





**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  $\pm$  SE of the indicated sample numbers.

sequences of 3'-acceptor site of intron 1 or the 5'-donor 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

**TABLE 1**  
Frequency of mutations in adiponectin gene

	Nondiabetic control subjects	Type 2 diabetic subjects	<i>P</i>
<i>n</i> (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 frequency of these mutations between diabetic and nondiabetic subjects. R221S mutation was identified only in one nondiabetic subject.

The subjects carrying I164T had markedly low plasma adiponectin concentrations compared with those without missense mutations (I164T mutation:  $2.0 \pm 0.5 \mu\text{g/ml}$  [means  $\pm$  SE], no mutations:  $6.9 \pm 0.2 \mu\text{g/ml}$ ;  $P < 0.001$ ) (Fig. 1B). 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:  $1.9 \pm 1.1$  vs.  $6.0 \pm 0.2 \mu\text{g/ml}$  and women:  $2.2 \pm 0.9$  vs.  $8.6 \pm 0.4 \mu\text{g/ml}$ ). 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 ( $6.3 \pm 0.3 \mu\text{g/ml}$ ,  $P < 0.001$ ), 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 ( $1.7 \pm 0.5 \mu\text{g/ml}$ ,  $P < 0.05$ ). On the other hand, plasma adiponectin levels of the subjects with H241P and R221S were similar to those of wild-type subjects (Fig. 1B).

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* Mean (SE)
	1	2	3	4	5	6	7	8	9	1	2	3	
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 ( $\mu\text{g/ml}$ )	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) 24.5 (0.2)
BMI ( $\text{kg/m}^2$ )	27.0	29.2	25.4	25.6	23.8	45.7	34.1	25.0	21.7	22.3	29.0	25.3	
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 <sub>1c</sub> (%)	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  $61.5 \pm 0.4$  years, BMI  $24.6 \pm 0.2 \text{ kg/m}^2$ ), 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  $62.0 \pm 0.7$  years, BMI  $24.9 \pm 0.3 \text{ kg/m}^2$ ) and 452 nondiabetic subjects (312 men and 140 women, aged  $61.2 \pm 0.6$  years, BMI  $24.4 \pm 0.2 \text{ kg/m}^2$ ). Criteria for diabetes were overnight fasting plasma glucose  $\geq 126 \text{ mg/dl}$  or plasma glucose at 2 h after 75-g oral glucose tolerance test  $\geq 200 \text{ mg/dl}$  or random plasma glucose concentration  $\geq 200 \text{ mg/dl}$ . 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'-GAAGTAGACTCTGCTGAGATGG-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'-CGGAGTCCCTTTGTAGGTCCTCACTG-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'-CAACTCCTAACCGTACTGAAAGCC-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  $\mu$ l 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<sub>2</sub>-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  $\mu$ l 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<sub>1c</sub> levels were determined by high-performance liquid chromatography.

**Statistical analysis.** The results were expressed as means  $\pm$  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.

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