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RAPID COMMUNICATION

Angiotensin converting enzyme gene polymorphism: Potential silencer motif and impact on progression in IgA nephropathy

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Angiotensin converting gene enzyme polymorphism: Potential silencer motif and impact on progression in IgA nephropathy. Since the renin angiotensin system (RAS) is established as an important factor in renal disease progression, we determined whether RAS alleles that have been linked to variability in outcome in several cardiovascular diseases also affect progression of IgA nephropathy. These genetic variants include: (1) angiotensin I converting enzyme deletion polymorphism in intron 16 (ACE I/D), reported to be associated with increased risk of myocardial infarction as well as left ventricular hypertrophy; (2) a point mutation in the angiotensinogen (Agt) gene resulting in a methionine to threonine substitution at residue 235 (M235T), reported to be associated with hypertension in Caucasians; and (3) an angiotensin receptor type I (ATR) A to C transition at bp 1166 (A1166C) which shows synergy with the deleterious effects of the ACE DD genotype in myocardial infarction. We examined these polymorphisms by PCR amplification of genomic DNA samples from 64 Caucasian patients in the USA (age 6 to 83 years) with biopsy-proven IgA nephropathy whose renal status was followed for an average of almost seven years. Patients who presented with and maintained normal serum creatinine (Cr, <1.5 mg/dl), had ACE genotype frequencies of II:35%, ID:61%, DD:4%. By contrast, in patients with progression (initially normal Cr increased to a mean of 4.5 ± 0.86 mg/dl), ACE genotype frequencies were II:22%, ID:44%, DD:33% ($P = 0.057$ by Fishers's exact test, vs. non-progressors). The association of the DD genotype with progression was even more striking when patients with other risk factors (hypertension and/or heavy proteinuria) were excluded. In this subgroup, the genotype frequencies in patients with stable creatinine versus those with deterioration in renal function was 53%, 47%, and 0% versus 0%, 40%, and 60%, respectively, for II, ID, and DD genotypes ($P = 0.009$ by Fisher's exact test, progressors vs. non-progressors). Further, sequence analysis of the I gene polymorphism revealed a potential 13 bp silencer motif. Neither the Agt 235T nor the ATR A 1166C gene variants, however, was associated with deterioration of renal function. Taken together, these results indicate that, although polymorphism in each of the three genes in the RAS system has been linked to cardiovascular diseases, only the ACE I/D polymorphism is associated with progressive deterioration in renal function in IgA nephropathy. Since previous observations link ACE polymorphism with ACE activity, these findings imply a widespread importance of ACE in modulating destructive processes in different organs.

Understanding genotype-phenotype correlations has rapidly expanded over the last decade. Single gene defects underlying many diseases, including cystic fibrosis, nephrogenic diabetes insipidus, and Huntington's chorea have been discovered. In addition, common diseases such as diabetes mellitus and systemic hypertension, long held to be strongly related to family history and now recognized as likely polygenic, are being defined at the gene level. The prominent role of the renin-angiotensin system (RAS) in cardiovascular regulation suggests that component gene abnormalities could modulate cardiovascular disease processes. Indeed, polymorphisms in several genes within the RAS system have been linked with cardiovascular disorders. A polymorphism of the angiotensin I converting enzyme (ACE) gene has been described consisting of the insertion or deletion (I/D) of a 287 bp fragment within intron 16. The homozygous deletion genotype is associated with increased risk for myocardial infarction and also with left ventricular hypertrophy [1, 2]. Moreover, the ACE (DD) polymorphism was shown to have synergy in its deleterious effects in myocardial infarction with a polymorphic locus in another RAS component gene, namely the angiotensin II type I receptor (ATR) gene. The latter consists of an ATR adenosine to cytosine transition at position 1166 (A1166C) in the 3′ untranslated region of the gene, which has also been associated with hypertension independent of ACE [3, 4]. Finally, a point mutation of the angiotensinogen gene (Agt), resulting in an amino acid substitution of threonine for methionine at position 235 (M235T), has also been associated with essential hypertension [5, 6]. These observations are of particular relevance in renal disease as the RAS is now firmly established as an important factor in progression of renal damage and development of end-stage renal disease. However, while the renin-angiotensin system is believed to be a pivotal mechanism in this destructive process, and manipulating the activity of this system is a major target for treatment, little information exists regarding the potential importance of genetic variation of the RAS as a determinant of progressive renal damage.

This potential impact of RAS genetic variations is of particular interest in diseases with a highly variable outcome, with incomplete understanding of risk factors for progression. IgA nephropathy, the most common primary glomerulopathy in many areas of the world, was believed to be a benign process when initially described by Berger and Hinglais in 1968 [7]. However, IgA nephropathy is now recognized to follow a variable course, with progressive loss of renal function occurring in up to 40% of patients [8–10]. Currently, risk factors recognized to predict poor

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outcome include hypertension, heavy proteinuria, and abnormal renal function at the time of presentation. However, each of these prognosticators reflects already existing and/or progressive renal damage. The current study was designed to determine whether genetic polymorphisms within the renin angiotensin system, which exhibit association with cardiovascular diseases, are also associated with progressive renal deterioration independent of the established clinical parameters which portend a poor outcome in this setting.

Methods

Study subjects

Only Caucasian patients with biopsy-proven IgA nephropathy were included in the study ($N = 83$). African Americans, who are known to have different ACE I/D and ATR-C frequencies compared with Caucasians (higher ACE D allele, lower ATR C allele [11, 12]) were excluded. Blood samples were obtained from patients who were consecutively recruited from our clinics between 1987 to 1991 to participate in a study of immune regulation in IgA nephropathy and tissue samples were obtained from newly cut tissue blocks from patients who had undergone renal biopsy for routine diagnosis. Only renal tissue preserved in paraffin block form was utilized to avoid DNA contamination (see below). Patients gave their written consent and the study was approved by the appropriate hospital ethics committee. Diagnosis of IgA nephropathy was based on established pathological criteria, including mesangial expansion and the diagnostic presence of IgA as the sole, predominant or co-dominant immunoglobulin [13]. Patients with other disease entities who had mesangial IgA deposition were excluded as were patients with insufficient clinical data ($N = 19$). Genotypes were thus evaluated in 64 patients (43 males and 21 females) who were aged 6 to 83 years at renal biopsy.

Follow up was obtained from the referring nephrologists. Since treatment was not standardized, the potential impact of treatment on patient outcome could not be evaluated. Hypertension was defined as systolic or diastolic blood pressure above 95th percentile for age [14]. Heavy proteinuria was defined as the presence of urinary protein excretion > 3.0 g/24 hr, or > 50 mg/kg/day, at any time during the follow-up period. Values of 24 hour collections for determination of proteinuria was available in 60 of the 64 patients.

Groups

First, all the individuals ($N = 64$) whose serum creatinine (Cr) at last visit was < 1.5 mg/dl, were compared with those whose last Cr was > 1.5 mg/dL. The average duration of follow-up in this group was 6.8 ± 0.6 years (range 1 month to 28.4 years, median 7.5 years). A subgroup of patients included only individuals whose creatinine at presentation was < 1.5 mg/dl ($N = 41$). These patients were subdivided into those who maintained a normal Cr throughout the follow-up period (< 1.5 mg/dl) and compared with those who developed progressive renal dysfunction. The average duration of follow-up for this group was 7.4 ± 0.8 years (range 1 to 15.3 years, median 8.3 years). A separate analysis was made in patients with normal initial Cr who did not develop known prognosticators of poor outcome in IgA nephropathy, namely, moderate or severe hypertension (defined by The Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure), or heavy proteinuria (> 3.0 g/24 hr) at any time during the follow up period ($N = 20$). The average time of follow up in this group was 6.9 ± 1.0 years (range 1 to 13 years, median 8.5 years).

DNA isolation from blood and tissue

Nuclear DNA was isolated from peripheral leukocytes in whole blood samples as previously described by us [15]. The concentration of DNA in each sample was determined by optical density of the purified DNA at 260 nM. Tissue sections (5 micron thickness) were cut for DNA isolation from formalin-fixed, paraffin-embedded renal biopsy specimens. Microtome surfaces were cleaned with 0.2 M NaOH prior to sectioning of each biopsy to eliminate DNA contamination. Tissue sections were deparaffinized with xylene, then washed with 100% ethanol. Resulting tissue samples were then incubated overnight at 55°C in 50 ml of a mixture of 0.05 M Tris, pH 8.3, 0.001 M EDTA, and 0.5% polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma Chemical Co., St. Louis, MO, USA) and 0.4 mg Proteinase K (Sigma Chemical). After digestion, samples were heated to 95°C for eight minutes to arrest proteinase activity.

Determination of ACE genotypes

PCR amplification to detect ACE I/D polymorphism was carried out in a 50 ml mixture of 1 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 mM deoxynucleotide triphosphates, 0.48 mM primers, and 1 U *Taq* polymerase (Promega, Madison, WI, USA), and 25 ng genomic DNA using the previously published primers, 5′-CTGGAGACCACTCCCATC-CTTTCT-3′ and 5′-GATGTGGCCATCACATTCGTCAGAT-3′. Amplification with this primer pair produces ~490 bp and ~190 bp products corresponding to the I and D alleles, respectively [16–18]. Thermocycling, performed with a PTC-100 apparatus in all PCR reactions (MJ Research, Watertown, MA, USA), consisted of denaturation at 94°C for one minute, annealing at 63°C for one minute, and extension at 72°C for two minutes for 30 cycles, followed by a final extension at 72°C for five minutes. Ten milliliters of PCR product were mixed with 2 ml of glycerol based loading buffer, electrically size fractionated in 0.8% agarose gel containing 1 mg/ml ethidium bromide, and visualized by ultraviolet transillumination (Fig. 1).

In view of preferential amplification of the D allele and inefficiency in amplification of the I allele, mistyping of ID heterozygotes as D homozygotes is possible [19]. To eliminate this possibility, all samples found to be DD after amplification with the above conventional primers were re-amplified with a recently reported insertion-specific primer pair which recognizes the inserted sequence, 5′TGGGACCACAGCGCCCGCCACTAC3′ and 5′TCGCCAGCCCTCCCATGCCCATAA3′ [20]. Briefly, insertion-specific amplification was performed in a 50 ml mixture of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 250 mM deoxynucleotide triphosphates, 0.4 mM primers, 3 U *Taq* polymerase, and 100 ng genomic DNA. Thermocycling consisted of 40 cycles of denaturation at 94°C for one minute, and annealing/extension at 78°C for one minute, followed by final extension at 72°C for 10 minutes. Eleven percent of the samples assigned the DD genotype by the initial screen were positive for the insertion by the additional insertion-specific method and were thus classified as ID. This rate of false positives is similar to the 10% recently found by other investigators [18].

Determination of angiotensinogen M235T genotype

Amplification of a 303 bp sequence encompassing the M235T point mutation was performed in a 50 ml mixture of 0.83 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 125 mM deoxynucleotide triphosphates, 1 unit *Taq* polymerase, 1 mg genomic DNA, and 1.3 mM of each of the primers 5′-GATGCGCACAAGGTCCTGTC-3′ and 5′-CAGGGTGTCCA-CACTGGGTCGC-3′ [6]. Thermocycling consisted of initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for one minute, annealing at 66°C for one minute, and extension at 72°C for one minute. A final extension period at 72°C for two minutes followed. The most 3′ guanosine residue in the latter primer, not matching genomic DNA, creates a SfaNI recognition site during amplification in the presence of the M235, but not 235T variant. Restriction endonuclease digestion of the resulting PCR product with SfaNI (New England Biolabs, Beverly, MA, USA) at 37°C was thus used for mutation detection. Digestion products were separated in 10% polyacrylamide TBE Ready Gel (Bio Rad, Hercules, CA, USA). For DNA visualization, completed gels were soaked in 0.8% ethidium bromide for one hour and ultraviolet transilluminated.

Determination of angiotensin II receptor type I A1166C genotype

An 856 bp sequence encompassing the A1166C polymorphism was amplified in a 50 ml mixture of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, 250 mM deoxynucleotide triphosphates, 3 U *Taq* polymerase, and 0.5 mg genomic DNA, and 0.4 mM of each of the following primers: 5′-AATGCTTGTAGCCAAAGTCACCT-3′ and 5′-GGCTTTGCTTTGTCTTGTTG-3′ [4]. ATR genotyping was done in blood and not tissue samples due to difficulties in reliably attaining the appropriate amplicon. Previous studies describe difficulties in attaining PCR amplification from tissues of DNA fragments which are in excess of 400 to 500 bps [21]. Thermocycling consisted of an initial denaturation of 94°C for two minutes, 40 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute, extension at 72°C for two minutes, and a final extension time of 72°C for 10 minutes. The 1166 C allele contains an additional recognition site for the restriction endonuclease DdE that is absent in the A 1166 allele. Accordingly, PCR amplification products were digested at 37°C with DdE I (Promega, Madison, WI, USA) and electrically separated on a 2% agarose ethidium bromide gel.

Sequencing the ACE insert fragment

To further characterize the ACE inserted fragment, we determined the sequence of PCR products in II, ID and DD individuals. The PCR products were initially cloned into a TA cloning vector (In vitrogen, San Diego, CA, USA), and the nucleotide sequence of the fragment was determined by the method of Sanger, using a sequenase kit (Sequenase, U.S. Biochem. Corp., Cleveland, OH, USA).

Statistics

Clinical parameters are reported as mean ± standard error. Statistical analyses were performed on the actual number of genotypes/alleles and not on the relative percent levels. Subgroup comparisons were made by Fisher's exact test. Possible interaction among clinical parameters was assessed by logistic regression by a logistic model (Poisson). Contingency analysis was applied to evaluate possible correlation amongst the genotypes.

Table 1. Genotype/allele frequencies for renin-angiotensin system polymorphisms in IgA nephropathy

		All patients		Nl initial Cr		Nl initial Cr No HTN/Uprot	
		Non-progress	Progress	Non-progress	Progress	Non-progress	Progress
N		24	40	23	18	15	5
ACE I/D genotype %	II	38	20	35	22[a]	53	0[b]
	ID	58	60	61	44	47	40
	DD	4	20	4	33	0	60
Agt M235T genotype %	MM	35	31	41	38	43	20
	MT	52	61	45	62	43	80
	TT	13	8	14	0	14	0
ATR A1166C genotype %	AA	35	61	32	63	14	80[a]
	AC	52	31	54	25	64	20
	CC	13	8	14	12	21	0

Frequencies are given as percent. Abbreviations are: Nl initial Cr, patients with normal serum creatinine at presentation; Nl initial Cr, No HTN/Uprot, patients with normal serum creatinine at presentation and no hypertension or heavy proteinuria at any time.
[a] $P \leq 0.05$, genotype distribution in non-progressors vs. progressors
[b] $P < 0.005$ genotype distribution in non-progressors vs. progressors

Results

Polymorphisms of ACE gene

In this population of American Caucasians with IgA nephropathy the ACE I/D, AtgM235T and ATR A1166C genotype frequencies were II:27, ID:59, and DD:14%; MM:32, MT:58, and TT:10%; AA:51, AC:39, and CC:10%, respectively (Table 1). The genotype frequencies for angiotensinogen and ATR polymorphisms in this population with renal disease approximate those reported for other Caucasian populations where only 10 to 20% are homozygous for the mutated allele [4, 22]. The ACE DD genotype frequency in the current cohort appears lower than the 25 to 30% reported for other Caucasian groups [2, 23]. However, it is important to note that the current 14% may be closer to the actual frequency of the DD genotype in the general population. This is because the newest methodologies for determining ACE genotype overcome the inherent inefficiency of I allele amplification in heterozygotes, which likely overestimated the DD genotype frequency in previous analyses (Fig. 1). Indeed, insertion specific PCR amplification reclassified 11% of DD genotypes as having an insertion and thus as actually being ID. These findings impact on the potential contribution of genetic polymorphism on disease incidence. In this population, no skewing of genotype frequencies toward the deleterious genotypes was detected, thereby reiterating that occurrence of IgA nephropathy *per se* is not influenced by polymorphism in these RAS genes.

To evaluate whether genetic polymorphism in RAS affects the course of IgA nephropathy, we looked for genetic differences in all 64 patients by comparing patients who, at last clinical evaluation, had normal versus those with abnormal serum Cr (average, 1.1 ± 0.1 mg/dl vs. 6.1 ± 0.7 mg/dl, $P < 0.05$). In those patients whose most recent Cr was, by definition, < 1.5 mg/dl, the ACE genotype distribution was 38, 58, and 4% for II, ID, and DD,

Table 2. Patient characteristics at presentation and outcome

All patients	(+) HTN	(+) Heavy proteinuria	(+) HTN & heavy proteinuria
64 (38)	28 (21)	13 (7)	4 (1)

Parentheses indicate number that progressed.

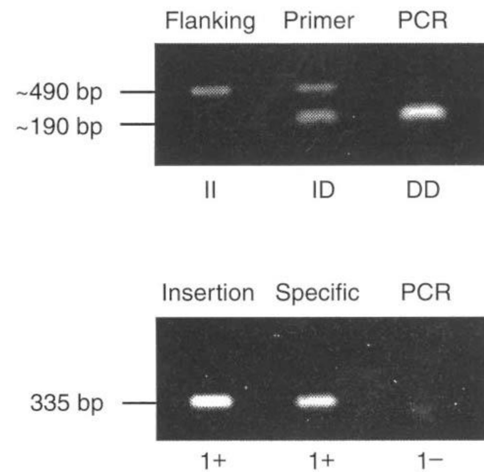

Fig. 1. *Ethidium bromide gel for PCR which used the outside flanking primers for ACE I/D polymorphism (top).* The longer band represents the I allele (~490), while the smaller band represents the D allele (~190). Ethidium bromide gel for PCR products using the insertion-specific PCR, which yields a 335 base pair amplicon (bottom).

respectively, and was not different than those with elevated Cr at last visit, where the distribution was 20, 60, and 20% (P = NS, non-progressors vs. progressors; Table 1). The allele frequency was also not different between progressors and non-progressors (I/D:67/33 vs. 50/50; P = NS, non-progressors vs. progressors). Thus, evaluation at last visit revealed no differences in the ACE genotype frequencies between patients who maintained normal Cr and those with renal dysfunction.

However, this type of analysis encompasses not only genetic predisposition but a variety of risk factors for progressive damage, including renal dysfunction at the time of presentation. Clinical parameters and outcome are given in Table 2. To determine whether outcome could be predicted at the time of presentation, we then analyzed only patients who presented with a normal creatinine ($N = 41$). In patients who presented with and maintained normal creatinine (0.9 ± 0.1 vs. 1.1 ± 0.1 mg/dl, P = NS) throughout follow-up, the ACE genotypes were 35, 61, and 4%. This distribution was different in those who progressed (Cr changed from 1.2 ± 0.0 to 4.5 ± 0.9 mg/dl, $P < 0.05$ where ACE genotypes were 22, 44 and 33% for II, ID and DD genotypes; $P = 0.057$ by Fisher's exact test, non-progressors vs. progressors; Table 1, Fig. 2). A predominance of the D allele was also observed in those with progression (I/D:65/35 vs. 44/56, $P = 0.07$ by Fisher's exact test, non-progressors vs. progressors). To further distinguish ACE D allele as a risk factor for progression, patients with known prognosticators of poor outcome in IgA nephropathy were excluded and the genotype frequency reassessed in the remaining population ($N = 20$). After exclusion of patients with hypertension or significant proteinuria, the association of the DD genotype as well as the D allele with progressive decline in renal function was even more striking. Thus, in patients with normal initial Cr who at no time developed hypertension or heavy proteinuria the ACE genotype frequencies were 53, 47, and 0% for II, ID and DD (Cr of 0.89 ± 0.06 mg/dl at presentation was 0.99 ± 0.06 at last visit, P = NS). By contrast, in patients with normal initial renal function and no hypertension or heavy proteinuria whose renal function declined, ACE genotype frequencies were 0, 40, and 60% for II, ID, DD ($P = 0.009$ by Fisher's exact test, non-progressors vs. progressors) (Fig. 2). Logistic model analysis revealed that the predictive value of the ACE genotype is not significantly modified by the addition of hypertension or proteinuria. Thus, patients without hypertension who have the DD genotype are 7.5 times more likely to develop progressive deterioration in renal function than patients who do not have this genotype. In addition, this analysis shows that genotype is an independent risk factor for renal progression after adjusting for proteinuria. This analysis revealed that the ACE genotype is an independent risk (incidence relative risk, IRR = 7.499821, with a 95% confidence interval of 1.25318 to 44.88365, $P = 0.027$). Proteinuria is also an independent variable after adjusting for the effect of ACE genotype (IRR of 13.22625 and 95% confidence interval of 1.199311 to 145.8618, $P = 0.035$). These additional analyses further strengthen our conclusion that ACE genotype impacts progression independently of these previously identified parameters of proteinuria and hypertension. Analysis of covariance revealed that ACE genotype was a prognosticator independent of proteinuria and hypertension, although hypertension at presentation was associated with increased creatinine throughout the course. No other clinical prognosticators were found. In those with progressive deterioration of renal function, Cr increased from 1.2 ± 0.1 mg/dl at presentation to 1.8 ± 0.3 at last follow-up ($P < 0.05$). Allele ratio for the non-progressors was I/D:77/23. An inversion of the allele frequency, with a D predominance was observed in those who progressed I/D:20/80, ($P = 0.002$ by Fisher's exact test, non-progressors vs. progressors). ACE genotype did not correlate with specific polymorphisms of the other RAS genes examined.

Sequence of the 'inserted' segment in the ACE gene

To gain further insight into the potential significance of ACE I/D alleles, we sequenced the polymorphic locus. Shown in Figure 3 is the I allele sequence. Presence or absence of the 287 bp fragment was the sole difference between the I and the D amplicons. The insertion matched the fragment described and registered into GenBank/EMBL Data Bank by Soubrier [16]. This insert has homology with the family of human alu repeat sequences. Interestingly, examination revealed a 14 base pair repeat sequence, 5′ATACAGTCACTTTT 3′ (boxed in Fig. 3). This sequence begins the insert, while the repeat abuts the end of the inserted fragment, immediately outside the insert.

Polymorphisms of the angiotensinogen gene

When these same analyses were performed with respect to the angiotensinogen M235T mutation, no genotype or allele frequency differences were identified between groups. Thus, in patients who, at the last follow-up visit, had normal Cr, genotype frequencies were 35, 52, and 13% versus 31, 61 and 8% for MM, MT, and TT, respectively in those with elevated Cr at last visit (P = NS). For those who presented with and maintained stable renal function throughout follow-up, genotype frequencies were 41, 45

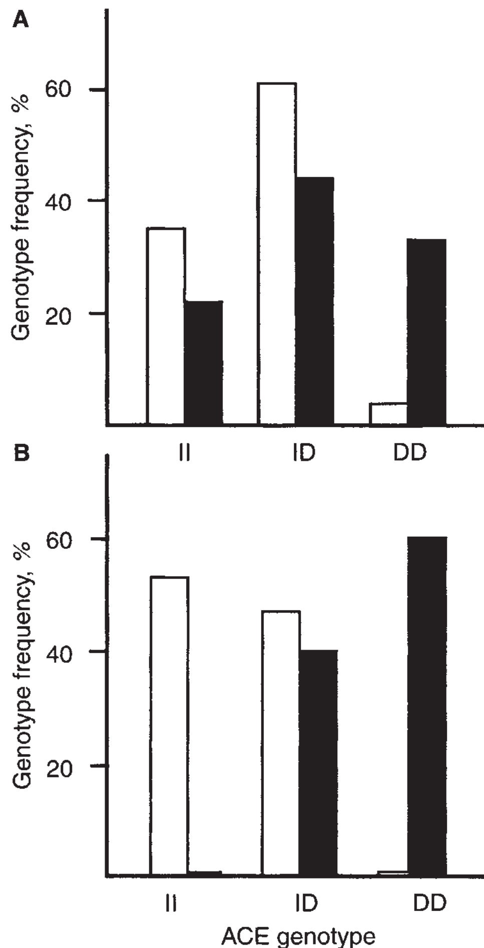

Fig. 2. *(A) Frequency of ACE genotypes in patients who maintained stable renal function (< 1.5 mg/dl, □) and those who developed progressive renal dysfunction (■).* (B) Frequency of ACE genotypes in patients without hypertension or heavy proteinuria who maintained stable renal function (□) and those who developed progressive renal dysfunction (■). Note, no II patient in this group progressed, while no DD patient remained stable.

and 14% versus 38, 62, and 0% for MM, MT and TT, respectively, in those who presented with normal Cr but experienced progressive decline in renal function (NS). Genotype frequencies for individuals with initially normal Cr without hypertension or heavy proteinuria were 43, 43, and 14% versus 20, 80, and 0% for MM, MT and TT, respectively, in those with initially normal Cr without hypertension or heavy proteinuria who progressed (NS). Similarly, no differences in the M or T allele frequencies were detected between those who did poorly and those who maintained normal function.

Polymorphisms of the angiotensin type 1 receptor gene

Analysis of genotype and allele frequencies were likewise performed with respect to the angiotensin II type I receptor A1166C polymorphism. Genotype frequencies for patients with normal versus elevated last Cr were not different, 35, 52, and 13% versus 61, 31, and 8%, respectively, for AA, AC and CC (NS). Similarly, there were no differences in the genotype frequencies between non-progressors and progressors who began with normal Cr, 32, 54, and 13% versus 63, 25, and 12%, respectively, for AA, AC and CC (NS). By contrast, analysis of individuals after exclusion of hypertension or proteinuria revealed an excess of the C allele in those with normal renal function who maintained normal Cr. Thus, the genotype distribution for these patients was 14, 64, and 21%. In patients with initially normal Cr without hypertension or heavy proteinuria who progressed, this frequency was different, 80, 20, and 0%, respectively, for AA, AC and CC.

Discussion

The study shows that in a cohort of American Caucasians with biopsy-proven IgA nephropathy, the distribution of genotypes for the ACE I/D polymorphism is remarkable for an increased frequency of the DD genotype and the D allele in those patients who ultimately experienced progressive decline in renal function during follow-up compared to those whose function remained stable over the same time. In contrast to the ACE I/D findings, the 235T mutation of the angiotensinogen gene was not associated with progression. Finally, the C allele of the A 1166C variant of angiotensin type I receptor, which has previously been associated with myocardial infarction, displaying synergy with the deleterious effects of the ACE DD genotype, was rather significantly underrepresented in our patients who progressed. Thus, the C allele did not appear to confer additional risk for poor outcome in patients with IgA nephropathy.

That abnormal genes may cause disease is now firmly established. Less fixed is the concept that genetic background may also affect progression of a disease, although support for such a notion has recently emerged. Thus, a variant of the interleukin-1 receptor antagonist gene is associated with increased severity in several immune mediated diseases such as systemic lupus erythematosus, alopecia areata, and inflammatory bowel disease [24–26]. In the current study, ACE I/D polymorphism was not predictive of incidence of IgA nephropathy. Indeed, the frequency of the DD genotype in this cohort was somewhat lower than that reported for other Caucasian groups, likely reflecting more specific PCR technique in the current study. However, the ACE gene DD genotype was significantly more frequent in IgA patients with progressive deterioration in renal function, supporting the idea that genetic background impacts disease outcome. Notably, exclusion of patients with known risk factors for progression, such as hypertension and significant proteinuria, strengthened the association of the DD genotype and progression of renal dysfunction in IgA nephropathy. The association between DD genotype and outcome in another setting, namely, cardiovascular disease, was also strengthened by the exclusion of known risk factors. Schunkert et al demonstrated that the association of the D allele with left ventricular hypertrophy was stronger in normotensive than hypertensive patients, reiterating the idea of ACE I/D polymorphism as a predictor of outcome, independent of known prognosticators of cardiac hypertrophy [2]. Similarly, the ACE DD genotype was a particularly potent predictor of myocardial infarction in middle aged men otherwise considered to be at low risk [1].

The impact of ACE I/D polymorphism in IgA nephropathy has been shown beyond the current population. Yoshida et al investigated the consequences of the ACE polymorphism in IgA nephropathy patients in Japan, which has the highest frequency of IgA nephropathy in the world [18]. As in the current study, significantly higher frequency of DD was present in patients with declining renal function. Moreover, excess D allele was also seen

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CTGGAGACCA  CTCCCATCCT  TTCTCCCATT  TCTCTAGACC  TGCTGCCTAT
ACAGTCACTT  TTTTTTTTTT  TTTGAGACGG  AGTCTCGCTC  TGTCGCCCAG
GCTGGAGTGC  AGTGGCGGGA  TCTCGGCTCA  CTGCAAGCTC  CGCCTCCCGG
TTCACGCCAT  TCTCCTGCCT  CAGCCTCCCA  AGTAGCTGGG  ACCACAGCGC
CCGCCACTAC  GCCCGGCTAA  TTTTTTGTAT  TTTTAGTAGA  GACGGGGTTT
CACCGTTTTA  GCCGGGATGG  TCTCGATCTC  CTGACCTCGT  GATCCGCCCG
CCTCGGCCTC  CCAAAGTGCT  GGGATTACAG  GCGTGATACA  GTCACTTTTA
TGTGGTTTCG  CCAATTTTAT  TCCAGCTCTG  AAATTCTCTG  AGCTCCCCTT
ACAAGCAGAG  GTGAGCTAAG  GGCTGGAGCT  CAAGCCATTC  AACCCCCTAC
CAGATCTGAC  GAATGTGATG  GCCACATC
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Fig. 3. *Inserted segment sequence.* The beginning and end of the 287 base pair insert are indicated by the double arrows. Repeat sequences are boxed; one begins the insert, while the other abuts the end of the inserted fragment.

in subanalysis of only normotensives who progressed, and was more apparent when patients without hypertension or impaired renal function at presentation were analyzed. Another recent report of IgA patients from Great Britain also found significant association of the DD genotype with progressive decline in renal function in IgA nephropathy [27]. Taken together, these observations suggest that while environmental and other genetic factors may contribute, similar ACE polymorphism distributions predict progressive IgA nephropathy in genetically distinct and geographically remote populations. These data point to a significant functional role for the deletion allele of the ACE polymorphism. Although the DD genotype is associated with poor outcome in a variety of settings, it is also possible that it is the corresponding decrease of the beneficial effects of the I allele, rather than the presence of the D allele, which impacts this finding. The decreased ACE levels seen in II genotype patients lends credence to this supposition [28].

Scrutiny of the sequence of ACE intron 16 encompassing the I/D segment revealed a repeated sequence, comprising 14 terminal bases of one end of the 'insert' and flanking the opposite end, comprising the next 14 bases which follow the insert in Figure 3. The arrangement of these two repeats suggests a possible origin of the D allele. During meiosis, one of these two repeats could align with the complement of the other, thus producing a 'loop-out' of the intervening 287 bp fragment. This observation suggests that the nature of the mutational event may actually be deletion rather than insertion. Interesting in this regard, is the positive correlation of circulating ACE levels with increasing preponderance of the D allele in normal Caucasians [28]. That is, those with the II genotype have the lowest serum ACE levels, DD subjects have the highest, and those with the ID genotype have intermediate levels, suggesting that the D allele contributes to the activation of ACE. This suggests that the 'insert' may contain a silencer motif, the absence of which activates the ACE gene. Of note, remarkable homology between a 13 base pair sequence of the ACE insertion fragment and a negative regulatory element in the renin *Ren-1* suggests possible broad implications of this sequence as a potential silencer motif in the regulation of RAS activity [29]. Further mechanistic potential of the ACE polymorphism comes from the observation of increased systemic pressor responsiveness to infusion of angiotensin I in normotensive men with the DD genotype when compared with II genotypes [30]. The plasma concentrations of angiotensin II were also higher across the dose range of angiotensin I infusion implying increased generation of angiotensin II in the DD individuals. Nonetheless, it is also possible that the identified polymorphic locus, while affecting ACE levels, may only be a marker for another gene variant which more directly contributes to renal functional deterioration.

The RAS affects progression in many settings [31, 32]. Further support for the role of RAS comes from observations of superiority of ACEI over other antihypertensive therapy in lessening renal damage and decreasing cardiac hypertrophy [33–35]. Of note, serum ACE levels are elevated in microalbuminuric diabetic patients who are likely to develop progressive renal loss [36, 37]. Further, interorgan variability in synthesis/availability of angiotensin II has been shown in animals, with plasma versus kidney Ang II/Ang I ratios, 0.3 versus ~ 2.0, indicating more ready conversion of available Ang I to Ang II in the kidney [38]. It is possible therefore, that the ACE I/D polymorphism may be particularly relevant at the tissue level, such that individuals with the DD genotype may, via enhanced Ang II activity, demonstrate even more striking renal Ang II predominance than the other genotypes.

In summary, these data indicate that among the three genes of the renin angiotensin system studied, only angiotensin converting enzyme, but not angiotensinogen or angiotensin type 1 receptor gene polymorphism was associated with progressive renal deterioration in patients with IgA nephropathy. This association was not predictive of the risk for acquiring IgA nephropathy, but instead was linked to progressive decline in renal function in patients presenting with normal creatinine. Notably, this association became even more significant when other known prognosticators were eliminated, suggesting that ACE polymorphism is an independent marker of poor outcome in this setting. These findings are consistent with the emerging role of angiotensin converting enzyme in modulating the slow, progressive destruction observed in many renal diseases. The exact mechanisms whereby this genetic polymorphism impacts the progression remain to be elucidated, although the implications of the described mutational loop-out of the ACE I/D are intriguing.

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