# Adiponectin Gene Polymorphism (G276T) Is Not Associated With Incipient Diabetic Nephropathy in Japanese Type 2 Diabetic Patients

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**A single-nucleotide polymorphism (SNP) G276T in the adiponectin gene has been associated with lower plasma adiponectin levels and insulin resistance, which are related to the prevalence of type 2 diabetes or diabetic complications of macroangiopathy. We performed a case-control study to examine whether the SNP276 of the adiponectin gene was also related to early diabetic nephropathy. SNP276 was examined with genomic DNA obtained from 108 type 2 diabetic patients with microalbuminuria (urinary albumin creatinine ratio [ACR] between 30 mg/g · Cr and 300 mg/g · Cr; case subjects), and 208 patients with normoalbuminuria (ACR < 30 mg/g · Cr; control subjects). The genotype distribution and G allele frequency of SNP276 in the case subjects (0.71) did not significantly differ from the control subjects (0.69). There were no differences among the genotypes of the adiponectin gene regarding age, duration of diabetes, body mass index (BMI), hemoglobin $A_{1c}$ ($HbA_{1c}$), serum lipids, serum creatinine, and plasma adiponectin levels. These data suggest that SNP276 of the adiponectin gene is not an independent risk factor for incipient diabetic nephropathy in Japanese type 2 diabetic patients.**


DIABETIC NEPHROPATHY is a serious complication of diabetic microangiopathies and the leading cause of dialysis in Japan. The risk of developing diabetic nephropathy may be partly determined by genetic factors,[1,2] as well as glycemic control. It is therefore important to identify genetic markers for diabetic nephropathy. It is suggested that insulin resistance may be a risk factor for diabetic nephropathy in type 2 diabetes.[3,4] A polymorphism in the genes involving in insulin sensitivity such as peroxisome proliferator-activated receptor γ (PPAR-γ)[5] has been associated with lower albumin excretion rate in patients with type 2 diabetes.[6] Adiponectin, an adipocytes-derived protein, also plays a major role in mediating insulin sensitivity, and lower plasma adiponectin concentration has been linked to macroangiopathies in type 2 diabetes.[7] Recently, a single-nucleotide polymorphism (SNP) G276T in the adiponectin gene has been associated with lower plasma adiponectin levels and hence insulin resistance in Japanese obese subjects.[8] We therefore investigated whether the SNP276 of the adiponectin gene was also related to early diabetic nephropathy in Japanese patients with type 2 diabetes.

## MATERIALS AND METHODS

A case-control study was conducted in 316 Japanese patients with type 2 diabetes participating in a miulticenter research protocol. Diabetes was diagnosed according to the 1999 World Health Organization criteria.[9] The diagnosis of diabetic nephropathy was based on repeated (2 separate days) measurements of the urinary albumin:creatinine ratio (ACR). After the pretest urination, patients were asked to avoid exercise for 1 hour, and spot urine was collected and measured for albumin by radioimmunoassay. Urinary creatinine concentration was also measured with the modified Jaffe method. Microalbuminuria (case subjects) was defined as urinary ACR between 30 mg/g · Cr and 300 mg/g · Cr, and normoalbuminuria (control subjects) as urinary ACR less than 30 mg/g · Cr. Fasting plasma glucose (coefficient of variation [CV], 1.1%), glycosylated hemoglobin ($HbA_{1c}$) (CV, 1.2%), total cholesterol (CV, 0.5%), triglyceride (CV, 0.7%), and serum creatinine (CV, 1.1%) levels were determined by routine automated laboratory methods, as described elsewhere.[10,11] Plasma adiponectin concentration (CV, 5.3%) was measured by human adiponectin enzyme-linked immunosorbent assay (ELISA) kits (Otsuka Pharmaceutical Co, Tokyo, Japan). The study protocol was approved by institutional ethics committee, and all patients gave informed consent. The patients were treated with diet alone (30 kcal/kg standard body weight per day containing of 60% carbohydrate and 25% fat), with diet in combination with hypoglycemic agents (sulfonylurea, α-glucosidase inhibitor, or biganide) excluding thiazolidinediones, or with diet in combination with insulin therapy.

### *Genotyping*

The genomic DNA was extracted from peripheral blood. The genotypes of SNP276 of the adiponectin gene were determined with a fluorescent allele-specific DNA primer assay system as described elsewhere.[12] Briefly, the polymorphic region of the adiponectin gene was amplified by polymerase chain reaction (PCR) with G allele-specific sense primers labeled at the 5′ end with fluorescein isothiocyanate (5′-TAGGCCTTAGTTAATAATGAACC-3′) or T allele-specific sense primers labeled at the 5′ end with Texas red (5′-CTAGGCCTTAGTTAATAATGAAAC-3′) and an antisense primer labeled at the 5′ end with biotin (5′-CATCACAGACCTCCTACACTGATA-3′). The reaction mixture (25 μL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, and 1 U of DNA polymerase (rTaq; Toyobo, Osaka, Japan) in either DNA polymerase buffer. For determination of the genotype, amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells at room temperature, and measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red. The

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**Table 1. Clinical and Laboratory Characteristics of Type 2 Diabetic Patients With Normoalbuminuria (control subjects) or With Incipient Diabetic Nephropathy (case subjects)**

| | Control Subjects | Case Subjects | *P* |
|---|---|---|---|
| n | 208 | 108 | |
| Age (yr)*† | 60.0 ± 9.9 | 64.1 ± 9.9 | <.001 |
| Sex (% men) | 61.8 | 55.0 | .204 |
| Known diabetes duration (yr) | 10.7 ± 8.0 | 13.8 ± 8.5 | .001 |
| BMI (kg/m$^2$) | 23.4 ± 3.3 | 23.9 ± 2.9 | .132 |
| SBP (mm Hg) | 130 ± 14 | 134 ± 17 | .039 |
| DBP (mm Hg) | 77 ± 10 | 79 ± 11 | .219 |
| FPG (mg/dL) | 143 ± 40 | 163 ± 53 | .002 |
| $HbA_{1c}$ (%)† | 7.0 ± 1.2 | 7.7 ± 1.4 | <.001 |
| Total cholesterol (mg/dL)* | 205 ± 34 | 208 ± 41 | .423 |
| Triglyceride (mg/dL) | 121 ± 75 | 125 ± 66 | .624 |
| Serum creatinine (mg/dL)*† | 0.7 ± 0.2 | 0.7 ± 0.2 | .685 |
| ACR (mg/g · Cr) | 11.6 ± 10.3 | 74.1 ± 58.3 | <.001 |
| Plasma adiponectin ($\mu$g/mL) | 6.2 ± 4.2 | 5.0 ± 2.2 | .096 |
| Retinopathy (NDR/NPDR/PDR) | 162/23/21 | 70/19/20 | .022 |
| SNP276 of adiponectin gene G carriers/noncarriers | 188/20 | 97/11 | .872 |
| PPAR-$\gamma$2 gene Ala carriers/noncarriers | 15/193 | 11/97 | .362 |

NOTE. Data are means ± SD. All Ala carriers of PPAR-$\gamma$2 gene are Pro/Ala heterozygous, and there were no Ala/Ala homozygous subjects in the present study.

Abbreviations: FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACR, albumin creatinine ratio; NDR, nondiabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

*†Significant differences between genders in the control and case subjects, respectively.

polymorphic region of PPAR-$\gamma$2 gene was amplified by PCR with G allele-specific sense primers labeled at the 5′ end with fluorescein isothiocyanate (5′-TGGGAGATTCTCCTATTGGC-3′) or C allele-specific sense primers labeled at the 5′ end with Texas red (5′-GGGAGATTCTCCTATTGCC-3′) and an antisense primer labeled at the 5′ end with biotin (5′-ACAGTGTATCAGTGAAGG- AATCG-3′). The following procedure was same as the adiponectin gene.

### *Statistical Analysis*

Statistical analysis was performed using StatView version 5.0 (Abacus Concepts, Berkeley, CA). Comparisons of clinical and laboratory characteristics between 2 different groups (eg, case and control subjects) were done by unpaired Student's *t* test or chi-square test as appropriate, and skewed data were logarithmically transformed before analysis. Genotype distribution in the case and control subjects was compared by chi-square test. A *P* value less than 5% was considered significant. Multiple logistic regression analysis was used to determine the independent factors associated with incipient diabetic nephropathy. Data are shown as the mean ± SD.

## RESULTS

Patients with microalbuminuria (case subjects) had older age, longer diabetic duration, greater levels of $HbA_{1c}$, higher systolic blood pressure, and greater ACR than normoalbuminuric patients (control subjects) (Table 1). Case subjects had a tendency toward lower plasma adiponectin levels than control subjects, but not the difference was significant. Diabetic retinopathy was also more frequent in case (35.8% with any retinopathy, 18.3% with proliferative retinopathy) than in control (21.3% with any retinopathy, 10.1% with proliferative retinopathy) subjects. Treatment of diabetes in case and control subjects consisted of diet (3% *v* 15%, $P < .05$) and insulin (27% *v* 17%, $P < .05$), respectively. The proportion (70%) of case subjects treated with oral hypoglycemic agents (57% sulfonylurea, 8% $\alpha$-glucosidase inhibitor, and 5% biganide) was identical to that (68%) of control subjects (53% sulfonylurea, 11% $\alpha$-glucosidase inhibitor, or 4% biganide). In control group, females' age (61.8 ± 8.2 *v* 58.6 ± 10.8 years, $P = .026$) and total cholestrol levels (214 ± 31 *v* 199 ± 34 mg/dL, $P = .001$) were significantly higher, and serum creatinine levels (0.7 ± 0.3 *v* 0.9 ± 0.2 mg/dL, $P < .0001$) were significantly lower than males' parameters. In the case group, higher age (66.4 ± 8.6 *v* 62.0 ± 10.9 years, $P = .026$), greater $HbA_{1c}$ levels (8.2% ± 1.5% *v* 7.4% ± 1.2%, $P = .007$), and lower serum creatinine levels (0.7 ± 0.2 *v* 0.9 ± 0.2 mg/dL, $P < .0001$) were observed in female subjects compared to male subjects. There was no difference in serum adiponectin levels between males and females in the control (male 6.1 ± 2.6 *v* female 6.6 ± 5.2 $\mu$g/mL, $P = .54$) and case groups (male 5.2 ± 2.2 *v* female 4.5 ± 2.1 $\mu$g/mL, $P = .40$). Other parameters did not show significant gender differences (data not shown).

The genotypic distribution of SNP276 in the adiponectin gene was in Hardy-Weinberg equilibrium (G/G, 49.2%, n = 174; G/T, 41.2%, n = 146; T/T, 9.6%, n = 34). The G allele frequency of the SNP276 in the adiponectin gene was 0.70, which was similar to that in a previous Japanese report.[8] The genotype distribution of G allele carriers and noncarriers and G allele frequency in case subjects were not significantly different from those in control subjects. There were no differences between the G allele carriers of the SNP276 in the adiponectin gene regarding age, duration of diabetes, body mass index (BMI), $HbA_{1c}$, serum lipids, serum creatinine, and plasma adiponectin levels (Table 2). Multiple regression analysis showed that diabetic duration (odds ratio [OR], 1.03; 95% confidence interval [CI], 1.002 to 1.066; $P = .035$) and $HbA_{1c}$ (OR, 1.43; 95% CI, 1.15 to 1.73; $P = .0006$), but not SNP276

**Table 2. Clinical and Laboratory Characteristics of Type 2 Diabetic Patients According to the Presence (G/G and G/T) or Not (T/T) of the G Allele in SNP276 of the Adiponectin Gene**

| Genotype | G/G and G/T | T/T | *P* |
|---|---|---|---|
| n | 285 | 31 | |
| Age (yr) | 61.3 ± 10.2 | 61.4 ± 8.5 | .977 |
| Known diabetes duration (yr) | 11.8 ± 8.3 | 11.4 ± 8.7 | .798 |
| BMI (kg/m$^2$) | 23.6 ± 3.3 | 23.5 ± 2.1 | .915 |
| SBP (mm Hg) | 131 ± 15 | 136 ± 17 | .106 |
| DBP (mm Hg) | 77 ± 10 | 80 ± 9 | .150 |
| FPG (mg/dL) | 151 ± 47 | 144 ± 39 | .421 |
| $HbA_{1c}$ (%) | 7.3 ± 1.3 | 7.0 ± 1.2 | .286 |
| Total cholesterol (mg/dL) | 207 ± 36 | 200 ± 37 | .306 |
| Triglyceride (mg/dL) | 124 ± 74 | 110 ± 52 | .319 |
| Serum creatinine (mg/dL) | 0.7 ± 0.2 | 0.7 ± 0.1 | .777 |
| ACR (mg/g · Cr) | 29.7 ± 44.3 | 31.8 ± 32.5 | .87 |
| Plasma adiponectin ($\mu$g/mL) | 5.9 ± 3.9 | 5.4 ± 2.5 | .690 |

NOTE. Data are means ± SD.

of the adiponectin gene (OR, 0.82; 95% CI, 0.49 to 1.41; $P = .78$) were independently associated with incipient diabetic nephropathy. The Ala allele frequency in the PPAR-γ2 gene in the case subjects (3.6%) was not significantly different from that (5.1%) in the control subjects.

## DISCUSSION

The risk of developing diabetic nephropathy may be in part genetically determined,[1,2] because not all patients progress to the same renal outcome even when they represent the same degree of glycemic control, and the condition has been linked to different chromosomes 13 including chromosome 3, on which the genes relating to insulin sensitivity such as adiponectin and PPAR-γ2 are located. It is suggested that insulin resistance is likely to be a risk factor for diabetic nephropathy in type 2 diabetes[3,4] since insulin-sensitizing compounds such as thizolidinediones, potent PPAR-γ ligands, have reduced microalbuminuria in patients with early diabetic nephropathy.[14,15] In the present study, we evaluated whether the SNP276 of the adiponectin gene was related to incipient diabetic nephropathy in Japanese patients with type 2 diabetes.

The results showed no association between genotypes of SNP276 in the adiponectin gene and incipient diabetic nephropathy. There were no associations between the genotypes of SNP276 of adiponectin gene and clinical parameters such as duration of diabetes, BMI, $HbA_{1c}$, and serum lipids levels. Recently, Hara et al[8] have reported that the G allele of SNP276 in adiponectin gene is associated with lower plasma adiponectin concentrations in diabetic subjects. In their study, however, only subjects with SNP276 G/G genotype of adiponectin gene whose BMI was greater than 26.7 $kg/m^2$ had significantly lower serum adiponectin levels compared to those with T/T genotype, but no significant difference was apparent between the 2 genotypes when BMI was lower range (BMI $< 26.5$ $kg/m^2$). The present study did not show that patients with SNP276 G allele carriers of adiponectin gene had lower adiponectin levels compared to those with G allele noncarriers. The lower BMI (23 $kg/m^2$) of subjects in our study may be attributable to no differences in adiponectin levels between the G allele carriers and noncarriers. In addition to the degree of obesity, plasma adiponectin levels have been influenced by pharmacological treatments with thiazolidinediones[16] and third-generation sulfonylurea.[17] Our recent cross-sectional study has shown that SNP276 of adiponectin gene was not associated with significantly different serum adiponectin levels in diabetic patients treated with hypoglycemic agents such as gliclazide or glimepiride.[18] The present study showed that the proportion (70%) of case subjects treated with hypoglycemic agents was identical to that (68%) of control subjects. Moreover, there was no significant difference between the case and control groups in the proportion of subjects treated with sulfonylurea, which may influence serum adiponectin levels. Therefore, these drugs could not have masked differences in adiponectin levels and hence the incidence of microalbuminuria between SNP276 G allele carriers and noncarriers of adiponectin gene.

In the current study, we selected and evaluated only the patients with incipient diabetic nephropathy but did not include patients with diabetic nephropathy that had progressed because it represents a more complex phenotype that is determined by different pathophysiological factors as well as genetic factors. Moreover, it has been reported that plasma adiponectin concentrations were elevated in the progression of diabetic nephropathy so that patients with macroalbuminuria had higher plasma adiponectin concentrations than those with normoalbuminuria and microalbuminuria.[19] The fact that renal impairment influences plasma adiponectin levels makes interpretation of the association between SNP276 of the adiponectin gene and diabetic nephropathy complex.

Several SNPs of other genes may interact and influence plasma adiponectin concentrations.[20] A polymorphism in PPAR-γ2 gene (Ala12Pro), another gene relating to insulin sensitivity, has been related to developing diabetic nephropathy.[21] Patients with type 2 diabetes carrying PPAR-γ2 Ala12 allele have a lower albumin excretion rate[6] and a low frequency of end-stage renal disease,[21] suggesting that this genetic variation in PPAR-γ2 has a protective role in relation to the risk of diabetic nephropathy in type 2 diabetic patients. In the present study, we also examined the polymorphism in the PPAR-γ2 gene (Ala12Pro), and found that the proportion of PPAR-γ2 Ala12 carriers in the case subjects was similar to that in control subjects. Therefore, genetic variation in PPAR-γ2 did not influence our results.

Multiple logistic regression analysis revealed that significant associations with incipient diabetic nephropathy in the present study were duration of diabetes and $HbA_{1c}$ level, but not SNP276 of the adiponectin gene. Cautious interpretation is necessary, because the present study is cross-sectional. The control subjects with normoalbuminuria had a shorter duration of diabetes in our findings, and it is not always the case that control subjects will maintain normoalbuminuria in 4 years. Nonetheless, it is also possible that we can prevent the development of incipient nephropathy in control subjects by maintaining their $HbA_{1c}$ at current levels, because the association between diabetic duration and incipient diabetic nephropathy is weak (OR, 1.03) and polymorphisms of polygenic genes other than adiponectin gene may contribute to the genetic background of diabetic nephropathy. More prospective genome-wide association studies will be needed to clarify the genetic aspects of diabetic nephropathy.

Recently, we have reported that SNP276 of the adiponectin gene is not an independent risk factor for retinopathy in type 2 diabetes.[22] In the current study, we conclude that SNP276 of the adiponectin gene is not associated with incipient diabetic nephropathy in Japanese type 2 diabetic patients.

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