequence of 5′- and 3′-flanking regions because these regions should contain the elements to determine the mRNA amounts of adiponectin. Indeed, we have screened the 2-kb promoter region of adiponectin gene, which might affect the adiponectin production, in 99 Japanese subjects, including 39 diabetic patients. Thirteen point mutations and a two-nucleotide insertion were identified, but none were in the putative transcription factor–binding sites. Furthermore, none were associated with the incidence of hypoadiponectinemia (data not shown). The sequence of 3′-flanking region remains to be examined. It would be also necessary to elucidate whether I164T mutation of adiponectin gene is associated with insulin resistance syndrome in larger scales of Japanese subjects and other ethnic populations.

We have been suggesting that hypoadiponectinemia induced by overnutrition might be causative for the development of metabolic syndrome associated with obesity (13,26). The present study revealed, for the first time, that genetic mutation in the coding region of the adiponectin gene (I164T) accompanying hypoadiponectinemia is causatively related to the development of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. A total of 670 subjects (449 men and 221 women, aged 61.5 ± 0.4 years, BMI 24.6 ± 0.2 kg/m^2), who received medical check in Osaka University Hospital or four other affiliated institutions, were recruited to our research protocol, which was designed for screening of the adiponectin gene. They included 218 patients with type 2 diabetes (137 men and 81 women, aged 62.0 ± 0.7 years, BMI 24.9 ± 0.3 kg/m^2) and 452 nondiabetic subjects (312 men and 140 women, aged 61.2 ± 0.6 years, BMI 24.4 ± 0.2 kg/m^2). Criteria for diabetes were overnight fasting plasma glucose ≥126 mg/dl or plasma glucose at 2 h after 75-g oral glucose tolerance test ≥200 mg/dl or random plasma glucose concentration ≥200 mg/dl. Patients with type 1 diabetes and other specific types were excluded from the study. Written informed consent was obtained from all subjects before enrollment in the study. The study was approved by the Osaka University Ethics Review Board.

Screening of mutations in the adiponectin gene. Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes. The entire translated regions of adiponectin gene were amplified by PCR using two pairs of specific primers: 5′-GAAGTAGACTCT GCTGAGATGG-3′ and 5′-TATCAGTGTAGGAGGTCTGTGATG-3′, which flank the region containing exon 2, and 5′-GATCTATAAGTCAAGAAGGTTGTGA-3′ and 5′-CAGGACTGGGAACATAGCATATGA-3′, which flank the region containing exon 3. The PCR products were directly sequenced on an ABI 377 automatic sequencer. Forward primer 5′-CGGAGTCCTTTGTAGGTCCCAA CTG-3′ and reverse primer 5′-TATCAGTGTAGGAGGTCTGTGATG-3′ were used for determination of the sequence of exon 2, and forward primer 5′-GTAACCAACCTAGGCAGGAGTTC-3′ and reverse primer 5′-CAACTTCCT AACCGTACTGAAAGCC-3′ were used for determination of the sequence of exon 3.

Determination of plasma adiponectin concentration. The concentration of plasma adiponectin was determined by the enzyme-linked immunosorbent assay system as previously described (13). Briefly, a 96-well plate was coated with 5 mg/ml mouse monoclonal antibody, ANOC 9108, at 4°C and blocked with 0.1% BSA and 0.05% sodium azide. Human plasma was diluted with five

volumes of the sample buffer (31.25 mmol/l Tris-HCl, pH 6.8, and 2.3% SDS) and boiled for 5 min. Each sample was then diluted with the sample buffer, and 50 μl of the sample, at a final 1:5000 dilution, was applied to each well of the antibody-coated plate and incubated overnight at room temperature. A recombinant adiponectin protein without a leader peptide, NH_2-terminal 11 amino acids, was used as the standard (13). The wells were washed three times with 5 mmol/l Tris-HCl (pH 8.0) containing 15 mmol/l NaCl and 0.05% Tween 20 and then 100 μl of a final 1:10,000 dilution of rabbit polyclonal antibody, OCT9104, was added and incubated for 3 h at room temperature. Each well was washed three times with wash buffer, and the binding of OCT9104 was determined by the *O*-phenylene-diamine dihydrochloride–horseradish peroxidase method.

Plasma glucose was measured by the glucose oxidase method, and serum total cholesterol and triglyceride levels were determined by enzymatic methods. HDL cholesterol was measured by the selective inhibition method. HbA_{1c} levels were determined by high-performance liquid chromatography.

Statistical analysis. The results were expressed as means ± SE. Statistical analyses were performed with unpaired *t* test. Intergroup differences in the frequencies of mutation were tested by Fisher's exact probability test. A P value <0.05 denoted the presence of a statistically significant difference.

ACKNOWLEDGMENTS

This work was supported in part by the "Research for the Future" program of the Japan Society for the Promotion of Science (JSPS-RFTF97L00801), Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (12307022, 12557090, 12671084, and 13204054), and the Fuji Foundation for Protein Research.

We are indebted to Sachiyo Tanaka for excellent technical assistance. We express special thanks to Drs. Kikuko Hotta, Kazuya Yamagata, and Toshiaki Hanafusa for their kind help in the study analysis and enrollment of subjects.

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