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Angiotensin-converting enzyme gene polymorphism in non-insulin dependent diabetes mellitus and its relationship with diabetic nephropathy

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Angiotensin-converting enzyme gene polymorphism in non-insulin dependent diabetes mellitus and its relationship with diabetic nephropathy. Previous studies have shown that the angiotensin-converting enzyme (ACE) gene polymorphism is associated with an increased risk of vascular disease in non-diabetic patients. The present study was conducted on 509 NIDDM patients who underwent a screening test to determine their ACE genotype for the Appropriate Blood Pressure Control in Diabetes (ABCD) Trial. Various baseline indices were correlated with the three ACE polymorphisms. The genotype was determined through polymerase chain reaction amplification of the angiotensin-converting enzyme polymorphism. The univariate relationship between the presence of the DD genotype with nephropathy as measured by urinary albumin excretion (UAE), and a history coronary artery disease (CAD) was then examined. Finally, a multiple model for each UAE and CAD was created so as to determine the independent effects of the presence of the DD genotype on each diabetic complication. Univariately, the presence of the DD genotype was associated with diabetic nephropathy. Furthermore, in a multiple model predicting diabetic nephropathy, the presence of the DD genotype was independently associated with diabetic nephropathy (odds ratio = 2.8, 95% confidence interval 1.4 to 5.5) but not CAD. Thus, the ACE DD genotype in 509 non-Hispanic white NIDDM patients in a metropolitan area in the U.S. was independently associated with the presence of diabetic nephropathy and, therefore, may be potentially used as a marker for NIDDM patients at risk for developing diabetic nephropathy.

Non-insulin dependent diabetes mellitus (NIDDM) accounts for approximately 90% of the 14 million diabetic patients in this country [1]. Moreover, this disease is responsible for over 90 billion dollars in annual health costs in the United States [2]. Diabetic nephropathy is the most frequent cause of end-stage renal disease (ESRD) in the United States, accounting for 33% of all cases. This complication costs the United States health system three billion dollars annually [3]. Insulin-dependent diabetes mellitus (IDDM) is estimated to account for 40%, while NIDDM diabetes causes 60% of the ESRD secondary to diabetic nephropathy. Prior studies have shown that the renin-angiotensin system may play an important role in the development of nephropathy and other diabetic complications in NIDDM [4], and thus the angiotensin-converting enzyme (ACE) polymorphism may be a potential predictor of NIDDM complications. The polymorphism consists of the presence (I allele) or absence (D allele) of a 287 bp *alu* repeat sequence, and the D allele is associated with higher serum ACE activity [5–7].

Previous studies in non-diabetic subjects have demonstrated that the presence of the DD genotype was associated with an increased risk for myocardial infarction [8] and dilated cardiomyopathy [9]. In NIDDM patients there are conflicting results regarding the relationship between the ACE genotype and diabetic complications. Fujisawa et al showed that expression of the DD genotype is related to myocardial infarction but not to diabetic nephropathy or retinopathy in 267 Japanese NIDDM subjects [10]. On the other hand Mizuiri et al, in 111 Japanese NIDDM subjects, showed that the II genotype was associated with a decreased risk of diabetic nephropathy [11]. To date there have been no studies performed in the U.S. evaluating the effects of the ACE genotype on NIDDM subjects.

The present study was therefore conducted on 509 non-Hispanic NIDDM patients who underwent a screening test to determine their ACE genotype for the Appropriate Blood Pressure Control in Diabetes (ABCD) Trial [12]. In these patients the study examined baseline characteristics across the three ACE genotypes as well as the effects of genotype on coronary artery disease and diabetic nephropathy in multiple models.

METHODS

Study population

The Appropriate Blood Pressure Control in Diabetes (ABCD) Trial is a randomized, prospective, blinded clinical trial designed to determine the effects of moderate versus intensive antihypertensive control on the outcome of diabetic complications. The ABCD trial has been described previously [13]. Subjects in the ABCD Trial were between the ages of 40 and 74 years and were identified from Diagnosis Related Group (DRG), pharmacy and billing lists from participating hospitals from March 1991 through January 1993. All patients enrolled in the ABCD Trial were diagnosed with NIDDM according to the criteria based upon the World Health Organization report of 1985 [14], which followed the National Diabetes Group criteria of 1979 [1].

Key words: angiotensin converting enzyme, blood pressure and diabetes, diabetic nephropathy, genotype ACE DD and diabetes, NIDDM.

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Six hundred ninety-seven NIDDM subjects had their angiotensin-converting enzyme insertion/deletion polymorphism determined in a single lab at the University of Colorado Health Sciences Center (Denver, CO, USA). Because of the smaller number of the other racial and ethnic groups, the study evaluated only the non-Hispanic white subjects. Informed consent was obtained on all patients. Accordingly, we determined the distribution frequency of angiotensin-converting enzyme insertion/deletion (I/D) polymorphism.

Genomic DNA preparation

Genomic DNA was extracted from peripheral blood as described previously [10]. Briefly, genomic DNA was extracted from leukocytes in dried blood spots collected on filter paper. Spots were excised using sterile biopsy punches, placed into microfuge tubes, and incubated at 56°C for 18 hours in lysis buffer (154 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM NaEDTA, pH 8.0, 5% proteinase K, 1% SDS). Proteins were removed by sequential phenol-chloroform and chloroform extraction. DNA was precipitated from the aqueous phase by addition of 1/10 volume ^{3}M sodium acetate (pH 5.0) and an equal volume of ice-cold isopropanol. Samples were held at −20°C overnight, and genomic DNA was collected by centrifugation.

Polymerase chain reaction amplification of the ACE polymorphism

The polymorphic region of the angiotensin-converting enzyme intron 16, consisting of the presence (I) or absence (D) of a 287 bp *alu* repeat sequence, was amplified from genomic DNA by polymerase chain reaction (PCR). The oligonucleotide primers used in the reaction were as previously published [4, 5]. Approximately 500 ng to 1 μg of genomic DNA was used per reaction, and the reaction conditions were as previously published [10]. The ACE genotype of each study participant was scored based on resolution of PCR products by gel electrophoresis. Amplification of the D allele resulted in a 190 bp DNA fragment and amplification of the I allele resulted in a 490 bp fragment. Homozygotes had a single 190 (DD) or 490 (II) bp band; heterozygotes had one 190 bp and one 490 bp band. Random replication of PCR amplification was used to verify all of the results.

Assessment of blood pressure

Blood pressure measurements were taken 8 to 11 weeks after the cessation of pre-existing antihypertensive medications during the pre-randomization placebo run-in period. Three blood pressure measurements were taken two minutes apart in the sitting position using a standard mercury sphygmomanometer at the randomization visit. The mean of these three measurements was used for analysis. Diastolic blood pressure was defined as the disappearance of the last Korotkoff sound and systolic blood pressure was defined as the first Korotkoff sound. All study nurses passed a standardized examination and were certified annually in the methods of obtaining proper blood pressure measurements by Shared Care, Inc. The recommendations for obtaining blood pressure measurements made by the American Heart Association (AHA) were used in the conduct of the ABCD Trial.

Assessment of metabolic parameters

Plasma glucose was measured in the fasting state and analyzed by a hexokinase enzyme reagent using an autoanalyzer (normal range 65 to 115 mg/dl; Olympus AU 5000; Olympus AU, Inc., Lake Success, NY, USA). Glycosylated hemoglobin was measured by affinity chromatography (normal range 5.5 to 8.2%; Helenda Labs, Beaumont, TX, USA) [15]. All lipid analyses were performed by a laboratory certified by the Cholesterol Reference Method Laboratory Network. Triglycerides were measured using the lipase glycerol phosphate oxidase method (Olympus AU 5000) [16]. Total cholesterol was measured using the cholesterol oxidase/hydrogen peroxide method [17]. HDL cholesterol was measured using a high performance enzymatic method (Sigma Diagnostics, St. Louis, MO, USA) [18].

Assessment of coronary artery disease

For purposes of statistical analyses, coronary artery disease (CAD) was considered to be present if a past history of myocardial infarction, positive cardiac catheterization results, percutaneous transluminal coronary angioplasty and/or coronary artery bypass surgery was noted. For participants who indicated that they had experienced one or more of these events, all efforts were made to obtain medical records.

Assessment of urinary albumin excretion/nephropathy

Urinary albumin excretion (UAE) was measured for individual patients on three separate occasions consisting of one 24-hour urine collection and two overnight collections. Patients were instructed to avoid strenuous exercise before providing urine samples. Urine albumin concentrations were measured within ten days of storage at standard refrigerator temperature. During the first eight months of the study UAE was measured by the nephelometric method [19], which has a sensitivity of 2 mg/dl, and thereafter UAE was measured using radioimmunoassay (Double Antibody Albumin #KHAD2; Diagnostic Products Corp., Los Angeles, CA, USA), which has a sensitivity of 0.3 mg/dl and an interassay coefficient of variation of 3%. The correlation coefficient for the two methods was $r^2 = 0.99$ ($N = 68$). For this study, the definition of diabetic nephropathy or overt albuminuria was an UAE measurement ≥200 μg/min.

Definitions

Hypertension was defined as a mean sitting diastolic blood pressure (DBP) ≥90 mm Hg and/or systolic blood pressure (SBP) ≥140 mm Hg. Duration of diabetes and hypertension were defined by personal history as the period between diagnosis of these disorders and the age at the time of the baseline examination. Minimal waist/maximal hip ratio was defined as the minimum circumference at or below the iliac crest. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

Statistics

The Statistical Analysis Software (SAS) system was used for all statistical analyses. When searching for differences in baseline characteristics across the three levels of genotype, two statistical procedures were used. When the baseline characteristic was continuous (age, duration of diabetes, SBP, DBP, duration of hypertension, glycosylated hemoglobin, glucose, serum creatinine, height, weight, BMI, cholesterol, HDL, uric acid and pack years

Table 1. Baseline characteristics of the ABCD Trial population according to ACE genotype

Variable	II (N = 162)	ID (N = 238)	DD (N = 109)
Gender *male/female*	103/59	157/81	65/44
Age *years*	57.8 ± 0.8	59.1 ± 0.5	58.9 ± 0.7
Duration of diabetes *years*	8.7 ± 0.7	8.5 ± 0.5	8.8 ± 0.6
Systolic blood pressure *mm Hg*	143.7 ± 1.8	145.9 ± 1.2	144.0 ± 1.4
Diastolic blood pressure *mm Hg*	89.6 ± 0.7	90.3 ± 0.6	90.2 ± 0.6
Duration of hypertension *years*	9.1 ± 1.0	10.1 ± 0.7	9.9 ± 0.8
Glycosolated hemoglobin %	11.3 ± 0.3	11.2 ± 0.2	11.5 ± 0.2
Fasting glucose *mg/dl*	187.4 ± 6.6	190.7 ± 4.3	194.3 ± 5.3
Serum creatinine *mg/dl*	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.02
BMI kg/m^2	31.8 ± 0.6	31.7 ± 0.4	31.7 ± 0.4
Cholesterol *mg/dl*	215.1 ± 3.6	212.1 ± 2.4	218.4 ± 3.7
HDL *mg/dl*	40.2 ± 1.2	39.5 ± 0.7	40.2 ± 0.9
Triglyceride *mg/dl*	253.3 ± 23.6	266.2 ± 16.0	282.9 ± 19.3
Uric acid *mg/dl*	5.5 ± 0.1	5.7 ± 0.1	5.6 ± 0.1
Pack years smoking	18.2 ± 2.6	21.6 ± 2.0	24.0 ± 2.6

All values are reported as means ± standard errors.

* Conversion factors for expressing conventional units in Système Internal (SI) units: fasting glucose, mg/dl × 0.05551 = mmol/liter; serum creatinine, mg/dl × 88.4 = μmol/liter; cholesterol/HDL, mg/dl × 0.02586 = mmol/liter; uric acid, mg/dl × 59.48 = μmol/liter.

smoking) an analysis of variance (ANOVA) was used. Chi-square analyses were used when the baseline characteristics were categorical (gender and race/ethnicity). A Chi-square analysis was also used for analyzing contingency tables exploring the univariate relationship between the genotype and each diabetic complication (diabetic nephropathy and coronary artery disease). Multiple logistic regression was employed when evaluating the effect(s) of a continuous and/or categorical covariate(s) on each diabetic complication. Odds ratios and their corresponding confidence intervals were then calculated from the logistic regression parameter estimates. When the outcome variable was clearly non-normally distributed, nonparametric analyses or parametric analyses on a suitably transformed variable were performed.

RESULTS

Baseline characteristics

The distribution of the I/D polymorphism is reported in Table 1. Baseline characteristics were compared across all three genotypes (Table 1). This table shows that these baseline characteristics were similar for the three different genotypes.

Coronary artery disease

Univariately, a Chi-square test revealed that there was no association between genotype and coronary artery disease (P = 0.23). Specifically, 54 (33%) of the patients with the DD genotype had coronary artery disease whereas 135 (39%) of the patients with the ID or II genotype had coronary artery disease.

Results of fitting the stepwise logistic regression model for coronary artery disease are presented in Table 2. Variables that are independently associated with coronary artery disease are age (OR = 1.2 per 5-year increase, 95% C.I. 1.1 to 1.4), duration of diabetes (OR = 1.5 per 10-year increase, 95% C.I. 1.1 to 2.0) and HDL [OR = 0.9 per 5 mg/dl (0.1293 mmol/liter) increase, 95% C.I. 0.8 to 1.0]. The presence of the DD genotype (OR = 0.8, 95% C.I. 0.5 to 0.2) was not statistically associated with coronary artery disease (P = 0.56).

Table 2. Logistic regression model for cardiovascular disease

Variable	Odds ratio	95% C.I.	P value	Change for continuous variables
Age	1.232	1.091–1.390	0.0008	5 years
Duration of diabetes	1.511	1.149–1.986	0.0031	10 years
HDL	0.912	0.832–0.999	0.0484	5 mg/dl (0.1293 mmol/liter)
Presence of DD genotype	0.782	0.518–1.181	0.2426	

Table 3. Relationship between genotype and diabetic nephropathy

	Genotype		
Diabetic nephropathy	DD	ID or II	Total
Present	23 (14.2%)	27 (7.8%)	50
Absent	139 (85.8%)	320 (92.2%)	459
Total	162	347	509

The Chi-square test was statistically significant (P = 0.023).

Table 4. Logistic regression model for diabetic nephropathy

Variable	Odds ratio	95% C.I.	P value	Change for continuous variables
Systolic blood pressure	1.476	1.225–1.778	0.0001	10 mm Hg
Uric acid	1.617	1.270–2.059	0.0001	1 mg/dl (59.48 μmol/liter)
Pack years smoking	1.127	1.030–1.232	0.0088	10 years
HDL	0.799	0.662–0.963	0.0187	
Duration of diabetes	2.216	1.446–3.394	0.0003	10 years
Presence of DD genotype	2.787	1.403–5.533	0.0034	

Diabetic nephropathy (overt albuminuria)

Initially, we performed the univariate analysis using DD, ID, and II genotypes as separate categories of the ACE polymorphism. The results revealed that 14.20% of the DD, 8.40% of the ID, and 6.42% of the II had overt albuminuria with a χ^2 of P = 0.065. We then used the ACE polymorphism as a dichotomous variable comparing the DD genotype versus the II/ID genotypes. Univariately, the Chi-square test revealed that there is an association between genotype and diabetic nephropathy (P = 0.023), which is demonstrated in Table 3.

Results of fitting the stepwise logistic regression model for diabetic nephropathy are presented in Table 4. The presence of the DD genotype (OR = 2.8, 95% C.I. 1.4 to 5.5) appeared to have the strongest association with diabetic nephropathy.

DISCUSSION

As the age of the U.S. population increases, so will the number of newly diagnosed NIDDM patients [20]. These patients and their complications will undoubtedly place a further burden on

the already stressed healthcare system in our country. In the present study, we evaluated the relationship of the ACE genotypes with diabetic nephropathy and coronary artery disease in 509 non-Hispanic white NIDDM patients. The results revealed that expression of the DD genotype is independently associated with an increased risk for diabetic nephropathy but was not associated with coronary artery disease.

In previous studies, there have been conflicting data regarding the association between the ACE gene polymorphism and cardiovascular disease in the diabetic and non-diabetic populations [9, 10, 21–25]. In a study with NIDDM subjects, Ruiz et al demonstrated an increasing relative risk in individuals heterozygous and homozygous for the D allele with regard to the presence of coronary artery disease [25]. Contrary to Ruiz's findings, the results of the present study revealed no association between the ACE genotype and coronary artery disease. Since the present study subjects were obtained from the ongoing ABCD Trial, the inclusion criteria of a diastolic blood pressure $\geq$ 80 mm Hg selected a population with a relatively high baseline blood pressure (145/91 mm Hg). Thus, the combination of hypertension and NIDDM in the present study population may overshadow the ACE genotype's effect on coronary artery disease for the present study.

We found an independent association between the DD genotype and the development of overt albuminuria. This association suggests that the angiotensin-converting enzyme polymorphism may be an important marker in the development of diabetic nephropathy in NIDDM. As such, patients with the DD genotype are more likely to develop diabetic nephropathy as compared to patients with the ID or II genotype independent of the effects of other known or suspected risk factors. Perhaps due to racial differences, the present finding is not consistent with the results of Fujisawa et al in Japanese diabetic patients [10], but is compatible with the results of another study suggesting that the presence of the D allele may increase the risk of diabetic nephropathy in IDDM [4]. In a study reported by Doria et al that evaluated the role of the ACE genotype in 151 IDDM subjects, the DD allele demonstrated a trend toward the presence of diabetic nephropathy but was not statistically significant (OR = 2.0, 95% C.I. 0.8 to 4.9) [26]. Schmidt et al evaluated 247 IDDM and 455 NIDDM patients with a diabetes duration of greater than 10 years [27] that revealed no association between the ACE genotype and diabetic nephropathy. However, the diagnosis of diabetic nephropathy was a UAE measurement >20 μg/min in this previous study; the definition of diabetic nephropathy in our study was overt albuminuria, that is, UAE >200 μg/min. When we performed the analysis using the same definition of diabetic nephropathy as the study by Schmidt et al, we obtained a similar non-significant association between ACE genotype and diabetic nephropathy. Thus, in the present NIDDM population the DD genotype is independently associated with overt albuminuria. The other risk factors found to be associated with diabetic nephropathy in the present study included an elevated SBP, elevated uric acid, and an increased duration of diabetes. These are similar risk factors found in earlier studies [28–30]. It should also be noted that this study population was generally in poor control with a mean glycosylated hemoglobin of 11.3%, and thus the effects of the DD genotype may be more pronounced in a NIDDM population with poorer glucose control.

Previous research has demonstrated that patients with the DD genotype have higher serum ACE activity than patients with the ID and II genotypes [4, 5]. Since ACE activity plays an important role in the renin-angiotensin and kallikrein-kinin systems in regulating systemic blood pressure, water balance and renal hemodynamics, increased ACE activity associated with the DD genotype in NIDDM patients may promote alterations in renal hemodynamics that can contribute to increased intraglomerular pressure and hyperfiltration [31, 32]. These alterations in turn may contribute to the development and progression of diabetic nephropathy.

Lastly, results of the present study may have an impact on the management of diabetic nephropathy, currently the most common cause of end-stage renal disease (ESRD) in this country. Moreover, nearly 50% of all new ESRD patients have diabetic nephropathy. The present results suggest that NIDDM patients with the DD genotype, especially in combination with the other known risk factors, are at higher risk for diabetic nephropathy. These NIDDM patients therefore warrant close follow-up, and perhaps when microalbuminuria is present to initiate ACE inhibitors. On the background of this information, interventional studies such as the ABCD Trial are needed to determine efficacious interventions for these NIDDM patients.

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