Original Article

Polymorphism of renin–angiotensin system genes in dialysis patients—association with cerebrovascular disease

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Abstract

Background. Polymorphisms of genes of the renin–angiotensin system (RAS) have been found in association with cerebrovascular and cardiovascular diseases in the general population. In dialysis patients, RAS gene polymorphisms have been studied in combination and separately and have yielded conflicting results.

Methods. In this study we have analysed, in 160 dialysis patients, the distribution of the following genetic polymorphisms: M235T and T174M of the angiotensinogen gene, A1166C of the angiotensin II type 1 receptor gene and the insertion/deletion (I/D) of the ACE gene. The association of these polymorphisms with cerebrovascular and cardiovascular diseases was also tested. Healthy blood donors and hospital staff (169) were the control group for the distribution of the polymorphisms.

Results. The distribution of the polymorphisms in dialysis patients as a whole did not differ significantly from that of healthy controls. However, for patients with severe cerebrovascular disease, 70% carried the D allele compared with 52% of patients without cerebrovascular disease (P = 0.035). We also found that the degree of carotid artery stenosis was significantly correlated with the presence of the ACE ‘D’ allele in subjects on dialysis (P = 0.0348).

Conclusions. The distribution of RAS genes in dialysis patients is similar to that of the normal population. The presence of the D allele of ACE gene is associated with cerebrovascular disease and the degree of carotid artery stenosis. We postulate that the ACE gene polymorphism is a risk factor for cerebrovascular disease in dialytic patients.

Keywords: dialysis; genes; polymorphism; renin–angiotensin

Introduction

Cardiovascular and cerebrovascular diseases are leading causes of mortality in chronic haemodialysis patients [1]. Some of the traditional risk factors identified in the general population, such as hypertension, smoking and positive family history, also contribute to the high prevalence of cardiovascular disease in dialysis patients. Other factors, such as hypercholesterolaemia, do not seem to play the same significant role. It has also been suggested that in haemodialysis patients, atherosclerotic lesions in blood vessels may be different from those in non-uraemic subjects. Therefore, many studies have investigated other non-traditional risk factors operating specifically in dialysis patients [2].

Some polymorphisms of the genes of the renin–angiotensin system (RAS) have been associated with atherosclerotic cardiovascular disease [3–5]. In haemodialysis patients, RAS gene polymorphisms have only been studied to a limited extent, and the investigations have produced conflicting results with respect to renal disease and its associated cardiovascular disease [6–9].

The aim of the present study was to investigate polymorphisms of the RAS genes in haemodialysis patients. In addition, we evaluated the prevalence and the severity of atherosclerotic cardiovascular disease and its association with individual genetic polymorphisms or with combinations of these polymorphisms.

Subjects and methods

Patients

The study was performed on 160 patients with chronic renal failure treated with haemodialysis in Perugia. To avoid selection bias, all patients treated by haemodialysis in our unit between 4 and 9 October 1999 were enrolled in the study. The cause of renal failure was established in 124 patients: chronic glomerulonephritis in 43 (26.8%), vascular disease in 23 (14.3%), diabetes in 22 (13.7%), nephrolithiasis, tubulointerstitial nephritis in 20 (12.5%), polycystic kidney disease in 10 (6.25%) and systemic disease (SLE, myeloma,
amyloidosis) in six (3.75%). In 36 (22.5%) patients the cause of renal failure remained undetermined. Demographic and clinical characteristics of the subjects are shown in Table 1.

The weekly time spent on dialysis was 11.99 ± 0.94 h. Of all hypertensive subjects, 73 were on pharmacological treatment. The following classes of drugs were employed (number of patients is given in brackets): β-blockers (9), α-adrenoceptor antagonists (9), calcium antagonists (39), clonidine (27) and angiotensin-converting enzyme inhibitors (24). Erythropoietin was used in 139 patients. The control group for genetic analysis comprised 169 healthy blood donors and hospital staff (age 61.9 ± 16.6; 105 males, 64 females), in whom the presence of cardiovascular disease was ruled out by periodic health check-ups.

**Laboratory measurements**

Venous blood samples were collected in the morning after an overnight fast on a mid-week dialysis day, before the dialysis session. For our study the following parameters were measured: haematocrit, serum albumin, serum cholesterol, HDL cholesterol, LDL cholesterol and triglycerides.

The adequacy of dialytic dose was assessed by the measurement of $K_t/V$ calculated using the Daugirdas equation [6].

**Cardiovascular status assessment**

Blood pressure was measured with a mercury manometer. Hypertension was defined as systolic blood pressure of 140 mmHg or greater, diastolic blood pressure of 90 mmHg or above, or taking antihypertensive medication [7].

Acute myocardial infarction was defined by a typical rise and fall of biochemical markers of myocardial necrosis (troponin or MB fraction of creatin kinase) with at least one of the following: ischaemic symptoms, development of abnormal Q waves, or ST elevation or depression on ECG. Clinical history and 12-lead ECG were employed in the diagnosis of chronic stable and unstable angina.

Cerebrovascular disease was assessed and classified according to the criteria of the ‘ad Hoc Committee on Cerebrovascular Disease’ [8]. In all patients with cerebrovascular disease, the diagnosis was completed with an imaging technique (computerized tomography or magnetic resonance imaging).

Peripheral vascular disease was assessed by clinical history and examination and confirmed by sonography. Clinical severity ranged from intermittent claudication to a gangrenous toe or knee amputation and was scored accordingly.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Number</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Body mass index (kg/m^2)</th>
<th>Blood pressure&lt;sup&gt;a&lt;/sup&gt; (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>Men 97; women 63</td>
<td>66.8 ± 13.6</td>
<td>24.8 ± 4.4</td>
<td>Systolic 140.0 ± 24.6, Diastolic 77.7 ± 12.3, Mean 98.5 ± 15.4, Time on haemodialysis (months) 76.8 ± 63, Interdialytic weight gain (kg) 2.91 ± 1.05, $K_t/V$ 1.33 ± 0.36</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pre-dialysis values, on anti-hypertensive treatment.

**Echocardiography**

Echocardiographic measurements were carried out according to the recommendation of the American Society of Echocardiography and left ventricular mass was calculated using the Devereux formula [9]. Left ventricular hypertrophy (LVH) was defined as a left ventricular mass indexed for body surface area (LVMI) greater than 125 g/m² in men or 110 g/m² in women. Body surface area was calculated according to the formula of Du Bois and Du Bois.

**Vascular ultrasonography**

Carotid and peripheral artery diseases were assessed by sonographic scans as previously described [10]. In addition, atherosclerotic cardiovascular disease in dialysis patients was catalogued using the index of co-existing diseases (ICED). With this coding system, also employed in the HEMO Study, the presence of 19 different diseases and 11 physical impairments is classified [11]. Comorbidity is also scored. The scoring ranges from 0 to 3 (0 indicates the absence of a condition; the increasing values indicate a more severe stage of the disease).

**Genetic analysis**

The following genetic polymorphisms were determined: M235T and T174M of the angiotensinogen gene, A1166C of the angiotensin II type 1 receptor gene and the insertion/deletion (I/D) of the ACE gene. The variant sequence of the angiotensinogen gene was amplified by PCR using 25 pmol each of primer set 5'-GATGCACAAGGTCTCG-3' and 5'-CAGGGTGCTGTCCACACTGGCG-3' in a total volume of 25 μl containing 2.5 mM magnesium chloride, 100 μM each of deoxynucleotide triphosphates and 0.5 U of Taq polymerase. Cycling conditions were: initial denaturation at 94°C for 10 min followed by 35 cycles of 1 min at 94°C, 1 min at 61°C and 1 min at 72°C, final extension was at 72°C for 10 min. The M235T variant was examined by restriction-endonuclease digestion of 3 μl of PCR product with 5 U of SspI. The T174M genotypes were determined by digestion of the same fragment with 5 U of enzyme NcoI. Digestion products were determined by electrophoresis on ethidium stained 2% agarose gels.

To examine the human AT receptor variant sequences, 25 pmol of primers 5'-ATAATGT5AAGCTCATCCACC-3' and 5'-GAGATTGCATTTCTGTCAGT-3' were used for PCR in a total 25 μl volume. There was an initial denaturation at 94°C for 10 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, final extension was at 72°C for 10 min. The PCR products were digested with 5 U of restriction enzyme DdeI and visualized on 2% agarose gels stained with ethidium bromide. The ACE I/D polymorphism was characterized as previously described, and all DD genotypes were checked with ‘I’ specific primers [15]. Genomic DNA of 169 healthy blood donors of the same ethnicity of patients was analysed as a control group.

**Statistical analysis**

Normally distributed data are presented as means ± SD. The comparisons were made with Student’s $t$-test and analysis of variance (ANOVA); simple linear regression was applied when appropriate. Ordinal data and proportion were compared with non-parametric tests: chi-square, Fisher’s exact test; the associations were measured by odds ratio (OR) with
95% confidence intervals (CI). Logistic regression analysis was employed to determine the relations of gene polymorphisms or other putative risk factors with atherosclerotic cardiovascular disease.

Results

Laboratory investigations gave the following results: serum albumin $3.73 \pm 0.96$ g/dl, serum cholesterol $190.7 \pm 49.9$ mg/dl, HDL cholesterol $38.3 \pm 7.7$ mg/dl, LDL cholesterol $128.8 \pm 45.6$ mg/dl, triglycerides $188.5 \pm 91.1$ mg/dl and haematocrit $33.1 \pm 4.0%$.

Cardiovascular disease

Hypertension was present in 101 patients (61 male, 40 female; age $65.3 \pm 14.8$, normotensive 70.0 $\pm 15.7$; $P = \text{NS}$). The prevalence of hypertension was higher in patients at early stages of dialytic treatment than in those on dialysis for a longer time. Blood pressure values and time on dialysis were negatively correlated. For mean arterial pressure the correlation coefficient was $-0.2785$, $r = 3587$, $P = 0.0004$.

A diagnosis of myocardial infarction and angina was made in 17 (10.6%) and 37 (23.1%) patients, respectively.

Transient ischaemic attacks (50 episodes) were recorded in 30 patients (18.3%). The prevalence of stroke was 11.8% (4.1% lacunar cerebral infarction, 1.87% haemorrhagic). The prevalence of atherosclerotic cardiovascular disease, according to the ICED, was: 42% coronary artery disease, 60.6% cerebrovascular disease and 49.7% peripheral vascular disease. The severity score (ICED) of all studied patients is shown in Table 2.

The mean LVMI, measured by echocardiography, was $146.8 \pm 47.9$ g/m$^2$. By the above criteria, LVH was detected in 107 patients (66.8%).

Sonographic examination of extracranial carotid arteries showed an average lumen narrowing of $21.8 \pm 21.3%$. A lumen narrowing of $>50\%$ was detected in 34 patients (21.2%). Patients who suffered a stroke had an average carotid lumen narrowing of $40.0 \pm 21\%$ vs $18.7 \pm 19.7\%$ in those without stroke ($t = 3.672$, $P = 0.0004$).

Genetic polymorphisms

The distribution of polymorphisms of the different genes of RAS was comparable between dialysis patients and controls (Table 3). In the healthy blood donors and in the dialysis patients, alleles were distributed according to the Hardy–Weinberg equilibrium. The frequencies of the analysed genotypes did not differ from those reported so far for the normal population. Analysis of the frequency of polymorphisms, separated for age and sex, showed no differences in their distribution.

Association of genotypes with comorbidities

ICED index and genotypes. No association was found between the angiotensinogen or the angiotensin II type 1 receptor polymorphisms and the observed comorbidities. Severe cerebrovascular disease was associated with the D allele of ACE gene. Of the patients with severe cerebrovascular disease (scores 2 and 3), 70% carried the D allele compared with the 52% with no disease (OR: 2.078; 95% CI: 1.049–4.171; Yates chi-square: $P = 0.035$). This association was stronger in patients with a cerebrovascular disease score of three (patients with a history of stroke with major residual neurological deficits). In this group, the prevalence of the D allele was 78% (OR: 3.14; 95% CI: 1.93–9.433; Yates chi-square: $P = 0.0197$). Patients with score 3 cerebrovascular disease were also older than those with score 0 ($74.2 \pm 5.5$ vs $61.2 \pm 15.8$ years; $P = 0.002$).

The degree of carotid artery disease was different in the three ACE genotypes. Average lumen narrowing was $29.2 \pm 17.5\%$ in DD carriers, $20.3 \pm 23%$ in ID carriers and $10.8 \pm 13.7\%$ in the II ($F = 3.493$, $P = 0.0348$). Logistic regression analysis produced a significant predictive model for carotid stenosis $>50\%$ composed by diabetes, interdiabetic weight gain, age, time on dialysis and D allele of the ACE gene (model chi-square 20.59, $P = 0.002$).

Hypertension. Although blood pressure values did not differ significantly between patients with different polymorphisms of the genes studied, some difference was noted in the distribution of the alleles of the ACE gene. In hypertensive subjects a higher prevalence of the DD and ID genotypes than II was observed ($P = 0.054$, NS). Applying logistic regression analysis we obtained a predictive model for hypertension.

Table 3. Distribution of gene polymorphisms in dialysis patients and in controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients ($n = 160$)</td>
<td>Controls ($n = 169$)</td>
</tr>
<tr>
<td>ACE</td>
<td>DD = 32.3%, ID = 50.9%, II = 16.8%</td>
</tr>
<tr>
<td>M235T</td>
<td>MM = 28.6%, MT = 45.4%, TT = 26.0%</td>
</tr>
<tr>
<td>T174M</td>
<td>MM = 0.61%, TM = 26.7%, TT = 72.6%</td>
</tr>
<tr>
<td>A1166C</td>
<td>AA = 45.2%, AC = 44.7%, CC = 10.1%</td>
</tr>
</tbody>
</table>

Table 2. Prevalence and severity of atherosclerotic cardiovascular disease in studied patients (ICED)

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease</td>
<td>57.9%</td>
<td>27.6%</td>
<td>12.5%</td>
<td>2%</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>39.3%</td>
<td>34.1%</td>
<td>15.4%</td>
<td>11.1%</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>50.3%</td>
<td>45.5%</td>
<td>2.1%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>
polymorphisms examined. We therefore analysed patients on dialysis for less than the average time (76.8 months) and found that hypertension was present in 72% of DD + ID carriers compared with 40% in II carriers (OR: 0.250; 95% CI: 0.065–0.911; Yates chi-square: $P=0.029$). The analysis of the effect of different combinations of polymorphisms on blood pressure and cardiovascular disease produced negative results.

Heart disease was not associated with any of the polymorphisms examined.

**Discussion**

Two major findings summarize the results of the present study: (i) the distribution of polymorphisms of the RAS genes in dialysis patients is not different from that found in healthy controls; and (ii) cerebrovascular disease and carotid artery disease in dialytic patients are more severe in carriers of the D allele of the ACE gene.

Previous studies have investigated the association of single genotypes of the RAS system with renal disease but the results were mostly negative. Our study targeted the terminal stage of renal diseases and several polymorphisms were tested, both separately and in association. The finding of a comparable distribution of the polymorphisms between our patients and the healthy controls may rule out a clear-cut association of the RAS genes with end-stage renal disease in the dialytic stage. We cannot exclude the possibility of premature deaths, at earlier stages of the disease, in patients carrying the adverse genotypes. This hypothesis could also explain the low prevalence of severe coronary heart disease and its lack of association with the D allele in our patients.

Studies on the general population and in selected families have shown that the angiotensinogen (AGT) gene polymorphism may account for changes in arterial blood pressure and may increase the susceptibility to essential hypertension [12]. A recent report has shown a rapid progression towards end-stage renal disease in individuals with the DD genotype, especially in those with the MM genotype of AGT as well [13]. In our patients we have not been able to show an association between AGT polymorphism and blood pressure. The use of antihypertensive medications may mask a possible association, but the analysis based on hypertension as a discrete variable also gave negative results.

Whether the high prevalence of hypertension in carriers of the D allele of ACE gene at an early stage of dialytic treatment is a significant finding, is unclear. It is known that subjects carrying the D allele have higher plasma levels of ACE, but experiments of gene titration in animals have shown that a 3-fold increase of plasma ACE does not affect blood pressure [14]. There is also no consensus on the effects of hypertension on the genetic variations in ACE levels in humans, which are associated with the I/D polymorphism. While an association between hypertension and ACE gene polymorphism has not been found in the general population, in some particular conditions, such as malignant hypertension, the D allele has been shown to be a significant risk factor [15]. In dialysis patients, blood pressure can be controlled by sodium and fluid removal. Carriers of the D allele seem to be less sensitive to sodium state than the I carriers and could therefore be less responsive to sodium removal by ultrafiltration in dialysis [16]. In previous studies of genetic association, hypertension at early stages of dialysis had not been analysed separately from late stages. A slight difference in pathophysiological mechanisms or patient population cannot be ruled out. Our finding of a negative correlation between time on dialysis and blood pressure is compatible with both cases, as suggested by Rahman et al. [17].

The finding of an association of the D allele with severe cerebrovascular disease may contribute to the understanding of the high prevalence of this complication in end-stage renal disease. Chronic renal failure has been defined as ‘a vasculopathic state’ and the relative risk of severe cerebrovascular disease in patients on dialysis is as high as 10.7, compared with the general population [18].

We also took into account the possibility that local environmental factors could contribute to the high overall incidence of cerebrovascular disease in our patients. We ruled out this hypothesis by examining the results of a regional survey, which showed that the adjusted rate for stroke in our local population is 1.55/1000/year, very close to the 1.56/1000/year of the European population [19].

In one study, the D polymorphism has been found in association with lacunar stroke but not with carotid disease [20], while in hypertensive subjects, the DD genotype is more frequent in those with a parental history of stroke [21]. In the present study we have found that both stroke and carotid artery disease are associated with the D polymorphism. Therefore, a concurrent action on microvascular and macrovascular disease leading to the more serious cerebrovascular events may be envisaged. The association between wall thickening of carotid arteries and the DD polymorphism of the ACE gene has been found in several clinical settings, but sometimes this gene effect was masked by stronger environmental factors such as smoking [22]. Only a few exceptions in high-risk conditions, such as diabetes, have been reported [23]. In non-diabetic dialysis patients, the D allele of ACE was significantly associated with an increase in carotid intima-media thickness [24]. In our patients diabetes was a coexisting risk factor with the D allele.

Our findings may be ascribed to the action of localized RAS on vascular function and structure: a hypertrophic effect including extracellular matrix production. The expression of vascular tissue ACE is associated with the I/D polymorphism and ACE exerts a regulatory effect on angiotensin II production in the vascular wall [25].
Most haemodialysis patients are chronically exposed to a complex stimulation of the RAS induced by dialysis, and this activation is deemed to produce significant effects on different target organs [26]. Those patients having genotypes associated with maximal expression of RAS components may therefore be more predisposed to organ damage, or even higher mortality, especially in the presence of other predisposing conditions [27].

Our findings suggest that the D allele of the ACE gene is associated with cerebrovascular events in dialysis patients. There are many variables operating in these patients that are not controlled due to the failure of homeostatic mechanisms produced by end-stage renal failure, and the interactions between genetic predisposition and environmental factors will undoubtedly be complex and require further study.

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