

ACE gene sequence and nucleotide variants in IgA nephropathy

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ABSTRACT

Introduction: Association studies with single nucleotide polymorphisms (SNPs) have been contradictory. Haplotypes may be more helpful. With gene sequencing, all SNPs can be found for construction of haplotypes.

Methods: The ACE gene was sequenced in four healthy Chinese subjects and 20 patients with IgA nephropathy (IgAN) to observe if differences exist among SNPs and haplotypes. 20 patients on angiotensin 1-converting enzyme inhibitor/angiotensin receptor antagonist (ACEI/ATRA) therapy were then compared with another 20 patients not treated with ACEI/ATRA to determine their renal outcome in response to ACEI/ATRA therapy and whether their genetic profile of ACE gene could play a role in determining their outcome to ACEI/ATRA therapy and progression to end-stage renal failure (ESRF).

Results: IgAN patients had 53 variants, of which 17 were unique, whereas normal subjects had 38 variants, of which two were unique (p less than 0.005). No unique variant was a significant risk factor for IgAN. Significant genotype and allele frequency differences in five variants were observed between IgAN patients with renal impairment and those with ESRF (p less than 0.02).

Conclusion: Our data suggests that at least in the ACE gene, haplotyping SNPs within a single gene seems to have no added advantage over genotyping the individual component SNPs. The D allele and haplotype 3 confer an adverse prognosis, while the I allele and haplotype 5 appear to be renoprotective. The data suggests that genotypes of the ACE gene are linked to certain haplotypes, which could influence IgAN patients' response to ACEI/ATRA therapy.

Keywords: haplotypes, IgA nephropathy, renal failure, single nucleotide polymorphism

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INTRODUCTION

IgA nephropathy (IgAN) is a complex human disease characterised by mesangial cell proliferation, mesangial deposition of IgA immune-complexes, proteinuria and haematuria. It is also the most common form of primary glomerulonephritis in Singapore and in many parts of the world,⁽¹⁻³⁾ contributing significantly to the population of patients with end-stage renal failure (ESRF) requiring expensive and life-long dialysis. First described by Berger and by Hinglais,⁽⁴⁾ the aetiology and pathogenetic mechanism of IgAN is still poorly understood today after more than three decades of studies.

In 1986, Anderson et al demonstrated that an angiotensin 1-converting enzyme inhibitor (ACEI) can reduce proteinuria and limit glomerular damage in rats with experimental reduction of renal mass.⁽⁵⁾ Since then, there have been numerous reports of ACEI therapy for retarding progression of renal failure in patients with various renal diseases,^(6,7) including IgAN.⁽⁸⁾ Another important discovery in ACE was the insertion/deletion (ID) polymorphism, which seems to account for half the variance of serum enzyme level in rats.⁽⁹⁾ This deletion polymorphism was subsequently shown to be a potent risk factor for myocardial infarction and left ventricular hypertrophy.⁽¹⁰⁾ The DD genotype has also been shown to be associated with the progression of diabetic and non-diabetic nephropathies.^(11,12) In IgAN, the ACE ID polymorphism and other single nucleotide polymorphisms (SNPs) in *angiotensinogen (M235T)* and *angiotensin type 1 receptor (A1166C)* have also been studied,^(3,8,11,13) but the results have been conflicting.

Recently, Narita et al reported that certain haplotypes (blocks of more than one SNP inherited together on the same chromosome) of the angiotensinogen gene can influence the therapeutic efficacy of renin-angiotensin system blockade in IgAN.⁽¹³⁾ It seems possible that more than one SNP of a haplotype acting in synergy may be a better predictor than the individual SNP.⁽¹⁴⁾ Nucleotide sequencing of a gene may reveal all available SNPs for the construction of haplotypes.

In the first part of this study, we determined the entire genomic sequence of the ACE gene (about 24,000 base pairs) in 24 Chinese subjects (20 patients with IgAN and

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four healthy controls). All identified nucleotide variants and haplotype constructs were tested for predicting a disposition to IgA nephropathy and for prognosticating disease progression to ESRF. In the second part of the study, 20 patients in the treatment group on ACEI/angiotensin receptor antagonist (ATRA) therapy were compared with another 20 patients who were not treated with ACEI/ATRA (control group). The two cohorts of patients were followed up for five years in order to determine their renal outcome (normal renal function, renal impairment and ESRF) in response to ACEI/ATRA therapy and whether their genetic profile of *ACE* gene could play a role in determining their outcome to ACEI/ATRA therapy and their progression to ESRF.

METHODS

The principles in the methodology of the study first involved the sequencing of the *ACE* genome. After this, SNPs were identified from the sequencing and the nucleotide diversity was studied. Thereafter, haplotype construction was undertaken to characterise any haplotypes that could be peculiar to IgA nephropathy. The presence or absence of these haplotypes was then examined in a group of patients ($n = 40$) with IgA nephropathy to determine if they had any predictive or two prognostic values in relation to the disease progression and therapy.

It is more cost effective to do the complete gene sequence in a small number of patients in an initial study, and then proceed to determine specific SNPs of interest in larger numbers using the rapid technique of Taqman[®] real-time polymerase chain reaction (PCR) (Applied Biosystems, Foster City, CA, USA) for allele discrimination. There is a higher chance of detecting rare functional variants (mutations) in diseased individuals than in healthy ones. Therefore, we enlisted 20 patients with IgAN and four healthy normals for the study. All the 24 subjects were Chinese. The 20 patients were the first from a cohort of 100 patients who formed the treatment group of a cohort of IgAN patients enrolled for a controlled clinical trial of ACEI/ATRA.

These 20 patients had their *ACE* gene sequenced to enable a study of nucleotide variants and haplotypes. Their *ACE* genes were genotyped in order to compare the effect of their genetic profile (SNPs and haplotypes) on response to ACEI/ATRA therapy. Entry criteria included proteinuria ≥ 1 g and/or renal impairment defined as serum creatinine > 1.6 mg/dl. All patients were advised a low salt diet. They were treated with ACEI/ATRA therapy and reviewed at six-monthly intervals. Patients were prescribed 5 mg enalapril (ACEI) or 50 mg losartan

(ATRA), which was increased to 10 mg or 100 mg, respectively, if proteinuria had not decreased to less than 1 g a day.

For the remaining 20 patients with IgA nephritis in the control group, the *ACE* gene was not sequenced, as this was a labour-intensive process. The respective nucleotides of patients in the control group were determined by Taqman[®] PCR, which was more readily performed. Control patients with hypertension were treated with atenolol, nifedipine or amlodipine. None of them were prescribed ACEI/ATRA. Routine tests for serum creatinine and proteinuria were performed at six-monthly intervals in all patients. All patients and the four normal healthy controls gave their informed consent to participate in the study, which was approved by the local institution review board and the hospital ethics committee.

Controlling for type I error of 5%, taking a power of 80% (i.e. type II error of 20%) and assuming the success would increase from 0% to 40% moving from the control to treatment groups, 16 patients would need to be recruited in each group. It was decided to recruit around 20 patients for each group to make up for patients who may drop out of the study.

For *ACE* Gene Sequencing, EDTA blood samples were collected from 20 IgA nephropathy and four unrelated local Chinese individuals for extraction of genomic DNA using the Qiagen QIAamp DNA blood extraction kit (<http://www.qiagen.com/>). Overlapping primer sets spanning the whole genomic DNA sequence of *ACE* were chosen on the basis of size and overlap of PCR amplicons. A total of 36 pairs of overlapping primers were designed to amplify 24 kb nucleotides, which covered the whole length of the *ACE* genomic DNA. The average size of the 36 PCR amplicons was 842 + 180 bp and the overlap length between the amplicons was 260 + 57 bp. The length of the primers was 21–28 bases and the primer annealing temperature was 53°C–68°C. Genomic DNA was subjected to PCR amplification carried out in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems).

The PCR reaction (50 μ l) consisted of 50 μ g DNA, 5 μ l 10X buffer, 0.4 μ M primer, 0.2 Mm dNTP and 1 unit Taq polymerase. The amplification programme consisted of an initial denaturation at 94°C for three minutes, followed by 35 cycles at 95°C for 45 seconds, 53°C–68°C for 45 seconds and 72°C for two minutes, with a final extension at 72°C for ten minutes. PCR amplicons were sequenced by using the BigDye Terminator Cycle Sequencing Kit and ABI 3100 Prism Automated DNA Sequencer (Applied Biosystems). Sequence data from every primer were aligned by using DNASTAR

software for visually inspecting the sequence variants. Furthermore, all variants were reconfirmed by additional PCR and sequencing. The complete gene sequence of the *ACE* gene consisted of 24,070 bp (base pairs).

Based on the sequence information of the seven SNPs, 300 bp DNA sequences flanking the SNPs point were submitted for Assays-by-Design Service (Applied Biosystems). Seven quality-controlled primer and probe sets for single nucleotide were then provided by Applied Biosystems. For every SNP, two TaqMan/MGB (a minor groove binder) probes for distinguishing between two alleles were designed. A report dye was situated at the 5'-end of each probe: VIC® dye label (Applied Biosystems) was linked to the 5'-end of Allele 1 probe and 6-FAM dye was linked to the 5'-end of the Allele 2 probe. A non-fluorescent quencher was placed at the 3'-end of the probe.

Optical 96 well reaction plates were used for the test. Firstly, 1 µl (10–50 ng) of each purified DNA sample was pipetted into the bottom of each well. The reaction master mixture was then pipetted into the wells, which included 5 µl of 2X TaqMan Universal PCR Master Mix (without AmpErase UNG), 0.25 µl of 40X working stock of SNP assay mix and 3.75 µl of Dnase-free water, with a total of reaction volume of 10 µl. The thermal cycling condition was 50°C for 2 minutes, 95°C for ten minutes and 40 cycles of denature and anneal/extend (95°C for 15 seconds and 60°C for one minute). Direct detection of PCR product with no downstream processing was accomplished within minutes of PCR completion, by measuring the increase in the fluorescence of dye-labeled DNA probes. This method permitted the analysis of large sample sizes with high sample-to-sample reproducibility.

After PCR amplification, endpoint plate read was performed. The SDS software (Applied Biosystems) calculated the fluorescence measurements made during the plate read and plotted Rn values based on the signals from each well. The presence or absence of the SNPs was detected in each sample. From the end point reading of fluorescence density for two probes with non template control, the homozygous or heterozygous alleles were clustered into three groups. The ABI Prism 7700 Sequence Detector (Applied Biosystems) generated multicomponented columns for no DNA, Alleles 1 and 2. The data was then normalised for each allele, and a genotype call was made for Allele 1 (homozygote 1), Allele 2 (homozygote 2) or Allele ½ (heterozygote).

The Statistical Package for the Social Sciences version 10.0 (SPSS Inc, Chicago, IL, USA) was used to calculate Pearson's chi square (χ^2) for comparing categorical data, student's *t*-test for calculating the significance of

difference between means of numeric data, and odds ratio for evaluating significance of difference in allele and haplotype frequencies between groups and correlation of two sets of coded values for genotyping. The Hardy-Weinberg (HW) principle states that gene and genotype frequencies remain constant between generations in an infinitely large, random mating population. Accordingly, the expected frequencies of genotypes (E_{AA} , E_{Aa} , E_{aa}) were given by p^2 , $2pq$ and q^2 . The allele proportions p and q were calculated from observed genotype frequencies ($p = [2O_{AA} + O_{Aa}]/2N$, $q = (2O_{aa} + O_{Aa})/2N$, where N is the total number of subjects studied). Departure from HW equilibrium was examined by the chi square test for goodness of fit between observed frequencies and calculated expected frequencies. Probability of deviation is looked up in a chi square distribution table at 1° of freedom for two alleles.⁽¹⁵⁾ Turet and Cambien⁽¹⁶⁾ had recommended that departure from HW equilibrium should be tested systemically in studies of association between genetic markers and disease. HW equilibrium should be demonstrated in the normal control group (not necessary in the case group) to confirm the genetic homogeneity of the study population and accuracy in the genotyping procedures. However, this test for HW equilibrium is not very sensitive and can only detect relatively large deviations.

The availability of *ACE* gene sequence data allows quantitative estimation of genetic variation at the nucleotide level. From the observed number of variant sites (S), the total length of nucleotide sequenced (L) and the number of chromosomes analysed (n), the level of nucleotide diversity may be normalised and estimated so that the degree of nucleotide diversity may be compared between gene regions, between different genes as well as between populations. The estimated θ equals the expected proportion of nucleotide polymorphic sites and is given by the following formula by Hartl and Clark:⁽¹⁷⁾

$$\theta = S / \sum_{i=1}^{n-1} i^{-1} L$$

Data obtained by sequencing genomic DNA from diploid individuals leads to ambiguity in assigning alleles to the right chromosome when there are two or more heterozygotic variants. In samples that are homozygotes or single-site heterozygotes, no ambiguity and haplotypes are established. In the remaining samples, efforts were then made to identify these known haplotypes by some combination of the ambiguous sites. Each time a new haplotype was found, it was added to the known pool of haplotypes to aid identification in samples yet unresolved. This inference process was continued until all the samples

Table I. Genotype and allele frequencies of variants in the ACE gene.

No.	Region	Nucleotide variants	Normal (n = 4)		IgAN (n = 20)	
			Genotype count	Allele 2 count	Genotype count	Allele 2 count
1	5' region	2400 A>T	3.1.0	1	7.9.4	17
2	5' region	2437 G>A	4.0.0	0	18.2.0	2
3	5' region	2547 T>C	3.1.0	1	7.9.4	17
4	Exon 1	2766 G>T	4.0.0	0	12.8.0	8
5	Exon 1	2801 A>G	4.0.0	0	19.1.0	1
6	Exon 1	2838 G>A	4.0.0	0	19.0.1	2
7	Intron 2	3872 G>A	0.1.3	7	5.8.7	22
8	Intron 2	4504 G>C	3.1.0	1	2.10.8	26
9	Exon 3	4633 C>T	4.0.0	0	18.2.0	2
10	Exon 4	5406 C>T	4.0.0	0	19.1.0	1
11	Intron 5	6435 A>G	0.1.3	7	5.8.7	22
12	Intron 7	7831 G>A	3.1.0	1	7.10.3	16
13	Exon 8	8128 C>T	0.1.3	7	4.9.7	23
14	Intron 9	8968 C>T	3.1.0	1	7.8.5	18
15	Intron 12	10305 G>A	4.0.0	0	18.2.0	2
16	Exon 13	10514 C>T ^a	0.1.3	7	5.7.8	23
17	Intron 13	10696 G>D ^a	3.1.0	1	8.7.5	17
18	Intron 13	10758 G>A ^a	0.1.3	7	5.7.8	23
19	Intron 14	10979 T>C ^a	0.1.3	7	5.7.8	23
20	Intron 14	11159 C>T	0.1.3	7	5.8.7	22
21	Intron 14	11376 A>G	0.1.3	7	5.6.9	24
22	Intron 14	11405 C>A	0.1.3	7	5.6.9	24
23	Intron 14	11447 G>A	0.1.3	7	8.4.8	20
24	Intron 14	11478 C>G ^a	0.1.3	7	5.7.8	23
25	Intron 14	11663 A>G ^a	0.1.3	7	5.7.8	23
26	Intron 14	11866 A>C ^a	0.1.3	7	5.7.8	23
27	Exon 15	12257 A>G	0.1.3	7	6.7.7	21
28	Intron 15	12429 A>G	4.0.0	0	16.4.0	4
29	Intron 15	12486 T>C	0.1.3	7	7.6.7	20
30	Intron 16	12727 T>C	0.1.3	7	5.8.7	22
31	Intron 17	12993 G>A	4.0.0	0	18.2.0	2
32	Intron 16	13145 A>C ^a	0.1.3	7	5.7.8	23
33	Intron 16	13230 A>G	3.1.0	1	9.6.5	22
34	Intron 16	13336 T>A ^a	0.1.3	7	5.7.8	23
35	Intron 16	13338 G>T ^a	0.1.3	7	5.7.8	23
36	Intron 16	13591 G>A	4.0.0	0	19.1.0	1
37	Intron 16	13730 A>T ^a	3.1.0	1	8.7.5	17
38	Intron 16 [†]	14094-381 I > D ^a	3.1.0	1	8.7.5	17
39	Intron 16	14480 G>C	1.0.3	6	20.0.0	0
40	Intron 16	14488 A>C	1.0.3	6	20.0.0	0
41	Exon 17	14521 A>G	3.0.1	2	9.5.6	17
42	Intron 18	15214 G>A	0.1.3	7	5.6.9	24
43	Intron 20	18222 G>A	0.1.3	7	6.8.6	20
44	Intron 20	18912 A>G	0.1.3	7	6.8.6	20
45	Intron 22	19942 C>T	4.0.0	0	19.1.0	1
46	Intron 23	20397 G>A	4.0.0	0	19.1.0	1
47	Intron 23	20833 C>T	3.1.0	1	5.9.6	21
48	Intron 23	21125 G>A	4.0.0	0	19.1.0	1
49	Intron 23	21148 G>D	4.0.0	0	19.1.0	1
50	Exon 24	22251 C>T	3.1.0	1	7.5.8	21
51	Intron 25	22933 C>T	4.0.0	0	19.1.0	1
52	Intron 25	22982 A>G	3.1.0	1	7.7.6	19
53	Exon 26	23162 A>T	4.0.0	0	19.1.0	1
54	3' region	23945-6 CT>D	3.1.0	1	8.5.7	19
55	3' region	23949-50 CT>D	4.0.0	0	19.0.1	2

^a Variant genotypes in absolute linkage with the Alu insertion/deletion (I/D) polymorphism; * common allele = 1, rare allele = 2; I 1.12.22 denotes common homozygote, heterozygote and rare homozygote; [†] location of (I/D) polymorphism

Table II. Nucleotide Diversity Index, q (mean + SE, x 10⁻⁴) in the ACE precursor gene.

Gene region	African-American (n = 10)*	European-American (n = 12)*	Normal (n = 8)	IgAN patients (n = 40)
Coding (4122 bp, 17%)	11.1 + 5.8 (S = 13)	5.6 + 3.2 (S = 7)	4.68 + 3.05 (S = 5)	6.27 + 2.66 (S = 11)
Noncoding (19,948 bp, 83%)	10.1 + 4.6 (S = 57)	6.1 + 2.7 (S = 37)	6.38 + 3.22 (S = 33)	4.95 + 1.67 (S = 42)
Overall (24,070 bp)	10.3 + 4.7 (S = 70)	6.1 + 2.7 (S = 44)	6.09 + 3.05 (S = 38)	15.18 + 1.70 (S = 53)

Note: q is calculated from the observed number of polymorphic sites (S) in the sequence length (L) and symbolises the proportion of nucleotide sites that are expected to be polymorphic in a sample of any number of chromosomes (n) from the region of the genome that is sequenced.

* Data from Rieder et al.⁽¹⁹⁾; n: number of chromosomes studied

Table III. ACE genes nucleotide variant, genotype and allele frequencies in association with renal functions.

No.	Region	Nucleotide variants Alu ID genotypes	Normal RF (n = 8)		ImpRF (n = 7)		ESRF (n = 5)	
			Genotype 11. 12. 22	Allele 2 freq (%)	Genotype 11. 12. 22	Allele 2 freq (%)	Genotype 11. 12. 22	Allele 2 freq (%)
1	5' region	2400 A>T	3.4.1	6 (38)	4.2.1	4 (29)	0.3.2	7 (70)
2	5' region	2547 T>C	3.4.1	6 (38)	4.2.1	4 (29)	0.3.2	7 (70)
3	Exon 1	2766 G>T	4.4.0	4 (25)	4.3.0	3 (21)	4.1.0	1 (10)
4	Intron 2	3872 G>A	1.4.3	10 (63)	2.1.4	9 (64)	2.3.0	3 (30)
5	Intron 2	4504 G>C	0.5.3	11 (69)	1.2.4	10 (71)	1.3.1	5 (50)
6	Intron 5	6435 A>G	1.4.3	10 (63)	2.1.4	9 (64)	2.3.0	3 (30)
7	Intron 7	7831 G>A	3.5.0	5 (31)	4.2.1	4 (29)	0.3.2	7 (70)
8	Exon 8	8128 C>T	0.5.3	11 (69)	2.1.4	9 (64)	2.3.0	3 (30)
9	Intron 9	8968 C>T	3.3.2	7 (44)	4.2.1	4 (29)	0.3.2	7 (70)
10	Intron 14	11159 C>T	2.3.3	9 (56)	1.2.4	10 (91)	2.3.0	3 (30)
11	Intron 14	11376 A>G	2.3.3	9 (56)	1.1.5	11 (79)	2.2.1	4 (40)
12	Intron 14	11405 C>A	2.3.3	9 (56)	1.1.5	11 (79)	2.2.1	4 (40)
13	Intron 14	11447 G>A	4.1.3	7 (44)	1.1.5 ^a	11 (79) ^f	3.2.0 ^a	2 (20) ^f
14	Exon 15	12257 A>G	2.3.3	9 (56)	2.1.4	9 (64)	2.3.0	3 (30)
15	Intron 15	12429 A>G	8.0.0	0 (0)	4.3.0	3 (21)	4.1.0	1 (10)
16	Intron 15	12486 T>C	2.3.3	9 (56)	2.1.4	9 (64)	3.2.0	2 (20)
17	Intron 16	12727 T>C	2.3.3	9 (56)	1.2.4	10 (91)	2.3.0	3 (30)
18	Intron 16	13230 A>G	3.3.2	7 (44)	6.0.1 ^b	2 (14) ^g	0.3.2 ^b	7 (70) ^g
19	Intron 16	14094-381 Alu>" "	3.3.2	7 (44)	5.1.1 ^c	3 (21) ^h	0.3.2 ^c	7 (70) ^h
20	Exon 17	14521 A>G	3.2.3.	8 (50)	6.0.1 ^d	2 (14) ⁱ	0.3.2 ^d	7 (70) ⁱ
21	Intron 18	15214 G>A	2.5.1	7 (44)	1.1.5 ^e	11 (79) ^j	2.3.0 ^e	3 (30) ^j
22	Intron 20	18222 G>A	2.3.3	9 (56)	2.2.3	8 (57)	2.3.0	3 (30)
23	Intron 20	18912 A>G	2.3.3	9 (56)	2.2.3	8 (57)	2.3.0	3 (30)
24	Intron 23	20833 C>T	3.3.2	7 (44)	2.3.2	7 (50)	0.3.2	7 (70)
25	Exon 24	22251 C>T	3.2.3	8 (50)	4.1.2	5 (36)	0.2.3	8 (80)
26	Intron 25	22982 A>G	4.2.2	6 (38)	3.2.2	6 (43)	0.3.2	7 (70)
27	3' region	23945-6 CT>" "	4.2.2	6 (38)	4.0.3	6 (43)	0.3.2	7 (70)

Note: Variants not in absolute linkage with the Alu variant and with counts of 3 or more of the rare allele were analysed.

Fisher's exact test: ^{a,c,e,h,j} p < 0.05; ^{b,d,f,g,i} p < 0.02

were haplotyped (Clark's inference algorithm.⁽¹⁸⁾) The frequency of each haplotype may then be tallied.

Alleles of different variants in random association are said to be in linkage equilibrium. Conversely, alleles that are inherited together are said to be in linkage disequilibrium (LD). From genotypic data, it is not

possible to determine exact values for LD. In studies of LD, gametic frequencies of alleles at two loci P_{11} , P_{12} and P_{21} , P_{22} can only be estimated using complex statistical procedures. However, an impression of the degree of linkage between alleles at the two loci may be gained by a simple correlation test. With common and rare alleles

Table IV. Significant-variants: association with renal function in haplotypes or individually.

	Nucleotide variants					Frequency count			p-value for odds ratio		
	11447 G>A	13230 A>G	Alu I/D	14521 A>G	15214 G>A	NRF (n = 16)	IRF (n = 14)	ESRF (n = 10)	NRF vs. IRF	NRF vs. ESRF	IRF vs. ESRF
Haplotype 1	1	1	1	1	2	2	0	1	ns	ns	ns
Haplotype 2	1	1	2	1	1	0	1	0	ns	ns	ns
Haplotype 3	1	2	2	2	1	6	2	7	ns	ns	14*
Haplotype 4	1	2	2	2	2	1	0	0	ns	ns	ns
Haplotype 5	2	1	1	1	2	6	11	2	6†	ns	0.07*
Haplotype 6	2	1	1	2	2	1	0	0	ns	ns	ns
SNP 11447 >A	2	-	-	-	-	7	11	2	ns	ns	0.07*
SNP 13230 >G	-	2	-	-	-	7	2	7	ns	ns	14*
Alu 14094-3811>""	-	-	2	-	-	7	3	7	ns	ns	8.6†
SNP 14521 >G	-	-	-	2	-	6	0	8	ns	ns	14*
SNP 15214 >A	-	-	-	-	2	10	11	3	ns	ns	0.12†

* p-value < 0.02; † p-value < 0.05

Abbreviations are: 1: common allele; 2: rare allele; n: chromosome number

NRF: normal renal function; IRF: impaired renal function; ESRF: end-stage renal failure; SNP: single nucleotide polymorphism

taking the value of 1 and 2, respectively, the sum values of 2, 3 and 4 for the common homozygote, heterozygote and rare homozygote, respectively at each locus may be correlated with similarly substituted values at the second locus.

RESULTS

The *ACE* gene was sequenced and genotyped in 20 IgAN patients and four normal subjects. Table I shows the total of 55 variants detected; 53 in the patients and 38 in the normal subjects. Among all the variants, 17 were unique to the patients, two were found only in the normal subjects and 36 were shared by both cohorts. Although the number of unique variants was significantly higher among patients than the normal subjects ($\chi^2 = 8.1$, $p < 0.005$), most unique variants had very low frequency (1–2) counts that did not contribute to significant association with the IgAN disease. HW equilibrium analysis showed no significant deviation in any of the variants among the four normal subjects. Between the two groups, there was also no significant difference in the genotype or allele frequencies in all the shared variants. Therefore, no new useful markers of IgAN were identified. Correlation statistics to estimate linkage between the Alu variant and each of the other variants showed 11 in absolute LD ($\rho = 1$ or -1). These absolutely linked variants were concentrated between exon 13 and the Alu variant site in intron 16, a span of about 4,000 bp (Table I).

Table II shows results of calculated nucleotide diversity index (θ). Overall, there was no significant difference in the coding or noncoding region between the normal subjects and the patients. There was also no

significant difference when compared to Rieder et al's data⁽⁹⁾ on five African-Americans and six European-Americans. Table III shows the genotype and allele frequencies of 25 variants, in which three or more counts of the rare allele among the 20 patients were studied (a frequency < 10% is too rare for statistical significance). In all 25 variants, there was no difference in genotype or allele frequencies between normal renal function (NRF) vs. impaired renal function (IRF) and NRF vs. ESRF. Between IRF and ESRF, the differences in genotype and allele counts were found only in five variants, namely 11447 G > A, 13230 A > G, 14094 Alu I > D, 14521 A > G and 15214 G > A, with p-values ranging from < 0.05 to < 0.02. These five variants were designated as significant-variants (or s-variants) for haplotype construction and further analysis.

Using Clark's inference algorithm,⁽¹⁸⁾ five-locus haplotypes were constructed with the five s-variants. Only six of the possible 32 haplotypes were found among the 40 chromosomes studied, as shown in Table IV. Two haplotypes were dominant: haplotype 3 (H3, 12221, 37.5%) and haplotype 5 (H5, 21112, 47.5%). H3 occurrence was 37.5%, 14% and 70% for the NRF, IRF and ESRF patients, respectively. The difference was significant only between IRF vs. ESRF patients (odds ratio [OR] 14.0, $p < 0.02$). H3 may be an indicator of risk for ESRF among the IRF patients. H5 occurrence was 37.5%, 78.6% and 20% for NRF, IRF and ESRF patients, respectively. The difference was significant in NRF vs. IRF (OR 6.0, $p < 0.05$) and IRF vs. ESRF (OR 0.07, $p < 0.02$). H5 could conversely be an indicator of protection from ESRF among the IRF patients. The OR for having

Table V. Clinical profile of the 20 patients with IgAN who were treated with ACEI/ATRA (treatment group) categorised into normal renal function, impaired renal function and end-stage renal failure.

No.	Age (yrs)	Gender	Hpt	Drug	Dosage (mg)	Trial (mths)	Initial Se creat (mg/dl)	Final Se creat (mg/dl)	Initial TUP (gm/day)	Final TUP (gm/day)	Alu I/D variant	Haplotype
Normal renal function												
1	47	M	Yes	At, ATRA	100,100	54	1.35	1.30	2.1	0.5	DD	3,3
2	46	F	No	ATRA	100	54	0.77	0.74	3.4	0.7	ID	1,3
3	35	F	No	ACEI, ATRA	5,50	60	0.92	1.33	2.3	0.9	ID	1,3
4	35	F	No	ATRA	50	60	0.93	0.80	1.1	0.2	II	5,5
5	27	M	Yes	ACEI, ATRA	5,100	66	1.71	1.49	1.5	0.3	ID	6,4
6	22	F	No	ATRA	100	60	0.88	0.84	3.1	0.3	II	5,5
7	46	F	Yes	Am, ATRA	5,100	66	1.64	1.53	2.4	1.1	DD	3,3
8	41	F	Yes	At, ATRA	100,100	54	0.97	0.77	2.1	0.1	II	5,5
Impaired renal function												
1	40	M	No	ATRA	100	65	1.71	2.38	3.3	0.5	II*	5,5 [†]
2	40	M	Yes	Am, ATRA	5,100	66	2.29	2.39	0.4	0.3	II	5,5
3	45	M	Yes	At, ACEI, ATRA	100,10,100	54	1.70	1.59	1.1	0.3	II	5,5
4	42	F	Yes	At, ATRA	100,100	60	1.99	2.58	2.1	1.0	II	5,5
5	18	M	Yes	Am, ATRA	10, 100	62	1.72	2.53	2.5	0.9	ID	2,5
6	41	F	No	ACEI, ATRA	5,100	60	1.36	1.75	2.6	1.4	DD	3,3
7	59	M	Yes	ACEI, ATRA	5, 100	60	1.80	1.92	1.5	0.4	II	5,5
End stage renal failure												
1	53	M	Yes	ACEI	20	65	2.00	5.67	2.6	2.2	DD*	3,3 [†]
2	43	F	Yes	ACEI, ATRA	10, 100	66	1.67	6.13	0.9	3.0	ID	5,3
3	46	F	Yes	Am, ATRA	5, 100	54	1.70	6.27	1.1	1.8	ID	5,3
4	36	F	Yes	Am, ACEI, ATRA	5, 10, 100	54	1.63	6.41	2.9	3.0	DD	3,3
5	44	M	No	At, ACEI	50, 15	60	1.71	5.88	1.5	0.7	ID	5,3

* versus * ($p < 0.05$); [†] versus [†] ($p < 0.01$)

At: atenolol; Am: amlodipine; ACEI: angiotensin I-converting enzyme inhibitor (enalapril); ATRA: angiotensin receptor antagonist (losartan); HPT: hypertension; TUP: total urinary protein; Se creat: serum creatinine

the rare allele of each s-variant alone was separately calculated and compared among the patient groups in Table IV. There was no significant difference between NRF vs. IRF and NRF vs. ESRF. Comparisons between IRF and ESRF were significant for all five s-variants, with an OR of 0.12–14.0 and a p-value ranging from < 0.05 to < 0.02 (Table IV).

Table V and VI show the clinical profiles of the 20 patients with IgAN who were treated with ACEI/ATRA (treatment group) and those of the 20 patients with IgAN who were not treated with ACEI/ATRA (control group). Table VII compares the data between the treatment and control groups at entry and at the end of the trial, their renal status (NRF, IRF or ESRF) with their ACE gene polymorphism and haplotypes. At entry, there were no significant differences between the treatment and control groups in the various parameters. However, post-trial, the mean serum creatinine in the control group was significantly worse than that in the treatment group ($p < 0.001$). The post-trial proteinuria in the control

group was also worse than that in the treatment group ($p < 0.002$). With regard to renal outcome, there were 11 patients with ESRF in the control group compared to five in the treatment group ($\chi^2 = 3.8$, $p = 0.053$). Thus, treatment does reduce the number of patients who progress to ESRF, albeit just outside the significance level.

The results of the ACE gene polymorphism study showed significantly more I allele and haplotype 5 among patients with renal impairment compared to those with ESRF who had significantly more D allele and haplotype 3 ($\chi^2 = 3.8$, $p < 0.05$). There was no difference in the distribution of the three genotypes and two haplotypes among the three patient groups. By combining the normal and renal impaired patients into a non-ESRF group and putting together patients with genotypes ID and DD into an ID/DD grouping, we reduced the χ^2 contingency table to 2×2 with improved numbers. The counts of II: ID/DD among non-ESRF vs. ESRF patients were 8:7 vs. 0:5, respectively ($\chi^2 = 4.4$, $p < 0.05$) and for haplotypes, also $p < 0.05$.

Table VI. Clinical profiles of the 20 patients with IgA nephritis who were not treated with ACEI/ATRA (Control Group) normal renal function, impaired renal function and end-stage renal failure.

No.	Age (yrs)	Gender	Hpt	Drug	Dosage (mg)	Trial (mths)	Initial Se creat (mg/dl)	Final Se creat (mg/dl)	Initial TUP (gm/day)	Final TUP (gm/day)	Alu I/D variant	Haplotype
Normal renal function												
1	21	M	Yes	At	50	65	0.77	1.14	2.2	1.2	II	5,5
2	54	M	No	Nil	-	58	1.07	1.37	5.2	0.8	II	5,5
3	29	M	No	Nil	-	50	0.96	1.28	1.0	0.2	ID	1,3
4	20	M	Yes	At	50	63	1.11	1.57	2.6	1.8	DD	3,3
Impaired renal function												
1	48	M	Yes	At, Am	50,10	60	1.58	2.81	3.9	2.5	II*	5,5 [†]
2	48	F	No	Nil	-	48	1.14	1.69	1.2	0.5	ID	3,5
3	25	M	Yes	At	50	65	1.57	2.10	7.8	1.8	ID	3,5
4	26	F	Yes	AT	100	49	0.95	1.98	1.5	1.6	ID	3,5
5	44	F	No	Nil	-	60	0.88	1.95	3.2	1.6	ID	3,5
End stage renal failure												
1	32	F	Yes	At	100	66	1.48	7.63	4.4	3.1	II*	3,3 [†]
2	31	F	Yes	At, Am	100, 10	49	1.61	8.61	1.0	1.7	ID	3,5
3	34	M	No	Nil	-	60	1.57	7.82	5.2	1.8	II	3,3
4	41	F	Yes	At	100	38	2.81	9.20	1.4	2.3	II	3,3
5	25	M	Yes	At, Am	100, 10	64	1.71	7.60	1.6	2.1	II	3,3
6	45	F	Yes	At, Am	100, 10	52	1.75	7.40	1.2	2.4	II	3,5
7	29	M	No	Nil	-	62	1.98	7.20	0.5	1.8	ID	5,5
8	40	F	Yes	Am	10	56	1.63	6.93	6.4	4.7	DD	5,5
9	44	F	Yes	At, Am	100,5	49	1.72	8.33	0.6	3.7	DD	5,5
10	47	F	No	Nil	-	58	1.28	6.45	1.4	2.9	DD	5,5
11	36	F	Yes	At, Am	100, 10	50	1.48	8.61	3.1	3.5	DD	5,5

* versus * (ns); † versus † (ns)

At: atenolol; Am: amlodipine; HPT: hypertension; TUP: total urinary protein; Se creat: serum creatinine

Thus, genotyping and allele counting confirms that the D allele and haplotype 3 confer poorer renal survival in treated patients, whereas I allele and haplotype 5 confer a better renal outcome. Of note, on examination of the clinical profile of the untreated control group in Table VI, we found no significant difference in the ID polymorphism and haplotypes 3 and 5 between patients with NRF, IRF and ESRF. This suggests that the *ACE* gene could influence the response of IgAN patients to ACEI/ATRA therapy.

DISCUSSION

Rieder et al reported sequence variations in the human ACE from a study of five African-American and six European-American samples. The latter sample had 44 varying sites, of which four were singletons (variant with a single occurrence only in the entire study). The African-American sample, however, had 70 varying sites, of which 22 were singletons. The difference in the proportion of singleton variants was significant ($\chi^2 = 7.7$, $p < 0.006$),

but not in their overall nucleotide diversity values of 9.7 ± 4.9 vs. 7.3 ± 3.8 , respectively (means \pm SD $\times 10^{-4}$).⁽¹⁹⁾ These data were similar to the values calculated for the four normal subjects (6.1 ± 3.1), 20 patients (5.2 ± 1.7) or 24 patients combined (5.1 ± 1.6) in our study.

Among the 55 variants, 11 were in absolute LD with the Alu I/D variant. This block of variants were concentrated in a region of about 4,000 bp between 10314 C > T in exon 13 and 14094 I/D, Alu variant in intron 16. The recombinant rate must be very low in a small region and hence, they stayed in absolute linkage. Each of these SNPs could replace the Alu variant as the genetic marker in the disease association study. With the advantage of high throughput and lower cost, SNPs may be genotyped within minutes of PCR completion by measuring the increase in fluorescence of the dye-labeled DNA probes with no downstream processing by using Taqman® real-time PCR for allele discrimination technique; whereas genotyping the Alu I/D polymorphism (distinguished by the presence or absence of a 287 base

Table VII. Comparison of data between treatment and control groups before and after trial.

Parameter	Treatment (n = 20)	Control (n = 20)	p-value
Gender			ns
Male	9	9	
Female	11	11	
Age \pm SD (yrs)	40 \pm 8	36 \pm 10	ns
Hypertension			ns
Yes	13	13	
No	7	7	
Blood pressure (mmHg)			ns
Systolic before	139 \pm 13	136 \pm 9	
Diastolic before	85 \pm 6	84 \pm 6	ns
Systolic after	138 \pm 9	136 \pm 8	
Diastolic after	83 \pm 5	80 \pm 6	ns
Trial duration (mths)	60 \pm 5	56 \pm 8	ns
Serum creatinine (mg/dl)			
Before	1.52 \pm 0.42*	1.45 \pm 0.47 [†]	ns
After	2.72 \pm 2.07*	5.08 \pm 3.15 [†]	< 0.01
Urinary protein (g/day)			
Before	2.0 \pm 0.9**	2.8 \pm 2.1	ns
After	1.0 \pm 0.9**	2.1 \pm 1.1	ns
Outcome			
ESRF	5	11	< 0.05
Non-ESRF	15	9	

Intra-group paired test : *..* p < 0.02; **..** p < 0.002; †..† p < 0.001

SD: standard deviation; ESRF: end-stage renal failure; ns: not significant

pair sequence) involved PCR amplification, followed by incubation with an enzyme, electrophoretic gel separation of cleavage products and final staining for identification.

Furthermore, DD cases needed confirmation with an insert-specific forward primer in a second PCR.⁽²⁰⁾ Presently, there is considerable redundancy in association studies with genotyping of polymorphisms that are highly linked or in absolute linkage. Therefore, prior to genotyping multiple polymorphisms in a particular gene, it is useful to sequence it in order to identify all variants and to determine the condition of linkage among them so as to avoid redundancy. The frequency of SNPs is high in the human genome, and it is not cost-effective to genotype all SNPs.⁽²¹⁾ From a block of common (absolute or closely linked) SNPs, a single haplotype-tagging SNP (htSNP) may be selected for genotyping without loss of power.⁽²²⁾ Our findings with the *ACE* gene were similar. Two of the six haplotype constructs from five significant-variants had predictive value for risk or low-risk of ESRF (haplotype 3 and 5 in our study). However, the predictive power of the individual component SNPs was quite similar. Thus, a single SNP may be selected for genotyping without loss of power for prediction. Again, the obvious reason for such redundancy was that these variants were highly linked. Between each of the four variants and the Alu variant, the correlation of genotype was highly significant, all r values were greater than 0.9 (p < 0.001). Keavney et al⁽²²⁾ reported similar findings.

Hence, a reasonable proposition for future development is finding a haplotype of non-redundant SNPs from many genes genomewide.⁽²³⁾ In this respect, Bantis et al reported a synergistic effect in the combined analysis of *AGT-M235T* and *ACE I/D* polymorphism.⁽²⁴⁾ Similarly, Yoon et al reported the interdependent effects of *ACE* and *PAT-AH* polymorphisms on the progression of IgAN.⁽²⁵⁾ It is possible that multiple interacting genes may be involved in the pathogenesis of IgAN.

Our data shows that patients with the DD genotype did not do as well as those with the II genotype when on ACEI/ATRA therapy. Those with the II genotype have a better prognosis, which seems to be more evident among Asians. Nonoguchi et al, in a recent study of 113 patients with chronic kidney disease, of which 75 had IgAN, reported that ATRA therapy extended the time to ESRF for patients with the II and ID genotype but not for those with the DD genotype,⁽²⁶⁾ thus suggesting that DD patients have diminished response to ATRA in terms of renoprotection.

Ng et al, in a meta analysis of 14,724 diabetic patients, reported the protective role of the II genotype in Asian patients with diabetic nephropathy, whereby there was a reduction in the number with ESRF when the patients were treated with ACEI/ATRA. In contrast, those with the D allele had a deleterious outcome in terms of ESRF.⁽²⁷⁾ Seki et al, another group of Japanese researchers, reported a similar renoprotective effect in 18 Asian type

II diabetes mellitus patients with II genotype, who were treated with ACEI/ATRA, in contrast to those with the DD genotype.⁽²⁸⁾ In a retrospective study conducted on 109 patients with IgAN, Woo et al showed that patients with the II genotype responded better to losartan 50–100 mg a day compared to those with the DD genotype in terms of developing ESRF.⁽²⁹⁾

The *ACE* gene was sequenced in 20 IgAN patients and four normal subjects in our current study. 55 variants were found, 19 of which were unique, but none were significant risk factors for the development of the disease. Each of the 11 variants in absolute linkage may replace the Alu I/D variant as a genetic marker, and may be more rapidly and cost-effectively genotyped by real-time PCR. Despite the limitation of the small number of patients studied, our data suggested that at least in the *ACE* gene, haplotyping SNPs within a single gene seems to have no added advantage over genotyping the individual component SNPs. It is possible that multiple interacting genes are involved in the pathogenesis of IgAN, and further genetic studies involving haplotyping SNPs across many genes and genome-wide may be more fruitful.

This is a preliminary study, and further studies of randomised clinical trials involving larger cohort of patients with control groups would be required in order to confirm the clinical significance of our study. We could, in future, proceed to determine specific SNPs of interest in larger numbers, using the rapid technique of Taqman® real-time PCR for allele discrimination. In previous studies of IgAN,^(30,33) in relation to *ACE* gene I/D polymorphism, a sample size of 100 patients or more are readily achievable, as polymorphism studies are less labour intensive and quite readily performed in contrast to gene sequencing work such as the *ACE* gene, which has 24,070 bp and is labour intensive.

Between patients and normal subjects, there was no significant difference in genotype or allele frequencies in all the shared variants. Therefore, no new useful markers of IgAN were identified. Among the patients with IgAN treated with ACEI/ATRA, those with the D allele and haplotype 3 had a higher of risk for ESRF, whereas those with the I allele and haplotype 5 had a better renal outcome. In the untreated control group, no significant difference in the ID polymorphism and haplotypes 3 and 5 between patients with NRF and those with renal failure was found. This may suggest that the *ACE* gene could have an influence on the response of patients with IgAN to ACEI/ATRA therapy.

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