ANGIOTENSIN-CONVERING ENZYME INSERTION/DELETION POLYMORPHISM IN TYPE I DIABETIC NEPHROPATHY

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Abstract: Angiotensin converting enzyme gene has been described with an insertion/ deletion polymorphism (I/D) of a 287-basepair sequence of DNA in intron 16 leading to three genotypes, DD and II homozygotes and ID heterozygote.

We examined the frequency of ACE I/D polymorphism in 217 patients, of which 59 with diabetes mellitus type I (controls), 37 with incipient diabetic nephropathy and 121 with end-stage renal disease (cases). The ACE I/D polymorphism was detected by PCR using three oligonucleotide primers in a single reaction. This study has found no evidence that the insertion/deletion polymorphism in the ACE gene plays a major role in the progression of diabetic nephropathy. In particular, the DD genotype, which has previously been implicated in diabetic nephropathy both type I and II, in our study, is not associated with an increased risk of developing type I diabetic nephropathy. Although this study found no association between ACE I/D polymorphism and diabetic nephropathy, we found that DN and ESRD patients have higher prevalence of dyslipidemia and blood pressure ($p < 0.001$), this parameters being major determinants of progression.

INTRODUCTION

Diabetic nephropathy is the most serious complication of diabetes mellitus and affects approximately a third of diabetic patients. Importantly, it is the leading cause of end-stage renal disease requiring dialysis or transplantation in developed countries (National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, 2003) as well as in rapidly developing countries in Asia (Singapore Renal Registry, 1997).

Genetic studies have revealed the the genes of renin-angiotensin system (RAS) are highly polymorphic, raising the possibility that in addition to environmental factors, the genetic make up of RAS affects the status of RAS in individuals. One of such is the insertion/deletion polymorphism of ACE gene. Studies of familial clustering have consistently demonstrated that genetic susceptibility plays an important role in diabetic nephropathy (Krolewski *et al*., 2001) and the gene encoding angiotensin-I converting enzyme (ACE) is a potential candidate gene in its etiology.

ACE, a potent vasoconstrictor, catalyzes the conversion of angiotensin I to angiotensin II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis (Crisan and Carr, 2000). ACE gene has been described with an insertion/ deletion polymorphism (I/D) of a 287bp sequence of DNA in intron 16 (Rigat *et al*., 1992) leading to three genotypes, DD and II homozygotes and ID heterozygote. The mean plasma/serum ACE level in the DD subjects is reported to be approximately double that of II subjects, with ID subjects having intermediate values (Rigat *et al*., 1990).

In a pioneering study, Marre *et al*. (1994) proposed a protective effect of the II genotype against the development of diabetic nephropathy in insulin-dependent diabetes mellitus. Thereafter, a sizeable number of association studies have investigated the possible role of ACE I/D polymorphism in the pathophysiology of diabetic nephropathy and most of them have recorded association of the D allele as a risk factor (Ng *et al*., 2005).

The goal of this study is to test the role of ACE gene, especially the insertion/deletion polymorphism, as a potentially reliable candidate gene for the progression rate in type I diabetic nephropathy. The relevance of this question has not only the clinical usefulness of identifying new prognostic indicators, but should help in dissecting some of the pathogenetic mechanism of diabetic renal diseases.

MATERIAL AND METHODS

Subjects and clinical data. A total of 449 patients with T1DM were recruited from the Diabetic and Nephrology Out-patients Clinics and from the Division of Transplantation at San Raffaele Hospital. Inclusion criteria were: a) established T1DM, 2) patient who underwent a kidney or simultaneous kidney-pancreas transplantation in the past 15 years, 3) absence of secondary nephropathy, 4) absence of chronic comorbidities other than diabetes mellitus. No restriction was adopted based on age, sex, BMI, blood pressure values, HbA1c levels, renal failure stage, disease duration, antihypertensive treatment and its duration.

Genotype was available for 217 patients. Informed consent was obtained.

Genotyping. DNA was extracted from venous whole blood by standard methods.

DNA was amplified by the PCR using three oligonucleotide primers in a single reaction. Two primers flanked the insertion-deletion site (5'-CTGGAGACCACTCCCATCCTTTCT-3'and 5'-GATGTGGCCATCACATTCGTCAGAT-3') and generated a product of 190bp from D alleles, and a product of 490 bp from I alleles. Due to preferential amplification of D allele, the 490bp band is not always visible in heterozygous samples. To avoid the resulting misidentification of ID heterozygotes as DD homozygotes, a third primer complementary to sequence within the insertion was included in all reaction (5'-TGGGATTACAGGCGTGATACAG-3') (Morgan *et al*., 1999).

This consistently generated a fragment of 159bp from I alleles only. The 159 and 190bp fragments were used for genotyping.

Amplification reactions were carried out in a total volume of 20 µl, using 100 ng genomic DNA (2 µl), 0.32 µl $MgCl_2$ (50 mM) (Bioline), 2 µl NH_4 (Bioline), 0.4 µl dNTPs (10 mM) (Promega), 0.8 µl (100 ng/µl) of each primer and 0.2 µl of 5U/µl Taq polymerase (Bioline). Samples were denatured at 96ºC for 5 min, followed by 34 cycles of denaturation at 94ºC for 30s, primer annealing at 58ºC for 1 min and DNA extension at 72ºC for 1 min, with a final 10 min extension stage at 72ºC.

Detection of ACE I/D polymorphism by electrophoresis. The reaction products were separated by electrophoresis in 2% agarose gels (SeaKem LE Lonza) and stained with ethidium bromide. Under ultraviolet light two bands, insertion (I) and deletion (D) were visible (Figure 1).

RESULTS AND DISCUSSIONS

Diabetic patients without nephropathy (DM) were 221, the genotype was determined in 59 of them, diabetic patients with incipient nephropathy (DN) were 94, 37 were genotyped, and diabetic patients with ESRD were 131, in 121 of them information about genotype was available.

Clinical and biological characteristics of each group are summarized in Table 1.

The mean age of the patients was 31.5 ± 0.8 years for DM, 40.8 ± 1.5 years for DN and 40.0 ± 0.7 years for ESRD group. Mean systolic and diastolic blood pressure were 120.0 ± 1.0 and 74.5 ± 0.7 mmHg for diabetic patients, 134.9 ± 2.1 and 81.3 ± 1.0 mmHg for DN group and 137.1 ± 1.7 mmHg for ESRD group respectively. Mean total cholesterol, triglycerides and HbA1c levels were: for cholesterol 171.9 ± 2.7 mg/dl (DM), 192.0 ± 5.4 mg/dl (DN), 214.0 ± 15 mg/dl (ESRD), triglycerides 79.5 ± 3.5 mg/dl (DM), 115.9 ± 7.7 mg/dl (DN), 214.0 ±15 mg/dl (ESRD) and HbA1c 8.3 ± 0.1 % (DM), 8.6 ± 0.2 % (DN) and 8.1 ± 0.6 % (ESRD) respectively.

As expected, patients with nephropathy and ESRD were older, with high levels of BP (both systolic and diastolic), and more severe metabolism alterations (cholesterol and triglycerides significantly elevated). Creatinine clearance values were estimated based on Cockcroft-Gault formula (Cockcroft and Gault,1976).

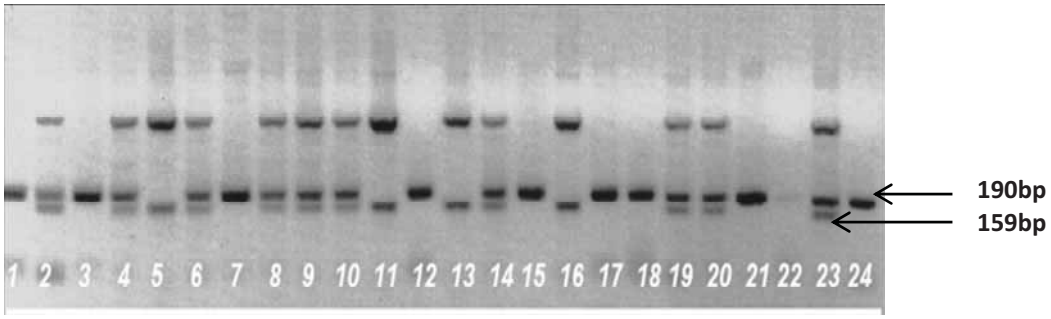

Figure 1. Agarose gel electrophoresis of PCR products of ACE gene. Lines 5, 11, 13, 16 homozygous II cases, 2, 4, 6, 8-10, 14, 19-20, 23 heterozygous ID and lines 1, 3, 7, 12, 15, 17, 18, 21, 24 homozygous DD cases

Patient characteristics (n = 217) according to ACE I/D genotypes are shown in Table 2. The distribution of patients as per genotyping was II (DM-19, DN-11, ESRD-45), ID (DM-27, DN-21, ESRD-63) and DD (DM-13, DN-5, ESRD-13). In each study group, the genotype frequency distributions of this polymorphism were in Hardy-Weinberg equilibrium.

In our study ID genotype was the most frequent, present in 45.8% (DM), 56.8% (DN) and 52.1% (ESRD), followed by II in 32.2 % (DM), 29.7% (DN) and 37.2% (ESRD) and DD was found in only 22% (DM), 13.5% (DN) and 10.7% (ESRD) (Table 2). There is a significant difference between DD genotype in cases (DN and ESRD) and controls

(DM). The DD genotype frequency decreases from 22% in diabetic patients without nephropathy to 13.5% and 10.7% in diabetics with nephropathy and ESRD patients, but there is no association between the D allele and diabetic nephropathy ($p > 0.05$).

Although this study found no association between ACE I/D polymorphism and diabetic nephropathy, we found that DN and ESRD patients have higher prevalence of dyslipidemia and blood pressure ($p < 0.001$), this parameters being major determinants of progression. Paradoxically, no correlation exists between HbA1c levels and BMI. This finding suggestthat the etiology of diabetes, dyslipidemia, hypertension and nephropathy may have a common factor(s), and it also provides clues for the high incidence of micro- or macrovascular complications in T1DM patients.

Table 1. Clinical features of patients with T1DM

	Diabetes (DM)	Diabetic Nephropathy (DN)	ESRD	p
Age	31.5 ± 0.8	40.8 ± 1.5	40.0 ± 0.7	< 0.001 DN & ESRD vs DM
Diabetes duration (years)	20.5 ± 0.6	22.6 ± 1.0	26.3 ± 0.7	< 0.05 vs all
BMI (kg/m^2)	22.9 ± 0.2	23.6 ± 0.4	23.2 ± 0.3	ns
SBP (mmHg)	120.0 ± 1.0	134.9 ± 2.1	137.1 ± 1.7	< 0.001 DN & ESRD vs DM
DBP (mmHg)	74.5 ± 0.7	81.3 ± 1.0	81.9 ± 1.0	< 0.001 DN & ESRD vs DM
Cholesterol (mg/dl)	171.9 ± 2.7	192.0 ± 5.4	214.0 ±15	< 0.001 DN & ESRD vs DM
Triglycerides (mg/dl)	79.5 ± 3.5	115.9 ± 7.7	165.5 ± 15	< 0.001 DN & ESRD vs DM
HbA1c (%)	8.3 ± 0.1	8.6 ± 0.2	8.1 ± 0.6	ns
Creatinine clearance	113 ± 2.4	114 ± 12	54.4 ± 1.3	-------

Table 2. Distribution of ACE genotype and allele frequencies in the three groups

	DM n=59 (%)	DN n=37(%)	ESRD n=121 (%)	p
Genotype frequency				
II	19 (32.2)	11 (29.7)	45 (37.2)	ns
ID	27 (45.8)	21 (56.8)	63 (52.1)	ns
DD	13 (22.0)	5 (13.5)	13 (10.7)	ns
Allele frequency				
I	46 (53.5)	32 (58.2)	108 (58.7)	ns
D	40 (46.5)	23 (41.8)	76 (41.3)	ns

Despite the huge amount of studies looking for candidate genes, the ACE gene remains the unique, well-characterized locus clearly associated with pathogenesis and progression of chronic kidney disease.

We examined insertion/deletion (I/D) polymorphism of theACE gene, one of the important genes in RAS, in T1DM patients with (DN and ESRD) and without nephropathy (DM). The primary objective of the study was to find the pattern of distribution of ACE I/D polymorphism in T1DM, in T1DM with incipient nephropathy and ESRD patients and to study the relation between DD gene polymorphism and DN. Although the data from Caucasian studies failed to confirm an increased risk for development of DN in T1DM and T2DM being associated with D-allele, a role of this genetic marker in Asian patients with T2DM cannot be ruled out (Kunz *et al*., 1998). A meta-analysis of 8663 type I and type II diabetics with incipient or overt nephropathy (defined, respectively, by the presence of microalbuminuria or macroalbuminuria/proteinuria, with or without renal insufficiency) and 6064 diabetic controls with no evidence of renal disease (defined as a urinary albumin excretion below the threshold for microalbuminuria) included in 47 studies published from 1994 to 2004, showed that those with the II ACE polymorphism had a 22% lower risk for nephropathy than homozygous or heterozygous carriers of the D allele. (Ng *et al*., 2005).

This study has found no evidence that the insertion/deletion polymorphism in the ACE gene plays a major role in the progression of diabetic nephropathy. In particular, the DD genotype, which has previously been implicated in diabetic nephropathy both type I (Ng *et al*., 2005) and II (Lee and Tsai, 2002), is not associated with an increased risk of developing diabetic nephropathy.

Thus, evaluating the ACE I/D polymorphism is by no means a reliable and cost-effective tool to identify patients at risk and those who may benefit the most of renoprotective therapy with ACE inhibitors or angiotensin II antagonists and, possibly, with other inhibitors of the RAS, such as renin and aldosterone antagonists. Several research groups have used control groups consisting solely of diabetic patients with normoalbuminuria despite a long duration of diabetes (Grzeszczak *et al*., 1998; Azar *et al*., 2001). This approach could yield clearer evidence for a true association between ACEI/D polymorphism and diabetic nephropathy, since the use of these controls can help reduce case misclassification.

Abreviation

ACE= angiotensin converting enzyme
BMI= body mass index
D=deletion
DN=diabetic nephropathy
DM= diabetes mellitus
ESRD= end-stage renal disease
I=insertion
PCR= polymerase chain reaction
RAS= renin-angiotensin system
T1DM= type 1 diabetes mellitus
T2DM= type 2 diabetes mellitus

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