Association Analyses of the Polymorphisms of Angiotensin-Converting Enzyme and Angiotensinogen Genes With Diabetic Nephropathy in Japanese Non-Insulin-Dependent Diabetics

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To investigate predictive genetic markers for diabetic nephropathy, we studied the genetic polymorphisms of angiotensin-converting enzyme (ACE) and angiotensinogen (AGN) in Japanese subjects with non–insulin-dependent diabetes mellitus (NIDDM) with and without nephropathy. Genotype distributions were studied in 132 unrelated NIDDM patients of three groups with normoalbuminuria ([Normo] $n = 53$), microalbuminuria ([Micro] $n = 54$), and macroalbuminuria ([Macro] $n = 25$). The ACE insertion/deletion (I/D) polymorphism of intron 16 was identified by polymerase chain reaction, and the AGN M235T polymorphism was identified by restriction fragment length polymorphism analysis. There were no significant associations between AGN 235 allele or genotype and diabetic nephropathy. The D allele of ACE was significantly more frequent in the Micro ($P = .003$) and Macro ($P = .009$) group than in the Normo group. Overall frequencies of the ACE genotype did not differ significantly between the Micro and Macro groups. There were significant relationships between I/D polymorphism and plasma ACE activity; the DD genotype had the highest activity. A multiple logistic regression analysis revealed that the D allele is a strong and independent risk factor for abnormal albuminuria in NIDDM patients. These results suggested that ACE I/D polymorphism, but not AGN M235T polymorphism, is a possible genetic risk factor for diabetic nephropathy in Japanese NIDDM patients.

THE TENDENCY for development and acceleration of diabetic nephropathy may be partly genetically determined in diabetic patients.[1] Recent observations indicate that the genetic predisposition to hypertension is the candidate factor for diabetic nephropathy.[2-8] The renin-angiotensin system is one of the important factors regulating blood pressure, as well as playing a cardinal role in salt and water homeostasis.[9]

Recently, angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism of intron 16 and angiotensinogen (AGN) M235T polymorphism were reported to be genetic markers of essential hypertension in Japanese subjects.[10,11] Moreover, it was reported that ACE I/D polymorphism is a potent genetic marker of diabetic nephropathy in white insulin-dependent diabetic (IDDM) patients.[12,13] ACE I/D polymorphism was also recognized as a risk factor for coronary heart disease,[14,15] restenosis after coronary intervention,[16] or sudden death as a result of cardiomyopathy.[17]

In this study, we investigated the association between diabetic nephropathy and ACE I/D polymorphism or AGN M235T polymorphism using Japanese non–insulin-dependent diabetic (NIDDM) patients. We found that ACE I/D polymorphism, but not AGN M235T polymorphism, was independently associated with incipient and established diabetic nephropathy in Japanese NIDDM patients.

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SUBJECTS AND METHODS

Patients

Japanese NIDDM patients attending the diabetic clinic of Gunma University Hospital, 132 unrelated NIDDM patients (72 men and 60 women; mean age, 60.8 ± 9.4 years) who were successively evaluated for more than 5 years were recruited for this study. All patients provided informed consent to participate. Patients testing positive for proteinuria, hematuria, or serum creatinine greater than 200 $\mu mol \cdot L^{-1}$ at the initial visit to our clinic were excluded, as were those with a possibility of other renal disease. Patients with cardiovascular disease, liver dysfunction, or clinical or laboratory evidence of other urological diseases and those receiving any drugs affecting renal function were also excluded from this study. All patients had no history of ketoacidosis and no use of insulin in the first year after diagnosis, and all fulfilled World Health Organization criteria for diabetes mellitus.[18] Any therapeutic interventions were applied to achieve better glycemic control after the initial visit to our clinic throughout the observation periods. Thirty-one patients were treated with diet only, 78 were taking an oral hypoglycemic agent, and 23 were treated with insulin.

Seventy-four normotensive, nondiabetic, healthy Japanese subjects were used to investigate the distribution of ACE and AGN genotypes in a normal population.

Determinations

Albumin concentrations in timed overnight urine collections were determined by radioimmunoassay and expressed as the albumin to creatinine ratio, ie, the urinary albumin index ([UAI] milligrams per gram of creatinine). Diagnosis of microalbuminuria was made when the average UAI determined at least two separate times was between 10 and 200 mg/g creatinine, and macroalbuminuria was diagnosed when the average UAI was over 200 mg/g creatinine on at least two separate occasions. According to the UAI, 132 patients were divided into three groups: normoalbuminuria ([Normo] n = 53), microalbuminuria ([Micro] n = 54), and macroalbuminuria ([Macro] n = 25). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters

squared. Blood pressure was measured at each visit by the same physician using a standard sphygmomanometer in the sitting position. Fasting plasma glucose (FPG) level was measured usually once per month by the glucose oxidase method, and mean FPG was calculated as the mean for the past 3 years. Hemoglobin A_{1c} (HbA_{1c}) level was routinely measured by a high-performance liquid chromatography method (Kyoto Daiichi Kagaku, Kyoto, Japan) and also used as the mean data for the past 3 years. Serum C-peptide concentration was measured by radioimmunoassay (Daiichi Radioisotope, Tokyo, Japan).

Plasma ACE activity was measured by spectrophotometric assay.[19] Patients who received ACE inhibitors were excluded from measurement of ACE activity. Diabetic retinopathy was classified as none, simple, or proliferative type by independent ophthalmologists.

DNA Analysis

Genomic DNA was isolated by phenol-chloroform extraction from whole blood drawn into tubes containing potassium EDTA.[20] Polymerase chain reaction was used to detect the two alleles of 490 and 190 bp corresponding, respectively, to the insertion (I) and deletion (D) fragments of ACE.[21] Genomic DNA (250 ng) was used in a final volume of 50 μL containing 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris hydrochloride (pH 8.4), 50 pmol of each primer (5′-CTGGAGACCACTCCCATCCTTTCT-3′ and 5′-GATGTGGCCATCACATTCGTCAGAT-3′), 250 μmol/L each of the four dNTPs, and 0.4 U *Taq* polymerase. DNAs were amplified on a programable thermal controller (MJ Research, Waterstone, MA) for 30 cycles with denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute.

AGN M235T polymorphism was investigated by polymerase chain reaction of genomic DNA followed by restriction endonuclease digestion.[22] Genomic DNA (250 ng) was used in a final volume of 50 μL containing 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris hydrochloride (pH 9.0), 50 pmol of each primer (5′-GATGCGCACAAGGTCCTG-3′ and 5′-CAGGGTGCTGTCCACACTGGCTCGC-3′), 250 μmol/L each of the four dNTPs, and 0.4 U *Taq* polymerase. There was an initial denaturation at 94°C, followed by 25 cycles of 1 minute at 94°C, 1 minute at 61°C, and 1 minute at 72°C. The longer oligonucleotide is mismatched with genomic DNA, which creates an *Sfa*N1 restriction site during amplification. After enzymatic amplification of genomic DNA, each product was digested with *Sfa*N1. If codon 235 is ATG (M235), digestion with *Sfa*N1 yields a 266-bp product relative to undigested 303-bp products (T235). The genotypes were analyzed by electrophoresis in 4% agarose gel and visualized by ethidium bromide staining.

Statistical Analyses

Results are presented as the mean ± SD. Allele frequencies were estimated by the gene-counting method, and the difference between groups was tested by χ^2 analysis. Hardy-Weinberg equilibrium was also checked by χ^2 analysis. One-way ANOVAs were used to compare group means for the different parameters studied. Student's t test was used to analyze differences for the two group comparisons. Simultaneous adjustments for qualitative and quantitative variables were made using a logistic regression model in which only variables showing a significant association with diabetic nephropathy in univariate analyses ($P < .05$) were introduced.

RESULTS

Figure 1 shows examples of three patterns of ACE I/D polymorphism and AGN M235T polymorphism. ACE I/D and AGN M235T genotype distributions did not differ significantly between NIDDM patients and healthy control subjects (Table 1).

Table 2 shows clinical characteristics of NIDDM patients in this study. Systolic blood pressure and HbA_{1c} were significantly higher in Micro and Macro groups than in the Normo group. There were no significant differences in variables such as FPG, fasting C-peptide, age, duration of diabetes, BMI, and lipids. The prevalence of diabetic retinopathy was significantly higher in the Macro group. There were no specific tendencies of therapeutic regimens between the three groups.

Table 3 shows distribution of ACE and AGN genotypes and alleles in the three groups. All groups were in Hardy-Weinberg equilibrium. The D allele of ACE I/D polymorphism was significantly more frequent in Micro ($P = .003$) and Macro ($P = .009$) groups than in the Normo group. Overall frequencies of ACE genotypes did not differ significantly between Micro and Macro groups. The II genotype prevalence was lower in Micro and Macro groups than in the Normo group. Concerning AGN polymorphism,

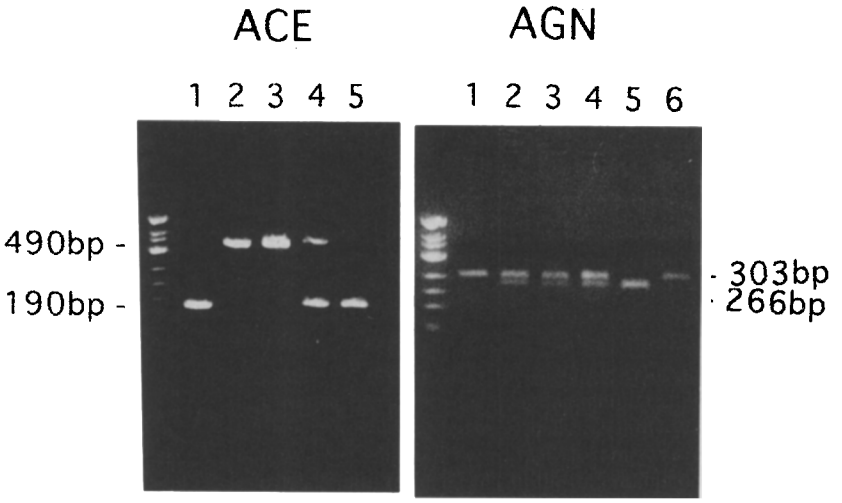

Fig 1. Examples of 3 patterns of ACE I/D polymorphism and AGN M235T polymorphism. ACE homozygotes (II) have a band of 490 bp (lanes 2 and 3), the other homozygotes (DD) have a band of 190 bp (lanes 1 and 5), and heterozygotes (ID) have both bands (lane 4). The variant AGN M235 was cut by digestion with *Sfa*N1 to form a 266-bp fragment relative to the 303-bp AGN 234T fragment. Homozygotes (MM) have a band of 266 bp (lane 5), the other homozygotes (TT) have a band of 303 bp (lanes 1 and 6), and heterozygotes (MT) have both bands (lanes 2 to 4). The first lane shows size markers (ϕX174RFIDNA/*Hin*f1).

Table 1. Distribution of ACE and AGN Genotypes in NIDDM Patients and Healthy Control Subjects

	ACE			AGN		
	DD	ID	II	MM	MT	TT
Control (n = 74)	8 (0.11)	34 (0.46)	32 (0.43)	1 (0.01)	32 (0.44)	41 (0.55)
NIDDM (n = 132)	20 (0.13)	53 (0.39)	59 (0.48)	4 (0.02)	55 (0.38)	73 (0.60)
χ^2		0.96			1.30	
P		.62			.52	

NOTE. χ^2 tests were performed for control *v* NIDDM subjects for analyses of genotype distributions. Frequencies are in parentheses.

there were no significant associations between AGN 235 allele or genotype and the three groups.

In view of the association between ACE polymorphism and diabetic nephropathy, values of different variables potentially related to nephropathy were compared among the three groups of ACE I/D genotypes. No difference could be detected between genotypes for FPG, HbA_{1c}, blood pressure, or plasma lipid variables (Table 4).

Mean plasma ACE activity in patients defined by I/D polymorphism, excluding those who received ACE inhibitors, was compared among the three groups. There were significant relationships between polymorphism and plasma ACE activity (Table 4). According to univariate analyses, the ACE deletion polymorphism was found to be a risk factor for abnormal albuminuria (Micro and Macro) in NIDDM patients (Table 3). A multiple logistic regression analysis was performed introducing the risk factors of abnormal albuminuria that were identified in univariate analyses. When abnormal albuminuria and Normo were compared, the D allele appeared to be an independent risk factor for abnormal albuminuria (odds ratio, 2.62; $P = .001$; Table 5).

Table 2. Clinical Features in NIDDM Patients

Characteristic	Normo (n = 53)	Abnormal Albuminuria: Micro (n = 54)	Abnormal Albuminuria: Macro (n = 25)
Age (yr)	60.3 ± 8.6	60.5 ± 7.2	62.4 ± 9.8
Sex (M/F)	30/23	29/25	13/12
Duration (yr)	12.8 ± 7.6	13.8 ± 6.7	13.1 ± 5.9
BMI (kg/m²)	22.7 ± 2.9	23.6 ± 2.2	22.1 ± 2.4
FPG (mg/dL)	143.9 ± 26.7	152.5 ± 32.0	148.4 ± 19.8
HbA_{1c} (%)	7.2 ± 1.1	7.7 ± 1.9*	7.9 ± 1.5*
TC (mg/dL)	202.6 ± 31.7	208.2 ± 36.8	206.8 ± 32.8
TG (mg/dL)	122.9 ± 73.2	135.7 ± 66.4	138.8 ± 78.9
HDL-C (mg/dL)	47.9 ± 12.4	45.2 ± 13.6	41.6 ± 14.9
Serum creatinine (μmol/L)	77.8 ± 18.6	78.8 ± 19.5	84.1 ± 38.0
Systolic BP (mm Hg)	131.6 ± 16.1	140.8 ± 18.3*	146.8 ± 18.1*
Diastolic BP (mm Hg)	77.2 ± 9.0	79.4 ± 10.1	81.0 ± 9.4
Fasting CPR (nmol/L)	0.54 ± 0.13	0.57 ± 0.16	0.56 ± 0.19
Retinopathy (N/S/P)	33/11/9	30/13/11	3/13/9†
Therapy (D/O/I)	16/32/5	13/31/10	2/15/8

NOTE. Data are expressed as the mean ± SD. Student's *t* tests were performed, except for retinopathy and therapy in Normo *v* Micro and Normo *v* Macro groups (*$P < .05$). χ^2 tests were performed in Normo *v* Micro and Normo *v* Macro groups for analyses of retinopathy and therapy (†$P < .05$).

Abbreviations: BP, blood pressure; N, none; S, simple; P, proliferative; D, diet; O, oral agent; I, insulin; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; CPR, C-peptide immunoreactivity.

Table 3. Distribution of ACE and AGN Genotypes and Alleles in NIDDM Patients

Parameter	Normo (n = 53)	Abnormal Albuminuria: Micro (n = 54)	Abnormal Albuminuria: Macro (n = 25)
ACE allele			
D	25 (0.24)	46 (0.43)	22 (0.44)
I	81 (0.76)	62 (0.57)	28 (0.56)
χ^2	—	8.72	6.73
P	—	.003*	.009*
ACE genotype			
DD	5 (0.09)	10 (0.19)	5 (0.20)
ID	15 (0.29)	26 (0.48)	12 (0.48)
II	33 (0.62)	18 (0.33)	8 (0.32)
χ^2	—	9.02	6.34
P	—	.01*	.04*
AGN allele			
M	25 (0.24)	22 (0.20)	16 (0.32)
T	81 (0.76)	86 (0.80)	34 (0.68)
χ^2	—	0.32	1.24
P	—	.57	.27
AGN genotype			
MM	2 (0.03)	1 (0.02)	1 (0.04)
MT	21 (0.40)	20 (0.37)	14 (0.56)
TT	30 (0.57)	33 (0.61)	10 (0.40)
χ^2	—	0.49	1.93
P	—	.78	.38

NOTE. χ^2 analyses were performed in Normo *v* Micro and Normo *v* Macro groups on each polymorphism. Frequencies are in parentheses.
*$P < .05$.

DISCUSSION

Familial clustering of diabetic nephropathy has been reported. In IDDM, 83% of diabetic siblings of probands with diabetic nephropathy have evidence of nephropathy, compared with only 17% of diabetic siblings of probands without nephropathy.[3] Familial influence on the development of nephropathy has also been described in Pima Indians with NIDDM.[6] These studies are consistent with

Table 4. Comparison of Several Variables Between ACE I/D Genotypes

Variable	ACE Genotype: DD (n = 20)	ID (n = 53)	II (n = 59)
FPG (mg/dL)	149.6 ± 18.6	148.8 ± 33.9	147.0 ± 25.6
HbA_{1c} (%)	7.6 ± 1.1	7.4 ± 1.3	7.7 ± 1.9
TC (mg/dL)	209.1 ± 46.8	201.2 ± 34.3	206.3 ± 49.4
TG (mg/dL)	140.4 ± 36.2	119.2 ± 87.4	136.3 ± 97.9
HDL-C (mg/dL)	44.4 ± 13.6	49.5 ± 13.7	48.7 ± 14.1
Systolic BP (mm Hg)	136.1 ± 21.1	139.7 ± 19.2	137.3 ± 16.4
Diastolic BP (mm Hg)	76.2 ± 9.7	79.3 ± 8.9	79.4 ± 9.9
ACE (IU/L)	18.8 ± 3.5 (n = 16)	18.2 ± 4.1 (n = 44)	16.6 ± 3.1* (n = 51)

NOTE. One-way ANOVAs were performed to compare group means for different parameters.

Abbreviations are as in Table 2.

*$P < .05$.

Table 5. Logistic Regression Analysis of Risk Factors Associated With Abnormal Albuminuria in Univariate Analysis in NIDDM Patients

Risk Factor	Odds Ratio	*P*	95% CI
Systolic BP	1.04	.001	1.02-1.05
D allele of ACE	2.62	.001	1.47-4.66
HbA_{1c}	1.36	.049	1.01-1.84

Abbreviation: CI, confidence interval.

the postulate that inherited factors play an important role in determining susceptibility to diabetic nephropathy.

A familial predisposition to hypertension has been suggested as a possible contributing factor to susceptibility to nephropathy in diabetes.[2-8] Recently, possible genetic markers of essential hypertension were reported. Namely AGN and ACE, which are involved in the renin-angiotensin system, were reported to be the possible candidate genes for essential hypertension.[10,11,21-23]

In this study, we found a significant and independent association between the D allele and diabetic nephropathy; however, there were no significant associations between AGN M235T polymorphism and diabetic nephropathy. Our finding that ACE I/D polymorphism and diabetic nephropathy are significantly associated is consistent with a previous report in which white IDDM patients were studied.[12,13] We could not find a significant association of AGN M235T polymorphism and diabetic nephropathy; however, these findings do not completely exclude the possibility of an association between the AGN gene and diabetic nephropathy, since one single polymorphism does not exhaustively assess the full information contained in a gene. Retrospective case-control studies are susceptible to experimental bias. ACE and AGN genotype distributions may have been different in NIDDM subjects as compared with the general population, but there were no significant differences between NIDDM and healthy control subjects. There were also no significant differences between Normo and abnormal albuminuric groups for the main clinical parameters (age, duration of diabetes, sex, BMI, and lipid profile) in this study. Moreover, the positive association between ACE I/D polymorphism and abnormal albuminuria in NIDDM is also strongly supported by multivariate logistic regression analyses. Survival bias cannot be avoided in a disease-association study; therefore, it is possible that an early mortality with abnormal albuminuria due to the ACE locus could lead to an underestimation of the association between diabetic nephropathy and this gene. Another problem is that Micro may be a less specific finding in NIDDM than in IDDM, because less than 70% of clinically proteinuric NIDDM patients showed histological evidence of diabetic nephropathy.[24] On the other hand, a renal biopsy is not feasible for making a diagnosis of diabetic nephropathy in a large number of subjects, because it is invasive and informed consent is not easy to obtain. To exclude misclassification of other renal diseases as much as possible, we studied only patients who had no proteinuria, hematuria, or elevation of serum creatinine at the first visit and who had relatively long observation periods (>5 years) in our clinic. Larger studies using patients with longer observation periods and advanced diabetic nephropathy are nevertheless required to confirm our results. ACE has been identified as a membrane-bound enzyme in several types of cells, including vascular endothelial cells, various adsorptive epithelial cells, neurons, and macrophages, and is also present in a circulating form in biological fluids such as plasma.[25-27] It was reported that the D allele was associated with a high plasma concentration of ACE in healthy subjects.[28,29] Costerousse et al[30] reported that not only plasma ACE activity but also the level of expression of membrane-bound ACE was associated with ACE I/D polymorphism. In this study using NIDDM patients, we also observed a significant relationship between ACE I/D polymorphism and plasma ACE level. It would be better to investigate membrane-bound ACE activity in the kidney, since it is possible that the D allele of ACE, which is associated with plasma ACE activity, can influence the progression of diabetic nephropathy. ACE plays a key role in the production of angiotensin II. Angiotensin II has been shown in many tissues to be important in cell growth and hypertrophy,[31-33] and there is a possibility of a direct hypertrophic effect on glomerular cells.[34] Recent study has demonstrated that infusion of angiotensin II results in a rapid increase in circulating levels of plasminogen activator inhibitor I, a finding that may help to explain clinical observations linking the renin-angiotensin system and thrombotic risk.[35] Attenuated fibrinolysis is one of the factors that accelerates renal damage. Several reports on ACE I/D polymorphism have demonstrated that the DD genotype is associated with an increased risk of cardiovascular disease.[14,15,36,37]

The D allele of ACE, which influences angiotensin II production, may affect renal damage from the progression of atherosclerosis and thrombosis. Further investigations are required to clarify the role of ACE activity and angiotensin II in the progression of diabetic nephropathy. In this study, there were no significant associations between ACE I/D polymorphism and FPG, HbA_{1c}, and lipid profiles in NIDDM patients, and we also did not find a significant association between ACE I/D polymorphism and blood pressure. Therefore, it is likely that ACE polymorphism acts as a risk factor for diabetic nephropathy by modulating renal hemodynamics without affecting systemic blood pressure, as has been hypothesized for myocardial infarction.[14] We could deal with this polymorphism as a self-contributing factor for diabetic nephropathy. We showed that frequency of the D allele was not significantly different in Micro and Macro groups, and there was a positive association between ACE I/D polymorphism and abnormal albuminuria by multivariate logistic regression analyses. This result suggests that ACE I/D polymorphism is a risk factor not only for established diabetic nephropathy but also for the incipient diabetic nephropathy that is often associated with dyslipidemia and coronary heart disease.[38-40]

In conclusion, our findings suggest that ACE I/D polymorphism, but not AGN M235T polymorphism, is independently associated with diabetic nephropathy in Japanese NIDDM patients. Further investigations are required to clarify the pathogenic role of the renin-angiotensin system in diabetic nephropathy.

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