Angiotensin-Converting Enzyme Gene Polymorphism Is Associated With Coronary Heart Disease in Non–Insulin-Dependent Diabetic Patients Evaluated for 9 Years

Xiao-Hong Huang, Vappu Rantalaiho, Ole Wirta, Amos Pasternack, Timo Koivula, Timo P. Hiltunen, Tapio Nikkari, and Terho Lehtimäki

The insertion/deletion (I/D) polymorphism of the human angiotensin-converting enzyme (ACE) gene is a major determinant of circulating ACE levels. Recent studies have found the ACE D allele to be associated with an increased risk for coronary heart disease (CHD) in diabetic and nondiabetic subjects. This association has not been evaluated in prospective studies. We therefore studied the relationship between ACE gene I/D polymorphism and CHD in patients with non–insulin-dependent diabetes mellitus (NIDDM) evaluated for 9 years. The I/D polymorphism was determined by polymerase chain reaction (PCR). Overestimation of the frequency of the DD genotype was eliminated by insertion-specific primers and inclusion of 5% dimethylsulfoxide (DMSO). Eighty-three patients were evaluated for a mean period of 9.1 years (range, 7.4 to 10.5). Among them, 64 patients showed no CHD at entry. During the follow-up period, 21 patients (37.5%) developed CHD. The systolic blood pressure ($P = .046$), fasting blood glucose ($P < .01$), and prevalence of hypertension ($P < .001$) increased, while high-density lipoprotein (HDL) cholesterol ($P < .001$) decreased. Patients who developed CHD were older than those who did not; the mean age was 59.3 and 53.2 years, respectively ($P = .003$). The prevalence of albuminuria at follow-up examination was higher in CHD subjects versus non-CHD subjects (61.9% v 20.9%, $P = .012$). The D allele of the ACE gene was significantly more frequent in subjects with CHD versus those without CHD in both follow-up ($P = .028$, χ^2 test) and cross-sectional ($P = .033$, χ^2 test) settings. No difference could be detected between the three genotypes in age, body mass index (BMI), blood pressure, or plasma lipid levels. In our logistic regression analysis, the best model selected the DD genotype ($P = .0105$) and age ($P = .0407$) as significant risk factors for CHD. This model classified 89% of the subjects correctly. In conclusion, this 9-year prospective study supports the hypothesis that the ACE I/D polymorphism is an important and independent risk factor for CHD in patients with NIDDM.

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IT IS WELL KNOWN that non–insulin-dependent diabetes mellitus (NIDDM) is associated with increased risk for the development of coronary heart disease (CHD).[1,2] However, the mechanism underlying the high incidence of vascular disease in diabetic patients is still not fully understood. Besides metabolic factors such as hyperglycemia and dyslipidemia, genetic factors might also contribute to its development.[3]

The nature of the genetic factors is unknown, but the genes encoding components of the renin-angiotensin system (RAS) present potential candidates to examine an association with CHD and myocardial infarction (MI). Angiotensin-converting enzyme (ACE) is a key component within the RAS system by converting angiotensin I to the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin.[4] The two peptide hormones have opposite effects on vascular tone and smooth muscle proliferation,[5,6] factors believed to be important in the pathogenesis of atherosclerosis. Pharmacological ACE inhibitors have been shown to reduce atherosclerosis in cholesterol-fed and Watanabe heritable hyperlipidemic rabbits,[7-9] supporting the potential pathogenic role of ACE and its substrates.

Plasma and tissue ACE levels are strongly genetically determined. The insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene accounts for as much as 50% of the variance in the enzyme level.[10] Subjects homozygous for the deletion (DD) show the highest values and those homozygous for the insertion (II) show the lowest, with heterozygotes (ID) showing intermediate values.[11] Cambien et al[12] first reported that the DD genotype was a risk factor for MI, especially in subjects thought to be at low risk according to plasma apolipoprotein B levels and the body mass index (BMI). Some subsequent case-control studies suggest that the D allele is also associated with atherosclerosis and nephropathy in diabetic patients,[13-15] whereas other studies do not,[16-17] and these data need to be confirmed by prospective studies. We therefore investigated the contribution of the ACE gene I/D polymorphism to the development of CHD in well-documented patients with NIDDM evaluated for 9 years.

SUBJECTS AND METHODS

Subjects

The clinical details of the patients have been described elsewhere.[18] Briefly, 150 consecutive patients with NIDDM, aged 40 to 65 years were recruited from the Primary Health Care Center of the city of Tampere, Finland, between 1985 and 1988. The patients fulfilled World Health Organization diagnostic criteria for NIDDM[19] and had a known disease duration of no longer than 1 year. Subjects with secondary diabetes or serious diseases with short life expectancy, eg, cancer, were excluded. Eighty-three patients were eligible at a mean follow-up duration of 9.1 years (range, 7.4 to 10.5), at which time clinical data were again recorded using the same methods.

The study was approved by the ethics committees of the University of

From the Departments of Clinical Chemistry and Internal Medicine, Tampere University Hospital, and Department of Medical Biochemistry, Medical School of Tampere University, Tampere, Finland.

Submitted December 20, 1997; accepted March 10, 1998.

Supported by grants from the Emil Aaltonen Foundation, the Finnish Foundation of Cardiovascular Research, the Elli and Elvi Oksanen Fund of the Pirkanmaa Regional Fund under the auspices of the Finnish Cultural Foundation, the Paulo Foundation, and the Medical Research Fund of the Tampere University Hospital.

Address reprint requests to Terho Lehtimäki, MD, Tampere University Hospital, Department of Clinical Chemistry, PO Box 2000, FIN-33521, Tampere, Finland.

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0026-0495/98/4710-0016$03.00/0

Tampere and the Health Care Center of Tampere. All subjects provided written informed consent.

Protocol

A detailed medical history was obtained. The use of antihypertensive and antiglycemic medication was reported and analyzed a categorical variable. At the time of entry, none of the patients received insulin treatment or ACE inhibitors. All blood samples were taken after an overnight fast. Height and weight were recorded, and the BMI was calculated (kilograms per meter squared). Blood pressure was measured to the nearest 2 mm Hg after 10 minutes' rest in the supine position. A subject was considered to have hypertension if he or she was taking antihypertensive medication or if the systolic blood pressure was 160 mm Hg or greater or diastolic pressure was 95 mm Hg or greater. A resting 12-lead electrocardiogram (ECG) was recorded and coded according to Minnesota criteria.[20] CHD was diagnosed if (1) the patient had a history of MI verified by hospital records or (2) the ECG showed ischemic abnormalities (Minnesota codes 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, and 7.1).

Hemoglobin A_{1c} was determined by liquid chromatography. Blood glucose levels were measured enzymatically. Serum cholesterol and triglycerides were determined by the dry-slide technique (Ektachenn 700 analyzer; Johnson & Johnson Clinical Diagnostics, Rochester, NY), and high-density lipoprotein (HDL) cholesterol was determined after precipitation[21] with the same technique. The urinary albumin excretion rate (UAER) was measured by immunonephelometry from 24-hour urine collections. Normoalbuminuria was defined as a UAER not greater than 30 mg/24 h, microalbuminuria as a UAER between 30 and 300 mg/24 h, and overt proteinuria as a UAER of at least 300 mg/24 h. For the present study, microalbuminuria and overt proteinuria were all combined into the group of increased UAER.

Determination of ACE Genotype

Genomic DNA was extracted from 200 μL whole blood with the QIAamp Blood Kit (Qiagen, Valencia, CA). The polymerase chain reaction (PCR) was used to detect ACE I/D polymorphism. The primers flanking the 287-base pair sequence were from the protocol of Rigat et al.[22] The PCR mixture contained 10 mmol/L Tris hydrochloride (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 200 μmol/L of each of the four deoxynucleotides, 1 μmol/L of each of the primers, 3 U Dynazyme (Finnzymes, Espoo, Finland), and 5% dimethylsulfoxide (DMSO) in a final volume of 50 μL. After an initial denaturation at 96°C for 3 minutes, the DNA was amplified by 30 PCR cycles: denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 5 minutes. Because the D allele in heterozygous samples is preferentially amplified, each sample found to be the DD genotype was confirmed by a second, independent PCR amplification with an insertion-specific primer and with inclusion of 5% DMSO as recently described.[23] PCR products were separated by electrophoresis on 2% agarose and visualized with UV light after ethidium bromide staining.

Statistical Analysis

Data are expressed as the mean ± SD unless otherwise specified. Non–normally distributed data were logarithmically transformed or compared by nonparametric tests (Mann-Whitney *U* test or Kruskal-Wallis test). Time-related changes within the group were analyzed by dependent *t* test. Genotype distribution and allele frequency were compared between groups using the χ^2 test. Variables between genotype groups were compared by ANOVA. When we searched for the set of variables that would classify the patients into subjects with or without CHD, we used logistic regression analysis. To find possible predictors, we used the following explanatory variables: age, ACE genotype, BMI, smoking status, systolic blood pressure, total cholesterol, HDL cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, and fasting blood glucose. A *P* value less than .05 was considered significant.

RESULTS

Eighty-three of the original 150 patients were evaluated for a mean period of 9.1 years (range, 7.4 to 10.5). Among them, 64 patients satisfied the criteria for having no CHD at entry. Table 1 lists the characteristics of the remaining 64 patients at entry and 9 years later. During the follow-up period, 21 patients (37.5%) developed CHD and/or MI. There were increases in blood pressure, fasting blood glucose, and the prevalence of hypertension, and a decrease in HDL cholesterol. The prevalence of albuminuria did not change significantly.

Table 2 shows the comparison of variables measured at baseline examination and 9 years later in subjects who developed CHD and those who did not. Age was associated with the development of CHD. There were no differences in the BMI, blood pressure, lipid levels, glycemic control, and prevalence of hypertension between these two groups either at baseline examination or 9 years later. The prevalence of albuminuria did not differ significantly at entry, but was higher in patients with CHD 9 years later. During the follow-up period, four patients who showed microalbuminuria at baseline examination reverted to normoalbuminuria.

The ACE genotype distribution and allele frequency in 64 patients who initially showed no evidence of CHD are shown in Table 3. The D allele of the ACE gene was significantly more frequent in patients who developed CHD versus those who did not ($P = .028$, χ^2 test). To investigate this further, we extended our analysis to include all 83 patients who were successfully evaluated for 9 years. Again, the D allele was more frequent in subjects with CHD versus those without ($P = .033$, χ^2 test; Table 4). The genotype distribution was in agreement with Hardy-Weinberg equilibrium, and the allele frequencies in the

Table 1. Characteristics of 64 NIDDM Patients at Baseline and After 9 Years

Characteristic	Baseline	9 Years	*P*
	Mean ± SD		
Age (yr)	55.2 ± 7.9	—	—
BMI (kg/m²)	30.6 ± 5.4	30.4 ± 5.2	
SBP (mm Hg)	154.2 ± 21.8	159.1 ± 19.5	.046*
DBP (mm Hg)	88.6 ± 10.1	89.1 ± 9.1	
Total cholesterol (mmol/L)	5.49 ± 1.13	5.33 ± 0.97	
HDL cholesterol (mmol/L)	1.23 ± 0.33	1.07 ± 0.33	<.001*
Triglycerides (mmol/L)	1.75 ± 1.01	1.94 ± 1.06	
LDL cholesterol (mmol/L)	3.46 ± 1.03	3.38 ± 0.86	
Fasting blood glucose (mmol/L)	7.8 ± 2.4	9.0 ± 2.6	<.01*
Hemoglobin A_{1c} (%)	7.5 ± 1.7	8.2 ± 1.6	
	No. %	No. %	
Hypertension	33 51.6	52 81.3	<.001†
Increased UAER	19 29.7	22 34.4	
Smoking	12 18.8	6 9.4	

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

*Dependent *t* test.

†χ^2 test.

Table 2. Clinical Characteristics at Baseline and After 9 Years in 64 NIDDM Patients According to Status at Follow-up Examination

Characteristic	CHD^-/CHD^- (n = 43)	CHD^-/CHD^+ (n = 21)
Baseline	Mean ± SD	
Age (yr)	53.2 ± 8.1	59.3 ± 5.8*
BMI (kg/m²)	31.1 ± 5.7	29.4 ± 4.3
SBP (mm Hg)	152.8 ± 24.1	157.0 ± 16.2
DBP (mm Hg)	90.1 ± 11.2	85.7 ± 6.5
Total cholesterol (mmol/L)	5.46 ± 1.18	5.55 ± 1.03
HDL cholesterol (mmol/L)	1.23 ± 0.34	1.23 ± 0.32
Triglycerides (mmol/L)	1.78 ± 1.05	1.69 ± 0.93
LDL cholesterol (mmol/L)	3.42 ± 1.09	3.55 ± 0.91
Fasting blood glucose (mmol/L)	7.9 ± 2.3	7.4 ± 2.6
Hemoglobin A_{1c} (%)	7.5 ± 1.8	7.5 ± 1.4
	No. %	No. %
Hypertension	19 44.2	14 66.7
Increased UAER	13 30.2	6 28.6
Smoking	5 11.6	7 33.3
9 Years	Mean ± SD	
BMI (kg/m²)	30.8 ± 5.8	29.6 ± 3.7
SBP (mm Hg)	156.7 ± 19.1	164.1 ± 19.9
DBP (mm Hg)	88.8 ± 9.1	89.7 ± 9.4
Total cholesterol (mmol/L)	5.28 ± 0.96	5.45 ± 1.02
HDL cholesterol (mmol/L)	1.08 ± 0.35	1.05 ± 0.28
Triglycerides (mmol/L)	1.82 ± 0.92	2.19 ± 1.29
LDL cholesterol (mmol/L)	3.37 ± 0.91	3.40 ± 0.77
Fasting blood glucose (mmol/L)	9.1 ± 2.5	9.4 ± 2.9
Hemoglobin A_{1c} (%)	8.1 ± 1.5	8.5 ± 1.8
	No. %	No. %
Hypertension	33 76.7	19 90.5
Increased UAER	9 20.9	13 61.9†
Smoking	5 11.6	1 4.8

Abbreviations: CHD^-/CHD^-, without CHD at baseline and follow-up examination; CHD^-/CHD^+, without CHD at baseline but with CHD at follow-up examination. Other abbreviations as in Table 1.

*P = .0031.

†P = .012.

subgroup of controls without CHD were similar to those reported in other European populations.[13,24] In addition, no significant differences were found between the genotypes for the blood pressure, prevalence of hypertension, or plasma lipid levels (data not shown).

The best model in the logistic regression analysis showed ACE genotype (P = .0105) and age (P = .0407) to be the

Table 3. Association of ACE Genotype and Allele Frequency With the Development of CHD in 64 NIDDM Patients Evaluated for 9 Years

	Genotype							
	II		ID		DD		Allele Frequency	
Condition	No.	%	No.	%	No.	%	I	D
CHD^-/CHD^-	8	18.6	24	55.8	11	25.6	0.47	0.53
CHD^-/CHD^+	2	9.5	7	33.3	12	57.2	0.26	0.74*

Abbreviations: CHD^-/CHD^-, without CHD at baseline and follow-up examination; CHD^-/CHD^+, without CHD at baseline but with CHD at follow-up examination.

*P = .028 by χ^2 test.

Table 4. ACE Genotype and Allele Frequency in 83 NIDDM Patients According to CHD Status at Follow-up Examination

	Genotype							
	II		ID		DD		Allele Frequency	
Condition	No.	%	No.	%	No.	%	I	D
CHD^-	8	18.6	24	55.8	11	25.6	0.47	0.53
CHD^+	3	7.5	17	42.5	20	50	0.29	0.71*

Abbreviations: CHD^-, without CHD; CHD^+, with CHD.

*P = .033 by χ^2 test.

significant risk factors for CHD (Table 5). In this model, the efficiency, ie, the percentage of subjects correctly classified as being with or without CHD, was 89%.

DISCUSSION

We have determined the distribution of the ACE genotype and allele frequency in Finnish patients with NIDDM evaluated for 9 years. Our data show that the DD genotype was the most common genotype in patients with CHD and the frequency of the D allele was significantly higher than that in subjects without CHD in both follow-up and cross-sectional settings. The best model in our logistic regression analysis confirmed that the D allele was a genetic risk factor for development of CHD in patients with NIDDM. In addition, no association between the ACE genotype and the number of known risk factors for CHD, such as hypertension and blood lipid levels, was identified. To our knowledge, this is the first prospective study investigating the effect of the ACE gene on CHD in diabetic patients.

Previous studies have shown that the DD genotype of the ACE gene was associated with an increased risk of MI[12] and CHD,[24,25] sudden death in hypertrophic cardiomyopathy,[26] left ventricular hypertrophy,[27] and restenosis after percutaneous transluminal coronary angioplasty.[28] Our present study strongly supports the observation by Ruiz et al,[13] who evaluated the effect of the ACE genotype in 316 unrelated white NIDDM patients and found that the D allele was a strong risk factor for CHD and was associated with early onset CHD (<65 years old) independently of hypertension and lipid values. Our findings are also in agreement with those reported by Hosoi et al[29] and Rasmussen and Ledet,[30] who showed an increased risk of carotid arterial wall thickness and aortic atherosclerosis, respectively, in diabetic patients with the DD genotype. However, our

Table 5. Risk Factors Predicting CHD in NIDDM Patients Based on Logistic Regression Analysis

Variable	P
ACE genotype	.0105
Age	.0407
BMI	.0854
SBP	.5516
HDL	.8447
LDL cholesterol	.4414
Triglycerides	.2075
Fasting blood glucose	.7158
Total mode: R^2 = 18.496, P = .02987, efficiency = 89%	

NOTE. Efficiency is the percentage of subjects correctly classified by the model as cases or controls.

results are in contrast to the report by Ringel et al,[17] who did not find an association between the ACE genotype and diabetic vascular complications. In their study, the definition of CHD was based on clinical findings such as a history of MI or angina pectoris. It is well recognized that angina, particularly in diabetes, is not reliable as an indicator of CHD.[31] The association of the DD genotype with CHD is concordant with the results of recent clinical trials that ACE inhibitors reduce the incidence of unstable angina and MI.[32]

In the present study, CHD was diagnosed according to a clinical history of MI verified by hospital records and/or ECG abnormalities (Minnesota codes 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, and 7.1). We cannot exclude the possibility that some diabetic patients were misclassified as non-CHD, especially at baseline examination, because a single ECG showed no evidence of ischemic changes. In view of this possibility, we extended our analysis to examine cross-sectionally all 83 patients who were evaluated for 9 years using ECG results both at baseline and at follow-up study, and again the result was positive (Table 4). However, we fully realize that ECG findings may not adequately separate case-patients into controls. Whether the ACE D allele is a risk factor for CHD should be addressed by future prospective studies with the diagnosis based on coronary angiography.

The mechanism(s) by which the ACE gene I/D polymorphism affects atherosclerosis and other cardiovascular diseases remains speculative. Given observations that the DD genotype is associated not only with elevated plasma[10,11] but also cardiac tissue[33] enzyme levels, it is tempting to speculate that it may mediate these cardiovascular effects through ACE and/or its substrates. This hypothesis was supported by a recent report[34] that in human coronary arteries, increased expression of ACE and colocalization with immunoreactive angiotensin II was found within atherosclerotic plaques. Increased cardiovascular ACE leads to a parallel increase in angiotensin II formation from angiotensin I in situ.[35] Experimental studies in several species suggest a role for angiotensin II in vascular remodeling and atherosclerosis.[5,6,36,37] Angiotensin II is shown to induce adhesion molecule expression in human endothelial cells[38] and to activate human monocytes,[39] resulting in increased adhesion to human endothelial cells. Angiotensin II also promotes growth, migration, and matrix production in smooth muscle cells[5,6,40] and may thus contribute to plaque formation. In addition, angiotensin II may play a role in the regulation of fibrinolysis by increasing the release of plasminogen activator inhibitor.[41,42] Finally, ACE inhibition has been shown to reduce atherosclerosis in hypercholesterolemic animals[7-9] and to prevent neointimal proliferation after vascular injury in rat models.[43,44]

In conclusion, this 9-year prospective study supports the hypothesis that the D allele of the ACE I/D polymorphism is a genetic marker for development of CHD in patients with NIDDM. The mechanism(s) of the ACE gene in the pathogenesis of CHD needs to be further clarified.

ACKNOWLEDGMENT

We thank Marita Koli for excellent technical help.

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