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No Association Between the Angiotensin-Converting-Enzyme Gene Insertion/Deletion Polymorphism and the Occurrence of Macroangiopathy in Patients with Diabetes Mellitus Type 2

Abstract

Previous studies have reported an association between the ACE-I/D-polymorphism and coronary heart disease (CHD) in patients with diabetes mellitus. However, ACE inhibitor treatment, which could have compensated for negative effects of the D/D form of the ACE gene polymorphism, was not considered in the studies. We investigated the influence of the ACE-I/D polymorphism and the ACE inhibitor treatment in patients with diabetes mellitus on the occurrence of CHD by multiple-regression analysis. Distribution of the ACE gene I/D-polymorphism was investigated in 691 patients with diabetes mellitus prospectively characterised for the presence/absence of coronary heart disease. The distribution of DD; ID; II genotypes was 105 vs. 202 vs. 102 (25.7% vs. 49.4% vs. 24.9) in the CHD^+ group and 55 vs. 160 vs. 67 (19.5% vs. 56.7% vs. 23.8%) in the CHD^- group, respectively ($p = 0.1$). A multiple logistic regression analysis introducing the typical risk factors for CHD (age, gender, smoking, BMI > 26 kg/m^2, LDL elevation, HbA1c > 7%) could not identify the ACE gene I/D-polymorphism as an independent risk factor for CHD ($p = 0.87$). Our data therefore suggest that the ACE gene I/D polymorphism is not associated with the occurrence of diabetic macroangiopathy in patients with or without treatment of ACE inhibitors.

Key words
Diabetes · Genetics · Complications of Diabetes

Introduction

Hyperglycaemia occurs in every patient with diabetes mellitus. It is one of the leading factors in the development of diabetic complications. However, even in patients with similar blood glucose control, onset, severity and the progression of macrovascular complications show large interindividual variations. The familial occurrence of diabetic nephropathy in type 1 [1] and type 2 [2] diabetes and the finding that only 25% – 40% of patients with diabetes mellitus develop diabetic nephropathy [3 – 4] both suggest a genetic predisposition towards diabetic nephropathy. Since cardiovascular complications of diabetes mellitus are the main reasons for diabetes associated deaths especially due to silent ischemia or sudden cardiac death [5 – 7], the identification of coronary heart disease risk stratification parameters in these patients is of great interest.

The introduction of angiotensin-converting-enzyme inhibitors into the therapy of hypertension and heart failure resulted in a 22% reduction in myocardial infarction, stroke or death from cardiovascular causes, especially in patients with diabetes mellitus [8 – 9]. ACE inhibitors also seem to prevent cardiovascular events in patients with diabetes mellitus [10]. However, 27% to 50% non-responders to the ACE-Inhibitor therapy, as determined by lack of blood pressure-lowering effects, have been reported [11 – 13]. A polymorphism in the angiotensin-converting-enzyme gene (ACE gene) results in either the presence (Insertion, I) or the absence (Deletion, D) of a 287 base-pair fragment in in-

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tron 16 of the ACE gene, thus resulting in three genotypes, DD or II homozygotes and ID heterozygotes. It is therefore of interest that the plasma ACE activity in subjects with the DD form is increased by 100% compared to those with the II form [14–15].

Results from studies on this ACE gene polymorphism vary. A strong association of the DD form with the development of ischemic coronary heart disease has been reported for patients both with type 2 diabetes [16] and without diabetes mellitus [17]. An association between acute myocardial infarction occurrence in patients with and without diabetes mellitus and the DD form of I/D polymorphism has been reported [17–18]. However, a large study (Physicians' Health Study) with a small subgroup of diabetic patients (n = 21 with and n = 56 without myocardial infarction) did not show any association between increased risk for ischemic heart disease or myocardial infarction and the ACE gene polymorphism [19]. With the exception of the Ruiz study, the small subgroups of patients with diabetes mellitus included in these studies were not separately investigated for their genetic risks. However, especially patients with diabetes mellitus have a markedly increased mortality due to cardiovascular events [6–7].

Also, patients with diabetes mellitus often suffer from hypertension. However, none of the previous studies considered the antihypertensive drug treatment. Therefore, we prospectively studied well-characterised diabetic patients with and without coronary artery disease for their ACE gene I/D genotypes. In a second step, the influence of the ACE inhibitor treatment on the genetic risk of the DD form was investigated.

Material and Methods

Patients

691 consecutive patients with diabetes mellitus type 2 of the III. Medical Department of the Leipzig University and the Leipzig Heart Centre respectively were recruited for the study. All patients gave their informed consent for participation in the study. The study was approved by the local ethics committee. The patients were divided into two groups, group 1 with coronary heart disease (CHD^+, n = 409) and group 2 (control group) without coronary heart disease (CHD^-, n = 282). The diagnosis of CHD was established by a history for myocardial infarction, coronary bypass operation or a significant stenosis (> 50%) of at least one coronary artery as defined by angiography. Patients without CHD had no history of acute myocardial infarction, ECG abnormalities, bypass surgery/coronary angioplasty, or typical angina or dyspnoea. Smoking was defined as at least 1 cigarette, cigar, or pipe per day. One package year is defined as 20 cigarettes per day for one year. Hypertension was established where blood pressure (in sitting position after 5 minutes of rest) was > 140/90 mmHg or where patients were treated with antihypertensive drugs. Also, a comprehensive history of the patient's present medical treatment including ACE-inhibitors, beta-blockers, nitrates, or lipid lowering drugs was taken. Further details for patients' data are given in Table **1**.

Genotyping of the ACE gene I/D polymorphism

Genomic DNA was extracted from 200 µl of whole peripheral blood with a QUIAmp Blood Kit (QUIAGEN). The I/D polymorphism of the ACE gene was determined according to the method described by Ribichini et al. [20]. The sense and antisense primers were 5′-CTG′GAG ACC ACT CCC ATC CTT TCT-3′ and 5′GAT GTG GCC ATC ACA TTC GTC AGA T-3′ respectively. The PCR was performed in a final volume of 50 µl containing 200 ng genomic DNA, 1 µl (10 mmolar) sense and 1 µl (10 mmolar) antisense primers, 1 µl (10 mmolar) dNTP, 1 U Taq DNA polymerase, and a variable amount of distilled water to reach 50 µl of total volume. Amplification was performed with an UNO cycler (Whatman, Biometra biomedizinische Ananlytik GmbH, Göttingen, Germany). Samples were denatured for 3 min at 94 °C, cycled 35 times for 45 s at 94 °C, 1 min at 62 °C and 1 min at 72 °C, followed by a final extension step of ten minutes. PCR products were electrophoresed in 1.5% agarose gel and visualised by ethidium bromide staining. The insertion allele (I) was detected as a 590-bp band, and the deletion allele was detected as a 330-bp band. In order to avoid overestimation of the D/D genotype, each D/D type was subjected to a second, independent PCR amplification with an insertion specific primer pair: 5′-TGG GAC CAC AGC GCC CGC CAC

Table **1** Clinical data for patients with and without coronary heart disease. A p-value < 0.05 is defined as significant difference between both groups (Mann-Whitney-U-test)

	Group 1: Coronary Heart Disease (CHD^+)	*Group 2: no Coronary Heart Disease (CHD^-)*	*p*
Patients (n)	409	282	
Age (mean [range])	67.6 (43–91)	64.8 (33–91)	0.003
Female/male (n)	148/261	145/137	
Duration of diabetes (mean years [range])	10.1 (0–50)	9.9 (0–46)	0.904
Mean HbA1c % (range)	7.31 (3.54–14.2)	7.61 (4.4–17.0)	0.49
Mean Body Mass Index (kg/m^2, [range])	28.46 (17.9–42.2)	28.96 (14.36–67.3)	0.68
Mean duration of CHD (years [range])	3.90 (0–59)	–	
Mean age of CHD manifestation (years [range])	63.7 (17–86)	–	
Hypertension (n)	178	102	0.02
ACE-Inhibitors treatment (n)	243 of 409	145 of 282	0.045
Smokers (average; range of pack years); n (%) smokers	5.4 (1–75) 139 (34%)	4.2 (0.5–80) 80 (28.5%)	0.2
Mean LDL (mmol/l [range])	3.2 (0.98–10.8)	3.46 (0.37–7.01)	0.53

TAC-3′; 5′-TCG CCA GCC CTC CA TGC CCA TAA-3′ with identical PCR conditions except for an annealing temperature of 67 °C.

Statistical analysis

The statistical analysis was performed with SPSS release 10.0.0 for Windows (SPSS Inc. 1989 – 1997). The χ^2 statistic test was used to test allelic frequencies of the ACE I/D alleles in groups 1 and 2. The Mann-Whitney U-test and one way ANOVA were used to test differences between two or three different variables where parametric distribution could not be definitely assumed, respectively. To obtain the Odds ratios for specific risk factors for CHD, multivariate-regression analysis was performed.

Results

Allelic frequencies

The I and D allele frequencies in both group 1 and group 2 were 50.3/49.7% for patients with CHD and 52.1/47.9% for patients without CHD. The total allelic frequency of I and D alleles in both groups was 50.7 and 49.3%, respectively. The distribution of DD; ID; II genotypes was 105 vs. 202 vs. 102 (25.7% vs. 49.4% vs. 24.9) in the CHD^+ group and 55 vs. 160 vs. 67 (19.5% vs. 56.7% vs. 23.8%) in the CHD^- group. The distribution of these three possible genotypes was not statistically significant between the groups with or without CHD (χ^2-test, $p = 0.1$). Subgroup analyses for additional characteristics like presence or absence of hypertension ($p = 0.1$ for absence of hypertension in group 1 vs. group 2; $p = 0.02$ for presence of hypertension in group 1 vs. group 2) show statistical significance for the DD; ID or II genotype of the ACE gene when compared patients with and without CHD.

Multiple logistic regression

Neither group showed statistically significant differences for HbA1c, LDL-cholesterol plasma concentrations, body mass index, percentage of smokers, or duration of diabetes (Table **1**). More patients in group 1 were treated with ACE inhibitors compared to group 2 (243 of 409 vs. 143 of 282; $p = 0.04$) (Table **1**). There was no statistically significant difference between the number of patients treated with ACE inhibitors with or without a history of myocardial infarction. The ACE gene polymorphism was not affected by the patients gender ($p = 0.46$; data not shown). Furthermore, it was also not affected by patients sex when female and male patients were analysed in two subgroups (presence or absence of CHD) $p = 0.66$ for females and 0.13 for males when tested by Pearson chi-square test.

One-way ANOVA showed a lack of statistically significant difference for the ACE gene variants between groups 1 and 2. Therefore, a multiple regression analysis was performed, introducing the typical cardiovascular risk factors like age, sex, LDL cholesterol plasma concentrations, smoking, HbA1c as a marker for diabetes control, and also the ACE-I/D polymorphism. This analysis showed that age > 60 and male gender are significant risk factors for CHD. The high body-mass index showed only a trend to be a risk factor for CHD ($p = 0.06$). Surprisingly, the ACE-I/D polymorphism was no significant risk factor (Table **2**). Moreover, when ACE inhibitor treatment was added to the multiple regression analysis, patients' age and sex remained statistically significant risk factors for CHD, the statistical relevance of the ACE-I/D polymorphism as a possible risk factor for CHD did not change (Table **3**).

Table **2** Logistic regression analysis for cardiovascular risk factors associated with CHD in univariate analysis (level of significance: $p < 0.05$)

Cardiovascular risk factors	*Total number of patients (n = 415)*		
	Odds ratio	*p-value*	*95% Confidence interval*
LDL (> 4.1 mmol/l)	0.96	0.89	0.52 – 1.77
Age (> 60 years)	3.30	< 0.001	1.99 – 5.47
Gender (male)	2.49	< 0.001	1.62 – 3.83
HbA1c (> 7.0%)	0.79	0.28	0.53 – 1.20
Smoking	0.89	0.69	0.50 – 1.58
ACE-I/D	1.04	0.87	0.63 – 1.70
BMI (> 26 kg/m^2)	1.56	0.06	0.98 – 2.48

Table **3** Logistic regression analysis for cardiovascular risk factors associated with CHD in univariate analysis adjusted for the treatment with ACE inhibitors (level of significance: $p < 0.05$)

Cardiovascular risk factors	*Total number of patients (n = 248)*		
	Odds ratio	*p-value*	*95% Confidence interval*
Age (> 60 years)	2.18	0.03	1.10–4.34
Gender (male)	2.17	0.007	1.23–3.82
HbA1c (7.0%)	0.69	0.18	0.40–1.19
Smoking	0.47	0.46	0.22–0.99
ACE-I/D	1.09	0.79	0.58–2.02
BMI (> 26 kg/m^2)	1.20	0.55	0.65–2.22

Discussion

In order to detect further determinants for the risk of patients with diabetes mellitus to develop macrovascular complications apart from the quality of blood sugar control genetic markers such as the ACE I/D polymorphism, polymorphisms in the Paraoxonase gene, and in the von Willebrand factor gene [21 – 26] have been investigated. As diabetic patients are at four times the risk of dying from silent myocardial ischemia/sudden heart death [6], a genetic marker for the prediction of the risk for cardiovascular complications would allow early selection of patients at risk for more aggressive prevention strategies. In this context, the ACE gene and its I/D polymorphism in intron 16 is of special interest.

The only studies performed exclusively with diabetic patients described the D/D form of the ACE gene polymorphism as an independent risk factor for CHD [16, 27]. In contrast to those findings, our logistic multiple-regression analysis only identified the known risk factors, such as age and male sex, as significant risks for CHD. The ACE gene polymorphism was no risk factor for diabetic macroangiopathy (Table **2**).

ACE inhibitor therapy was introduced in 1982, and its efficacy has been documented by a number of studies (AIRE 1993; CONSENSUS 1987; SOLVD 1991) [28 – 30]. The number of patients treated with ACE inhibitors was neither specified in the Ruiz study published in 1994 [16] nor in the study of Keavney published in 1995 [27]. Keavney only matched patients with and without antihypertensive drug treatment. Adjustment of our analysis for the influence of ACE inhibitory drug treatment did not change the influence of the I/D polymorphism on CHD (Table **3**). One reason for the missing effect of the ACE inhibitor treatment on diabetic macroangiopathy could be lack of response to this drug treatment described in up to 50% of treated patients as determined by improvement of blood pressure [11 – 13].

The ACE plasma levels of subjects with the D/D form are increased by 100% compared to the values of those with the I/I form. Patients with the I/I variant of the ACE gene polymorphism have a better efficiency and response of muscular contraction to physical training [31 – 32]. One possible reason for this finding is the lower plasma angiotensin level associated with the I/I form of the polymorphism, even in patients with normal body mass index [31]. This could be a possible reason for the beneficial effects of ACE inhibitor treatment on cardiovascular outcome. However, in our study, this does not influence the lack of the I/D polymorphism as a risk factor for CHD as determined by multiple logistic regression analysis. This result suggests that there are most likely other mechanisms by which the DD form is associated with diabetic macroangiopathy. Moreover, other studies performed with very large study groups of CHD patients (Lindpaintner et al., n = 3590; Keavney et al., n = 11000; Pfohl et al., n = 1131; Friedl et al., n = 464; Arca et al., n = 630; Agerholm-Larsen et al., n = 10150) [19, 32 – 36] also did not find an association of the D/D variant with CHD in patients mainly without diabetes.

Our patients with coronary heart disease were three years older than the patients without CHD ($p = 0.003$). Therefore, they had additional three years to develop their CHD. The age of the patients is therefore the main risk factor for developing CHD, besides gender. However, even when comparing age matched patient groups (data not shown), this result did not change significantly.

In conclusion, our data suggest that the ACE gene I/D polymorphism is not associated with the occurrence of diabetic macroangiopathy. Only age (CHD patients in our study were three years older) and gender were stable cardiovascular risk factors in our investigation.

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