Angiotensin-converting enzyme (ACE) haplotypes and cyclosporine A (CsA) response: a model of the complex relationship between ACE quantitative trait locus and pathological phenotypes

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It is highly controversial to define the role of angiotensin-converting enzyme (ACE) polymorphisms in essential hypertension. We studied a group of patients in whom hypertension was the major side effect of treatment by cyclosporine A (CsA). This study group comprised 227 Italian patients with nephrotic syndrome, 103 of which were treated with CsA and had different outcome. Forty-nine patients developed serious hypertension that was reversed after withdrawal of drug. ACE haplotypes were determined by a combination of molecular and statistical methods after verifying genotypes of six intragenic single nucleotide polymorphisms in 304 Italian blood donors and assembling them in clades (A, B, C) that include 95% of observed haplotypes. The association between ACE clade combinations and serum enzymatic levels confirmed the previous results about a role of an unidentified genetic variant at the 5’ of the intragenic recombination site located near intron 7. ACE clades were then determined in patients, and regression methods were used to analyze variables associated with CsA responsivity and progression to renal failure. ACE genotype and responsiveness to CsA were strictly associated, because homozygosis for ACE B clade was able to influence CsA sensitivity. This highlights the role of 5’ variants, which differentiate clades B and C. Other genetic markers were tested to search for possible additive effects. We found that PAI-1 4G allele was associated with progression to renal failure in the group of CsA-treated patients. Our results are in agreement with the hypothesis, raised after experimental results obtained in mouse models, that the effect of ACE polymorphisms on blood pressure is detectable once environmental factors, like CsA treatment in our case, overcome physiological homeostatic mechanisms.

INTRODUCTION

Angiotensin II (AngII) is a potent vasoactive and fibrogenic peptide with wide implications in human physiology and pathology. Almost 60% of the biologically active AngII is derived from the conversion of AngI into the active peptide by ACE (1), a large protein encoded by the ACE gene (MIM106180). AngII levels depend therefore, to some extent, on ACE levels that are, in part, determined by genetic factors. Definition of ACE genotypes, which influence levels, and their association with human diseases such as hypertension, myocardial infarction, renal fibrosis and so on were a logical consequence of the above assumption, but clear proof for a direct relationship is still lacking. The starting point was the recognition of a major quantitative trait locus (QTL) in ACE gene in 1990 where the presence of a 287 bp
insertion/deletion polymorphism in intron 16 (I/D; rs1344744) was identified: the D allele was codominant in raising ACE plasma levels in Europeans (2–4). New polymorphisms at the 5′ in strong linkage disequilibrium with the I/D locus were identified and it was proposed a bifunctional QTL model in which two different causative variants act together in determining ACE level variability (5). Finally it was introduced a haplotype-based model in which an ancestral recombination breakpoint at intron 5–exon 8 interval determines a haplotype cluster carrying the D variant but still differing at the 5′ of the gene (6–8). Data on ACE haplotypes have been extended to different populations in the last few years, considering several single nucleotide polymorphisms (SNPs) with the conclusion that a cladistic approach better fits statistical analysis in Europeans. An approach based on a simple cladistic structure was first proposed by Keavney et al. (7) in a study on a British white population. Clades A, B and C were defined on the basis of evolutionary information and they account for about 90% of the observed haplotypes. This concept was further extended to Africans and led to consider a few other SNPs as causative of ACE levels: at the promoter region (rs4291), at exon 17 (rs4343) (9) and at the 3′ SNPs as causative of ACE levels: at the promoter region about 90% of the observed haplotypes. This concept was on the basis of evolutionary information and they account for a British white population. Clades A, B and C were defined on the basis of evolutionary information and they account for about 90% of the observed haplotypes. This concept was further extended to Africans and led to consider a few other SNPs as causative of ACE levels: at the promoter region (rs4291), at exon 17 (rs4343) (9) and at the 3′ end of intron 25 (rs4363) (9–11). Cox et al. (12) defined a multiple, independent effect of four variants in Nigerian families, a minor one (rs4459609) in the 5′ flanking region at −5499 from the ATG starting codon and three located downstream of the recombination site (rs4343, 31839delC, rs4363). Taken together, the aforementioned studies demonstrated that the I/D polymorphism is indeed associated with serum level, but a cladistic approach based on haplotype analysis represents a more efficient determinant of ACE-related phenotypic traits.

Although D/D genotype is invariably linked to high circulating levels, data associated with cardiovascular and renal diseases are controversial (13). Moreover, association of ACE clades with human pathology is not a direct consequence of levels, as hypothesized in the past. Results by Zhu et al. (9) contradicted, in fact, this possibility because they found an epistatic interaction between the aforementioned variants located in the promoter (rs4291 A/T) and in exon 17 (rs4343 A/G), which affects blood pressure. Furthermore, the authors observed that the allelic effect of the promoter variants is opposite in direction for ACE concentration and blood pressure. This probably reflects a complex interaction between genetic loci not necessarily linked to ACE levels.

Beside hypertension, ACE I/D has been implicated in the clinical outcome to renal failure in patients who develop tubulo-interstitial fibrosis. Several genetic variants of molecules synergic with AngII in determining fibrosis may play additive effects to ACE locus (14). We study the effect of three functional polymorphisms that belong to the renin-angiotensin system (RAS), i.e. M235T (C4027T) of angiotensinogen (AGT) (MIM106150), A1166C of angiotensin II type 1 receptor (AT1) (MIM106165) (15,16) and the K528R (A1583G) (17) in exon 11 of adipocyte-derived leucine aminopeptidase gene (ALAP) (MIM606832) plus the SNP 4G/5G (four or five guanines) in the promoter region of plasminogen-activator inhibitor type 1 (PAI-1) gene (MIM173360). The former three regulate AngII levels (AGT and ALAP) and function (AT1), whereas PAI-1 regulates removal of extracellular matrix (18).

This work has two major aims: the first was to consider the complex interaction among the ACE genetic locus, enzymatic levels and human hypertension in a clinical condition such as cyclosporine-induced hypertension in which AngII appears more directly involved in regulating blood pressure. A vast area of clinical and experimental observations in animals and humans support this possibility (19–25). The second was to consider the additive effects of synergic polymorphisms in determining evolution to end-stage renal failure (ESRF).

For this reason, we have evaluated ACE haplotypes in a group of patients with nephrotic syndrome, part of them treated with cyclosporine. We found a strong association of different ACE clades with either protection or intolerance to the drug, always manifested as severe hypertension and worsening of renal function.

RESULTS

Population genetics of ACE haplotypes

The starting choice of genetic markers for defining ACE haplotypes was based on a critical review of data reported by Rieder et al. (8) who defined haplotypes in Europeans and African-Americans on the basis of 52 SNPs. This original report, in agreement with the study by Keavney et al. (7) on Europeans, supported the idea of an ancestral recombination in ACE gene and validated the association of ACE haplotypes in three major clades (A, B and C) that include the five most frequent haplotypes as follows: H6 and H8 in clade A, H1 in clade B, H7 and H9 in clade C. Six of the original 52 SNPs are sufficient to define haplotypes and therefore clades: three markers (rs4424958, rs4309 and rs4311) flank the recombination site, one upstream in intron 2 (rs4295) and two downstream, in introns 16 (I/D) and 25 (rs4363) (see Table 1). The combination of single marker genotypes, which characterizes haplotypes and clades, is given in Table 2. In agreement with other studies in Europe (7,26,27), we could confirm an overall frequency of the haplotypes described by Rieder in the 94% of a normal Italian population. The finding of H1-6 and the reciprocal C2 possible products of recombination between H1 and H6 haplotypes (see Table 2) allows to state that recombination has occurred between rs4309 and rs4311, as already hypothesized (6). Recombination between rs4424958 and rs4309 could also have occurred although the recombination products reciprocal to H7 and H9 were not found. As described in other studies (27,28), the presence of this ancestral recombination breakpoint does not influence the high level of linkage disequilibrium across the gene (Table 3). Using the Arlequin 2000 software, we tested and excluded significant departure from the Hardy-Weinberg equilibrium for both genotypes and haplotypes frequencies.

ACE haplotypes and levels

ACE levels were determined in normal population with different combinations of haplotypes belonging to A, B, C clades. In agreement with Danilov et al. (29), we could confirm a strong correlation between serum ACE activity and protein levels ($r = 0.9$) in a sample of 16 individuals representative...
of different haplotype combinations. ACE levels in relation to different genotypes and haplotypes are reported in Figure 1 and Table 4. Basically, serum ACE levels were different among carriers of I/I, I/D and D/D genotypes, however the stratification in haplotypes splits the two simple I/D and D/D genotypes of the previous classification in five possible combinations (2 for I/D and 3 for D/D) that present different ACE levels (Figure 1). The most remarkable difference is between B/B and B/C, both included in the previous D/D genotype. This finding supports the hypothesis that differences in ACE serum levels are associated to the I/D alleles, with an additional contribution of a variant located at the 5′ of the recombination site.

Determinants of CsA responsivity and clinical outcome

In a recent analysis of factors that influence survival in patients with steroid-resistant nephrotic syndrome, CsA was found as the major modifier, improving the outcome in a portion of patients with worse prognosis (30). We have evaluated 99 patients treated with CsA considering several variables, which included genetic factors, by logistic regression analysis. Results relative to genetic variables were given following a dominant model of allelic effect on phenotype except for ACE haplotype in the form B/B versus others that gave most significant result in contingency table analysis (see below). Table 5 shows that homozygosis for ACE B clade was able to influence CsA sensitivity (\(P = 0.0139\)) in the overall cases and such influence was confirmed when considering only steroid-resistant patients. CsA response was also influenced by response to steroid treatment (\(P = 0.0001\)) but this analysis highlights the independence of the ACE haplotype for other clinical variables. Contingency table analysis on all clade combinations shows a five times higher frequency of B/B in unresponsive versus responsive patients. This is counterbalanced by a higher A/C (19% versus 11%) and C/C (8% versus 2%), respectively, in responsive and unresponsive to CsA patients (Table 6). Overall, the difference is statistically significant. In untreated patients, frequencies of different clades were the same. The concomitant higher C and lower B frequencies in responsive patients explains lack of significance using the I/D marker because both clades C and B include the D allele.

Almost all patients who were treated and had a good response to CsA had a genotype different from B/B (23 out of 24) whereas in the subgroup of intolerant to CsA 13 out of 45 were B/B. Overall, 13 out of 14 B/B in the category
of patients treated with CsA were intolerant. Therefore ACE genotype and responsiveness to CsA were strictly associated.

We then looked at several clinical and genetic parameters influencing progression to ESRF in the context of a multivariate approach (Table 7): the variables CsA treatment, ACE haplotype and PAI-1 and AT-1 genotypes were selected on the basis of a stepwise method. Also in this case, genetic variables were given following a dominant model of allelic effect on phenotype with exception of ACE haplotype, also in this case, in the form B/B versus others. This grouping was based on both results from logistic regression and on the observation that differences in ACE levels become remarkable upon splitting the D/D subgroups in B/B, B/C and C/C.

Confirming previous data (30), progression was strongly influenced by CsA response. In the subgroup with intolerance to CsA, the outcome was not influenced by ACE genotype but only by PAI-1 4G allele considered as dominant (Table 7 and Figure 2). This is most likely due to the strong association between diplotype and CsA responsivity, which masks the significance of the B/B diplotype in the survival regression for the treatment group. In the untreated patients, the CsA responsivity cannot be considered and the variable ACE haplotype, although not significant, shows some relation with the failure time.

Therefore, ACE is strictly associated with responsiveness and/or intolerance to CsA and, without any treatment, is only weakly associated to progression. PAI-1 is associated with progression to renal failure in the group of CsA-intolerant patients.

**DISCUSSION**

Regulation of blood pressure in humans is a classical example of complex trait resulting from the interplay of several homeostatic mechanisms. Clinical and experimental evidences support the general idea that hypertension arises when significant environmental factors overcome the homeostatic potential in a specific genetic background. This makes it difficult to approach the genetic basis of hypertension in large population studies where environmental factors are not uniform.

The definition of a homeostatic key role of the kidney in regulation of blood pressure and in hypertension represented a milestone in the understanding of pathogenetic mechanisms. This is essentially based on the regulation of sodium balance...
and is particularly evident in case of renal diseases and renal failure. Elegant experiments with cross-renals transplants in animals (31,32) and in humans (33) first demonstrated the key role of kidney and recent genetic advances on tubular salt transporters (34) contributed to provide further rational proofs. Current view on the interplay between hypertension and the kidney considers the possibility that acquired subtle renal defects confer sensitivity to salt overload through the basic mechanism of local vasoconstriction. Johnson et al. (35) recently reviewed the putative causes of initial subclinical damage and proposed two general physiology imbalances such as hyperactive sympathetic nervous system and stimulated RAS and, in parallel, two environmental causes such as low-potassium diet and CsA use. CsA is an immunosuppressive drug used in therapy of autoimmune diseases, but this treatment is commonly associated with the development of hypertension and nephrotoxicity: evidence of the literature indicates that hyperactivity of RAS and related systems is implicated in such events. When we analyzed patients receiving CsA for response and adverse effects, we realized that investigating the genetic background of these effects was worth to be done, based on the above assumption that any implication of genetic variants is amplified in such a particular environment. In other words, the systems directed at maintaining blood pressure homeostasis (36,37) are altered with CsA use and intolerance to CsA. Therefore, we consider unlikely that the difference in ACE levels. The mutual aspect is the association with high ACE levels such as B/C and C/C are neutral or positively linked with drug responsiveness.

As clades B and C are differentiated by the presence of a variant localized upstream of the recombination site, one possible interpretation of our findings is that this variant is a determinant of CsA hypertensive response in spite of moderate difference in ACE levels. The mutual aspect is the association between clade C and CsA responsiveness because clade C is, respectively, more and less frequent in patients with sensitivity and intolerance to CsA. Therefore, we consider unlikely that the effects of genetic factors characterizing the different clades can be explained by differential ACE enzymatic activity and rather advance other hypothesis: (a) the 5’ variant confers functional properties at the level of tissue(s) which influence blood pressure control; (b) more than one variant in the 5’ region could independently influence blood pressure and ACE activity; (c) the 5’ functional variant, in linkage disequilibrium with those analyzed, is external to ACE. Regarding hypothesis (c), based on reports about linkage disequilibrium in the genomic region of interest, the location of such variant can be approximately limited to a region extending up to about 729 kb from the ACE gene.
Such region includes two genes and a pseudogene: one is cytochrome b-561 (CYB561), the second is a putative, uncharacterized, ankyrin-repeat containing protein (DKFZP564D166) and the third is a pseudogene similar to cyclophilin (LOC342541). Further studies are requested to clarify any involvement of these genes or the included non-coding sequences to the CsA-related phenotypes. Hypothesis (b) is in agreement with a previous study (9) which described an association of rs4291- \( A \) allele with ACE levels, while \( T \), at the same site, was associated with hypertension. In general, our findings suggest that ACE QTL influences hypertension only in concomitance with environmental factors (CsA in this case) that act together and overcome the capacity of homeostatic mechanisms as suggested by Krege et al. (36).

A second major finding reported in this paper suggests that PAI-1 4G allele is associated with long-term outcome in patients treated with CsA. PAI-1 genotype was evaluated together with genetic markers of several other substances that play additive effects on AngII side functions that converge to renal fibrosis. It is known that the 4G allele, in PAI-1 promoter, is associated with high levels of an inhibitor of plasminogen activator (t-PA, u-PA) with the final effect of inhibition of extracellular matrix removal and renal fibrosis, which is the pathological substrate of renal failure. This mechanism appears even more deleterious because it is reported that CsA treatment induces factors, such as AngII and TGF-\( \beta_1 \), that influence PAI-1 expression (14,47).

In conclusion, our data underscore the role of genetic factors, ACE for response to CsA treatment and PAI-1 for disease progression, in a specific model of hypertension caused by CsA in humans.

**SUBJECTS AND METHODS**

**Populations studied**

Patients. We retrospectively studied 227 Italian patients affected by sporadic nephrotic syndrome with dependence or resistance to steroids, who had been followed at different clinical institutions and for whom DNA was available. Only patients who resulted dependent (n73) and resistant (n154) to steroids were enrolled (Table 8).

Inclusion criteria. Major inclusion criterion was the presence of nephrotic proteinuria (>40 mg/kg/day) for which at least the first therapeutical approach had been completed following the scheme below. Only patients with blood pressure levels up to 90th percentile of normal range for age.
and sex and adjusted for the percentiles of height were considered using data from the Task Force Reports on High Blood Pressure in Children and Adolescents (48,49). Range of variations considering age between 1 and 17 years were: systolic blood pressure (SBP), from 94 to 140 mmHg; diastolic blood pressure (DBP), from 50 to 90 mmHg. As a rule, therapy of nephrotic syndrome started with steroids associated or substituted with cyclophosphamide (2 mg/kg for 60 days) and/or with CsA (5 mg/kg starting dose, followed by tapering to reach the minimum dose required for maintaining cyclosporine serum at levels between 50 and 100 ng/ml). In case of persistent steroid-cyclosporine resistance, methyl-prednisolone was given in pulses (10 mg/kg, six cycles). Steroid resistance was considered the failure to achieve remission of proteinuria (<2 mg/kg/day) after 4 weeks of steroid treatment. CsA resistance was the failure to modify proteinuria after 8 weeks of CsA at starting dose of 5 mg/kg. CsA intolerance was considered the worsening of renal function (50% reduction of creatinine clearance of pre-CsA values) and/or the increment of mean arterial blood pressure (±50%) after the start of therapy. Increments of blood pressure over this limit required withdrawal from the CsA treatment and combined treatment with one or more drugs (calcium antagonists, β-blockers and ACE inhibitors) if requested.

Exclusion criteria. They included positivity for auto-antibodies (antinuclear, nDNA, ENA and ANCA) as evaluated with indirect immunofluorescence; molecular defects in one of the slit-diaphragm genes are responsible for familial nephrotic syndrome (NPHS1, NPHS2, exon 8 hot spot of alfa-actinin4). There was no exclusion criterion related to age. Availability of renal morphology was not an exclusion criterion under 16 years; after this age, a renal biopsy excluding all kinds of primary and secondary renal diseases other than FSGS was required for enrolment.

Renal biopsies were processed by standard procedures that included ordinary histological preparation and immunofluorescence of common antigens and were evaluated by a pathologist who discussed all relevant aspects with referent clinicians. Clinical and pathological features (i.e. gender, age at onset of proteinuria, evolution towards renal failure, renal transplant, etc.) and demographic characteristics of patients are reported in Table 8. Informed consent for DNA analysis and for reviewing their clinical parameters on statistical basis were obtained and handled as already described (30). General clinical parameters included blood cell counts, serum creatinine and urea levels, cholesterol, triglycerides and liver function tests performed according to international standardized procedures.

Control population. For genetic population studies on ACE haplotypes, we overall used 304 normal Italian blood donors afferent to our hospital (males 76.5%, females 23.5%, median age 30 years, interquartile range (IQR) 14.6).

Genotyping

ACE haplotypes. For ACE haplotypes the following SNPs were evaluated: (1) rs4295 (G/C) and rs4363 (A/G) were determined by allele-specific primers combined in a multiplex-PCR (Figure 3a); (2) rs4424958 (A/G), rs4309 (C/T) and rs4311 (C/T), which are located nearby the ancestral recombination site, were simultaneously determined by allele-specific primers combined in a multiplex-PCR (Figure 3a); (3) rs4295 (G/C) and rs4363 (A/G) were determined by allele-specific primers combined in a multiplex-PCR (Figure 3a).
At least two primer matching alleles in a single chromosome. This results in the formation of two fragments of 863 and 265 bp only with a C allele in the rs4309 SNP (Figure 3b). In any case in which allele-specific primers were used for discriminating a transition (purine/purine), a mispairing was introduced in the second or in the third base from 3’ end to increase specificity of annealing (53). All primer pairs and annealing temperature were determined with Oligo 4.02 (National Biosciences, Inc., Plymouth, MN) and Amplify1.2 programs. The I/D polymorphism was determined following the method described previously (3,54). Primer list and conditions for each PCR are available upon request.

AT1. The A1166C polymorphism was determined according to Bonnardeaux et al. (16).

ATG. The C4027T (M235T) polymorphism was determined according to Russ et al. (55).

PAI-1. The promoter 4G/5G polymorphism was determined according to Margaglione et al. (56).

ALAP A1583 (K528R) at exon 11 of ALAP was determined by restriction fragment length polymorphism analysis.
(PCR-RFLP) utilizing the MnI restriction enzyme as described by Yamamoto et al. (17), with some modifications.

For all bi-allelic genotypes, the observed frequencies have been compared with the expected frequencies in conditions of Hardy-Weinberg equilibrium, using the Chi-square test with one degree of freedom.

Haplotype analysis

For individual haplotype reconstructions, we used PHASE 2.1.1 program (57,58) with the –k option, 100 cycles and 100 iterations. Results were confirmed with the Arlequin 2000 software (59). Measure of pairwise linkage disequilibrium by the D' parameter was obtained by Arlequin (60), as well as testing of departure from the Hardy-Weinberg equilibrium.

After characterizing ACE haplotypes in control individuals, three SNPs (rs4295 (G/C), rs4363 (A/G) and rs13447447 (I/D)) were selected, which can identify patients reduced haplotypes belonging to ACE clades. Overall, clades A, B and C included 97% of the patient population whereas rare haplotypes were excluded from the analysis.

Serum ACE levels

Serum ACE levels were determined in a part (n170) of normal controls used for genotyping (males 79.5%, females 20.5%; median age 30.3, IQR 14.4) with ACE haplotypes combinations of A, B, C clades (see Figure 1). Enzymatic activity of ACE was evaluated in triplicate following the method described by Lieberman (61). The average intra-assay coefficient of variation was 6.2 (S.D. 4). Blood samples were kept in ice and processed within 4 h from drawing. In a sample of 16 sera, ACE protein levels were determined with a commercial ELISA technique (Chemicon International, Inc., Temecula, CA, USA).

Statistical analysis

ACE serum levels/ACE haplotypes correlations. To analyze the relationship between ACE levels determined as enzymatic activity and by ELISA, we used the least-square method and the linear correlation coefficient (r). Analysis of variance tested the influence of genotypes on ACE levels (Figure 1). Post hoc test (Fischer’s PLSD) tested pairwise differences between each combination of haplotypes (Table 4) and significant differences were also tested with the non-parametric Mann-Whitney test.

Survival analysis. The influence of any clinical and genetic variables on the progression to ESRF was tested using the multivariate Cox regression model (proportional hazards). General and clinical variables were considered as follows: dichotomous in case of CsA response (responsive, intolerant/resistant), sex, cyclophosphamide treatment (yes/no) or continuous (age at onset of proteinuria). Genetic variables were considered following a dominant, recessive or additive model of allelic effect on phenotype. Both classical I/D ACE genotypes and haplotypes (clades combinations) were separately used in tests. Available variables (CsA sensitivity, sex, age at onset, ACE haplotype or I/D polymorphism, PAI-1, AT-1, AGT, ALAP) were selected with a backward stepwise selection method, performed separately in steroid-resistant (SR) subgroups (67 CsA-treated, 84 CsA-untreated) and relevant parameters are reported in Table 7. The global significance of each set of selected variables was tested by the likelihood test, whereas the Chi-square test was associated with either the hypothesized exclusion of individual continuous covariates or with each level of nominal covariate. Relative risk was determined as the e^b coefficient with 95% confidence interval. For the association between an effect of ACE haplotype or PAI-1 and CsA treatment on outcome a “dummy” variable was created and analyzed with the Kaplan–Maier analysis and log-rank test (Figure 2).

CsA responsivity, genotypes and clinical variables. The relationship between the clinical outcome in relation to CsA responsiveness and independent variables (all those described in the previous section, plus “Steroid Responsiveness”) was examined with logistic regression technique. The significance was tested by the Wald Chi-square statistic (Table 5). Contingency tables were used for comparing frequencies among groups and versus controls.

StatView version 5.0.1 (SAS Institute, Inc., Cary, NC, USA) was used in the statistical approach.

Links to database information


Entrez Nucleotides database: http://www.ncbi.nlm.nih.gov/entrez (for annotated ACE sequences submitted by Rieder et al. [AF118569; AY436326]).


Linkage disequilibrium map of chromosome 17: http://snp.wustl.edu/snp-research/ld-blocks/(Table S1c for SNPs description and S7c for LD statistics; ACE intragenic SNPs: rs4335, rs4353).

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