Brief Genetics Report

The Angiotensin-Converting Enzyme DD Genotype Is Associated With Glomerulopathy Lesions in Type 2 Diabetes

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Genetic factors are important in conferring diabetic nephropathy (DN) risk. The insertion/deletion (I/D) polymorphism of the ACE gene has been described to be associated with DN risk and progression. The renal lesions underlying DN in type 2 diabetes are heterogeneous; only a subset of patients, characterized by a faster decline of renal function, have diabetic glomerulopathy. This study explored the relations between diabetic glomerulopathy and the ACE genotype distribution in 77 type 2 diabetic patients with an albumin excretion rate ≥20 μg/min. Using morphometric analysis of kidney biopsies, mesangial and mesangial matrix fractional volumes [Vv(mes/glom) and Vv(MM/glom)] and glomerular basement membrane (GBM) width were estimated. We found that 13 patients were II, 30 were ID, and 34 were DD. Clinical features and renal function were similar in the three groups; in contrast, the DD patients had the highest Vv(MM/glom) and GBM width. Subdividing patients in tertiles of GBM width and Vv(MM/glom), from the lowest (I) to the highest (III) values, the DD carriers had an odds ratio of 6.11 (95% CI 1.84–20.3) and 10.67 (2.51–45.36), respectively, for the likelihood of being in tertile III than I for GBM width and Vv(MM/glom). Multiple regression analysis revealed the I/D polymorphism as an independent determinant of GBM thickening in addition to diabetes duration and HbA_{1c}. In conclusion, the ACE DD genotype is associated with diabetic glomerulopathy lesions, making the study of this polymorphism helpful in identifying those type 2 diabetic patients at higher risk of fast DN progression. *Diabetes* 51:251–255, 2002

Only a minority of type 1 and type 2 diabetic patients develop diabetic nephropathy (DN) (1,2). Environmental factors (3) are not sufficient to entirely explain DN risk variability; moreover, the demonstration that DN clusters in families (4–8) suggests that genetic factors are important in conferring DN risk or protection (9). These family studies in type 1 and type 2 diabetes have encouraged the search for potential candidate genes; the most extensively studied so far is the ACE gene. A positive association between the DD genotype and DN risk has been described in both type 1 and type 2 diabetes by some but not all studies (9–12). The D allele seems to be associated more with progression of renal disease than with nephropathy risk in both type 1 (13) and Japanese type 2 diabetic patients (14). Although there is considerable evidence to support the association between the ACE insertion/deletion (I/D) polymorphism and nephropathy risk/progression in type 1 diabetes (10,13), data on this association in type 2 diabetes are less conclusive. This is not surprising because abnormalities in the albumin excretion rate (AER) in type 2 diabetes may be an expression of different phenotypes. Indeed, progression of nephropathy varies greatly among type 2 diabetic patients, likely because of the heterogeneity in renal lesions. Heterogeneity in renal structure has been described in type 2 diabetic patients with both proteinuria and microalbuminuria (15–16); only a subset of them have typical diabetic glomerulopathy, whereas a substantial proportion has less specific renal lesions, such as relatively advanced tubulo-interstitial and vascular changes, despite mild or absent diabetic glomerulopathy (16). Moreover, structural heterogeneity has a prognostic impact in that the loss in renal function is strongly related to the degree of glomerulopathy (17). It is currently unknown whether the structural heterogeneity in type 2 diabetes is associated with different genetic backgrounds.

This study explored the relations between the degree of diabetic glomerulopathy and the ACE I/D polymorphism in 77 type 2 diabetic patients (55 men and 22 women aged 56 ± 7 years, known type 2 diabetes duration 10.6 ± 7 years, HbA_{1c} 8.3 ± 1.6%). The AER was 113 μg/min (20–1925); 54 patients had microalbuminuria and 23 had macroalbuminuria. The glomerular filtration rate (GFR)

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AER, albumin excretion rate; ANCOVA, analysis of covariance; ANOVA, analysis of variance; DN, diabetic nephropathy; GFR, glomerular filtration rate; GBM, glomerular basement membrane; OR, odds ratio; PCR, polymerase chain reaction; Vv(mes/glom), mesangial fractional volume; Vv(MM/glom), mesangial matrix fractional volume.

TABLE 1
Clinical features and morphometric measures of glomerular structure of type 2 diabetic patients divided in three groups on the basis of their genotype

ACE genotype	II	ID	DD	*P*
n (men)	13 (7)	30 (22)	34 (25)	
Age (years)	55.5 ± 8.5	56.3 ± 7.7	57.2 ± 6.8	NS
Diabetes duration (years)	7.7 ± 5.9	10.4 ± 7.7	11.4 ± 5.6	NS
HbA_{1c} (%)	8.2 ± 1.8	8.1 ± 1.5	8.4 ± 1.6	NS
BMI (kg/m^2)	28.1 ± 4.2	29.3 ± 4.2	29.2 ± 4.9	NS
GFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	107 ± 26	99 ± 24	101 ± 33	NS
AER (μg/min)	106 (20–982)	74 (20–721)	151 (22–1925)	NS
SBP (mmHg)	145 ± 15	148 ± 17	149 ± 15	NS
DBP (mmHg)	86 ± 9	85 ± 9	87 ± 10	NS
GBM width (nm)	406 ± 94	433 ± 111	503 ± 131*†	0.016
Vv(mes/glom)	0.24 ± 0.06	0.26 ± 0.07	0.28 ± 0.08	0.08
Vv(MM/glom)	0.121 ± 0.04	0.125 ± 0.044	0.149 ± 0.04*	0.04

Data are means ± SD or median (range). DBP, diastolic blood pressure; SBP, systolic blood pressure. *$P < 0.05$, DD vs. ID; †$P < 0.05$ DD vs. II (Duncan's test).

was 101 ± 28 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$, and systolic and diastolic blood pressure were 148 ± 16 and 86 ± 9 mmHg, respectively. All patients were receiving antihypertensive therapy (all but eight received ACE inhibitors). Patients were divided on the basis of the ACE I/D polymorphism into three groups that were not significantly different with respect to age, diabetes duration, BMI, HbA_{1c}, blood pressure levels, GFR, or AER (Table 1); however, the DD patients tended to have longer diabetes duration and higher AER values.

All estimates of glomerular structure were significantly higher in the three groups than in control subjects ($P <$ 0.01 for all comparisons). Group differences in glomerular structure were found, with DD patients having the highest mesangial matrix fractional volume [Vv(MM/glom)] and GBM width. There was no difference between ID and II patients (Table 1 and Fig. 1). Patients were then subdivided into tertiles of GBM width and Vv(MM/glom), with tertile I and III having the lowest and the highest values, respectively. For GBM width, the DD carriers represented 27, 36, and 80% of patients in tertiles I, II, and III, respectively, having a 6.11 (odds ratio [OR]; 95% CI 1.84–20.3) higher risk to be in tertile III than in tertile I. Similarly, for Vv(MM/glom), the DD carriers (13, 54, and 62% in tertiles I, II, and III, respectively) had a 10.67 (OR; 2.51–45.36) higher risk to be in the tertile with the highest mesangial matrix expansion.

All structural parameters were significantly related to renal function; both mesangial fractional volume [Vv(mes/glom)] and Vv(MM/glom) correlated with AER ($r = 0.45$ and 0.46, respectively; $P < 0.001$ for both) and GFR ($r = -0.44$ and -0.40; $P < 0.001$); GBM width was related to

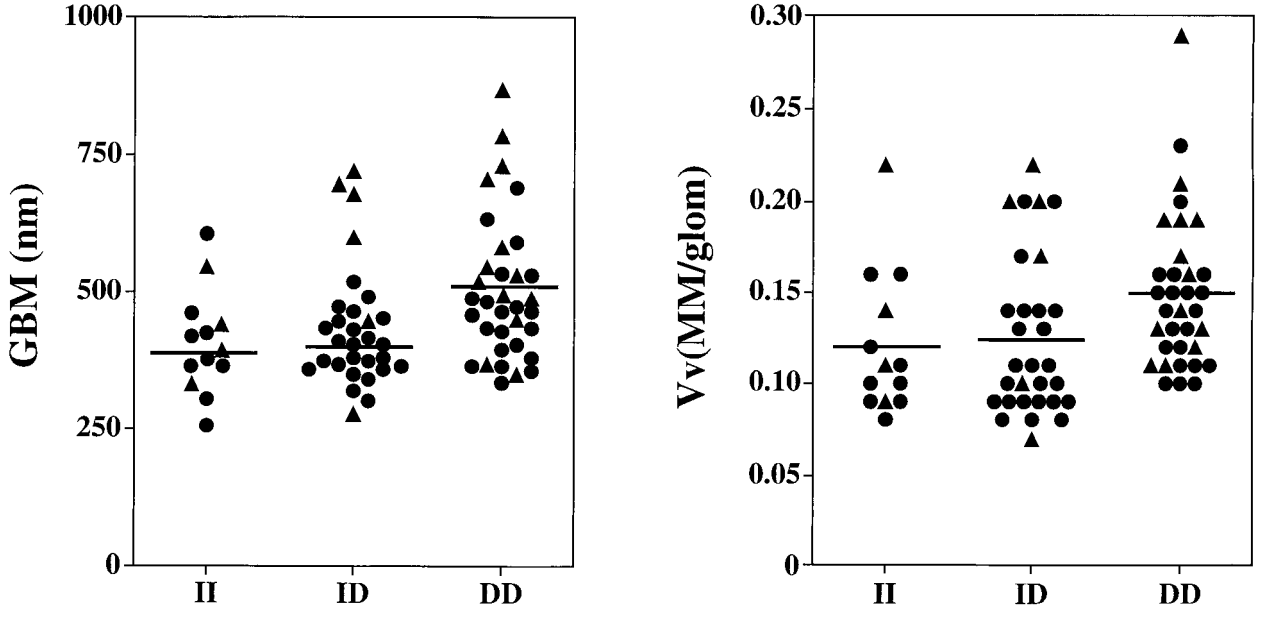

FIG. 1. *A*: GBM width values in the three groups of patients (II, ID, and DD). ●, Microalbuminuric patients; ▲, proteinuric patients. The lines represent the mean values. ANOVA = 0.016; DD vs. ID and II, $P < 0.05$ for both (Duncan's test). *B*: Vv(MM/glom) values in the three groups of patients. ●, microalbuminuric patients; ▲, proteinuric patients. The lines represent the mean values. ANOVA = 0.04; DD vs. ID, $P < 0.05$ (Duncan's test).

TABLE 2
Multiple regression analysis with GBM width as dependent variable

Independent variable	β	*P*
Known diabetes duration	0.35	0.001
HbA_{1c}	0.27	0.007
ACE genotype	0.24	0.02
Systolic blood pressure	0.04	0.67

The model explained 35% of GBM width. β, standardized partial regression coefficient.

AER ($r = 0.52$; $P < 0.001$). In univariate analysis, GBM width correlated with diabetes duration ($r = 0.49$; $P < 0.001$), HbA_{1c} ($r = 0.36$; $P < 0.005$), and systolic blood pressure ($r = 0.21$; $P < 0.05$). In a multiple regression analysis (Table 2), the ACE genotype resulted an independent determinant of GBM width, although diabetes duration and HbA_{1c} were stronger factors. In addition, analysis of covariance (ANCOVA) revealed that after adjustment for diabetes duration, GBM width was still significantly different among groups ($P < 0.05$).

Using light microscopy, we found that among the II subjects, there were 7 (58%) category CI (normal or near normal renal structure) subjects, 2 (17%) category CII (typical diabetic nephropathology) subjects, and 3 (25%) category CIII (atypical patterns of renal injury) subjects. Among the ID subjects, there were 11 CI (37%), 6 CII (20%), and 13 CIII (43%) subjects; among the DD subjects, there were 5 CI (16%), 16 CII (52%), and 10 CIII (32%) subjects (χ^2; $P < 0.02$). Thus, the prevalence of CIII subjects was not different in the three groups, whereas among the DD carriers, there were more CII and fewer CI subjects.

This study describes for the first time an association between the ACE gene I/D polymorphism and the severity of glomerulopathy lesions in type 2 diabetes. Diabetic glomerulopathy was more advanced in DD than in II and ID patients; also, the DD patients had a significantly higher risk to be in tertile III than in tertile I of distribution for GBM width and Vv(MM/glom). The ACE genotype emerged as an independent determinant of GBM thickening, in addition to other well-known factors, i.e., diabetes duration and metabolic control. Because glomerulopathy is related to renal function and is a determinant of progression (17), it is reasonable to hypothesize an association of the DD genotype with a high risk of developing DN and with faster progression of renal disease in type 2 diabetes. This hypothesis needs to be verified in prospective longitudinal studies. To date, only one study, performed in 30 young microalbuminuric type 1 diabetic patients, has shown that the D allele carriers had more rapid progression of some glomerulopathy parameters (18). The reasons for this association are unknown. However, carriers of the DD genotype have higher ACE plasma levels (19) and an increased activity of the renin angiotensin system; thus, the angiotensin-induced increase in intraglomerular pressure and the effects of angiotensin on both mesangial cell replication and, especially, extracellular matrix accumulation could contribute to the development of diabetic glomerulopathy. Most patients (90%, equally distributed in the three groups) were receiving ACE inhibitors; the finding of worse diabetic glomerular lesions in the DD carriers is consistent with the concept that in these latter patients, ACE inhibitors might be less renoprotective, as previously suggested in type 1 diabetes (20).

These findings might in part explain the contradictory results of case-control studies on the association between the ACE I/D polymorphism (and other genes) and nephropathy risk in type 2 diabetes. In fact, although all of our patients had abnormal AER, only a subset had advanced diabetic glomerulopathy, and these patients were more frequently DD carriers; without kidney biopsies, these associations would not have easily emerged. Thus, when planning to study the association of candidate genes with nephropathy risk/progression in type 2 diabetes, it is necessary to define the patients' phenotype as precisely as possible. The AER phenotype in type 2 diabetes is probably too heterogeneous and imprecise to be used in genetic association studies.

The clinical features were similar, irrespective of the genotype, as were the renal functional parameters. Although not significant, there was a trend for higher AER values in DD patients; also, the II carriers showed a trend toward shorter diabetes duration, this being in keeping with recent data from Krolewski (9). This suggests that either the II patients have a higher mortality rate or that the II genotype primarily contributes to early onset of DN. These data thus support the heterogeneous nature of abnormal AER in type 2 diabetes: in the II carriers, microalbuminuria and early overt nephropathy occur earlier and with less glomerular lesions than in the DD patients, in whom the increased AER appears to be consequent to diabetic glomerulopathy and has a later onset.

In conclusion, this study demonstrates that among microalbuminuric and proteinuric type 2 diabetic patients, the presence of the DD genotype is associated with a high risk for the development of advanced diabetic glomerulopathy. Genotyping patients for the ACE gene might be helpful in clinical practice in identifying those type 2 diabetic patients at greater risk of serious renal lesions, so they could be targeted for intensive metabolic and blood pressure control.

RESEARCH DESIGN AND METHODS

This study was designed to explore the associations between the polymorphisms of candidate genes and diabetic glomerular lesions in type 2 diabetes. Thus, blood samples for DNA extraction were collected from type 2 diabetic patients, who also underwent a kidney biopsy and renal function tests. Inclusion criteria were type 2 diabetes (diagnosed according to World Health Organization criteria [1985]) for at least 2 years, age ≤70 years, serum creatinine <2 mg/dl, and persistent microalbuminuria or macroalbuminuria (albumin excretion of 20–200 μg/min or >200 μg/min, respectively, in at least two of three 24-h urine collections). In addition, the renal tissue obtained from the biopsy had to be adequate for electron microscopic morphometric analyses. Excluded were patients with renal biopsy contraindication (single kidney, serious stone disease, anticoagulant therapy, severe and uncontrolled hypertension, known renal artery stenosis, or known nondiabetic renal disease) and serum creatinine ≥2 mg/dl. In no case were renal biopsies performed for clinically indicated diagnostic purposes. These studies were approved by the ethical committee of the University of Padova, and each patient gave written informed consent before each study. Altogether, 83 consecutive Caucasian patients met the inclusion criteria and agreed to participate; in 6 patients, renal tissue was not adequate for electron microscopic morphometric analysis, and those patients were excluded. Thus, 77 type 2 diabetic patients were included in the present study. These patients were participants in an ongoing study on renal structural-functional relations in type 2 diabetes and were recruited in diabetes clinics in northeast Italy. They were admitted in the Division of Internal Medicine at the University of

Padova Hospital, where percutaneous renal biopsy and renal functional studies were performed. In patients on antihypertensive treatment, the therapy was withdrawn 3–5 days before admission and was not given during the study; after the study was completed, previous therapy was given. During admission, patients underwent at least three 24-h urine collections for measurements of AER (by immunoturbidimetric method) after urine cultures were determined to be sterile. Although ACE inhibitors were stopped for ~1 week before urine collections, we cannot exclude that AER in our patients was reduced by a persistent effect of ACE inhibitors. Nevertheless, this consideration should not affect our results, given that all but eight patients were receiving these drugs. The GFR was determined by the plasma clearance of ^{51}Cr-EDTA (16,17). HbA_{1c} was measured by high-performance liquid chromatography (DIAMAT Analyzer; BIO-RAD, CA). Blood pressure was measured at least 10 times in supine position.

Renal structure. Kidney biopsies were performed under ultrasound guidance by an experienced investigator (P.F.). After the kidney biopsy, tissue was immediately examined under a dissecting microscope to insure adequate numbers of glomeruli and then processed for light, electron, and immunofluorescence microscopy.

Electron microscopic morphometric analysis was performed on three open glomeruli per biopsy. Glomeruli were photographed with a Hitachi H600 electron microscope at ×3,900 to obtain photomontages of the entire glomerular profile to estimate Vv(mes/glom), the fraction of the glomerulus occupied by mesangium, as previously described (21). Normal values are 0.19 ± 0.03. Another set of micrographs, photographed at ×12,000 by entering the glomerulus at its lowest segment and systematically sampling ~20% of the glomerular profile, was used to estimate glomerular basement membrane (GBM) width (21). Normal values are 310 ± 38 nm. The same micrographs were used to estimate the fraction of the glomerulus occupied by mesangial cells [Vv(MC/glom)] and matrix [Vv(MM/glom)]. Normal values for both are 0.09 ± 0.03. The normal ranges were obtained from a group of 27 normal kidney donors (14 mol/l, 13F) matched for age (56 ± 10 years) with the patients studied.

Light microscopy. Most of the core was placed in Zenker's fixative, embedded in paraffin, and processed for light microscopy. Adequate tissue for light microscopy evaluation was available in 73 patients. PAS sections (2-μm thick) from all patients were blindly evaluated and categorized as previously described in detail (16) into the following three categories. Category CI: normal or near normal renal structure. These patients had biopsies that were normal or showed very mild mesangial expansion, tubulo-interstitial changes, or arteriolar hyalinosis in any combination. Category CII: typical diabetic nephropathology. These patients had established diabetic lesions, with balanced severity of glomerular, tubulo-interstitial, and arteriolar changes. This picture is typical of that seen in type 1 diabetic patients. Category CIII: atypical patterns of renal injury. These patients had absent or only mild glomerular diabetic changes, with disproportionately severe renal structural lesions including *1*) tubular atrophy, tubular basement membrane thickening and reduplication, and interstitial fibrosis (tubulo-interstitial lesions); *2*) advanced glomerular arteriolar hyalinosis, commonly associated with atherosclerosis of larger vessels; and *3*) global glomerular sclerosis (>25%). None of the patients had immunofluorescent or electron microscopy findings of any definable renal disease other than typical diabetic nephropathy or the patterns described above.

Determination of genotypes. Genomic DNA was extracted from leukocytes by phenol-chloroform extraction. A DNA fragment on intron 16 of the ACE gene was amplified by polymerase chain reaction (PCR) to determine the ACE genotype. The sequences of flanking primers used were 5′-CTGGAGAC CACTCCCATCCTTTCT-3′ for the sense primer and 5′-GATGTGGCCATCA CATTCGTCAGAT-3′ for the antisense primer.

PCR amplification products were obtained as previously described (12). To avoid ID-DD mistyping, a pair of primers that amplify a region inside intron 16 was also used to analyze all samples showing DD genotype (12). Genotype distribution was similar to allele frequencies obtained in 181 randomly ascertained healthy subjects from the same geographical area.

Statistical analysis. Data are expressed as the means ± 1SD. Values for AER, not normally distributed, were logarithmically transformed before analysis and are expressed as the median and range. Data were analyzed by using the statistical package SPSS for Macintosh. Differences among mean values have been evaluated by analysis of variance (ANOVA) or ANCOVA, as appropriate, and then with Duncan's multiple range tests for multiple comparisons. The ORs were used to compare frequencies in the extreme tertiles of distribution of glomerular parameters in the DD carriers compared with the ID + II carriers. Univariate and multiple regression analyses were used to test the relations between independent variables (e.g., diabetes duration, HbA_{1c}, age, and blood pressure) and the dependent variables (the parameters of diabetic glomerulopathy). Values for $P < 0.05$ were considered significant.

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