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No Association of Angiotensin-Converting Enzyme 2 Gene (*ACE2*) Polymorphisms With Essential Hypertension

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Recent intriguing findings from genetic linkage, knockout, and physiologic studies in mice and rats led us to conduct the first investigation of the novel angiotensin-converting enzyme 2 gene (*ACE2*) in human hypertension (HT). We genotyped four single nucleotide polymorphisms (SNP) (A→G at nucleotide 1075 in intron 1, G→A at nucleotide 8790 in intron 3, C→G at nucleotide 28330 in intron 11, and G→C at nucleotide 36787 in intron 16) in HT (n = 152) and normotensive (NT, n = 193) groups having inherently high biological power (>80%) due to our inclusion only of subjects whose parents had the same BP status as themselves. The SNPs were in linkage disequilibrium (D' = 54% to 100%, P = .05 to 0.0001). Because *ACE2* is on the X chromosome, data for each sex were analyzed separately. Minor allele frequencies in HT versus NT were as follows: for the intron 1 variant 0.21 versus 0.17 in female subjects (P = .31) and 0.25 versus 0.29 in male subjects (P = .60); intron 3 variant 0.22 versus 0.18 in female subjects (P = .35) and 0.15 versus 0.20 in male subjects (P = .47); intron 11 variant 0.39 versus 0.46 in male subjects (P = 0.17) and 0.31 versus 0.30 in male subjects (P = .96); intron 16 variant 0.20 versus 0.19 in female subjects (P = .72) and 0.17 versus 0.17 in male subjects (P = .95). Haplotype analysis was also negative. These data provide little support for *ACE2* in genetic predisposition to HT. Am J Hypertens 2004;17: 624–628 © 2004 American Journal of Hypertension, Ltd.

Key Words: Angiotensin-converting enzyme 2, association study, blood pressure, essential hypertension, X chromosome.

Many association studies of renin-angiotensin system gene variants have been conducted in essential hypertension. In the case of the angiotensin I–converting enzyme gene (*ACE*), despite occasional reports of a possible role the *ACE* locus in blood pressure (BP) variation[1] or association with hypertension (HT),[2,3] by far the majority of studies have been negative.[4–9]

Recently, a homologue, ACE2,[10,11] has been discovered, whose catalytic domain is 42% identical with ACE.[11] Whereas *ACE* is expressed ubiquitously in the vasculature, *ACE2* is expressed predominantly in the kidney,[10,11] but also in cardiovascular and gastrointestinal[12] tissues. Notably ACE2 cleaves a single residue from angiotensin I (Ang I) to generate angiotensin 1–9 (Ang 1–9), which has no known function. Angiotensin 1–9 cannot be converted to Ang II by ACE2, but can be converted by ACE to Ang 1–7, a vasodilator.[11] Angiotensin 1–7 can also be generated directly from Ang I by endopeptidases other than ACE2 and ACE, including neprilsyn, prolyl endopeptidase, and thimet oligopeptidase.[13] In contrast to ACE, ACE2 does not hydrolyze bradykinin,[14] nor is it inhibited by typical ACE inhibitors.[10,15] Moreover, ACE2 is clearly multifunctional, having been reported recently to be the functional receptor for the severe acute respiratory syndrome (SARS) coronavirus.[16]

It has been suggested that ACE2 might negatively regulate the activated renin-angiotensin system by diverting the generation of vasoconstrictor Ang II towards the inactive Ang 1–9 and vasodilatatory Ang 1–7 peptides.[17] Competition between ACE and ACE2 for the same substrate, Ang I, could thereby serve a counterbalancing function.

The gene *ACE2* maps to a quantitative trait locus (QTL) for BP on the X chromosome in three strains of genetically HT rats—SHR, SHR-SP, and Sabra SBH/y.[18] In each strain ACE2 mRNA and protein levels were greatly reduced in the kidney and inversely correlated with elevation in BP.[18] Moreover, in the SBH/y strain, salt loading reduced ACE2 further and raised BP even more, whereas

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Table 1. Demographic characteristics

Characteristic	NT	HT
n	193	152
Male:female (%)	52:48	36:64
Age (y)	45 ± 12	56 ± 12
BMI (kg/m^2)	25 ± 4	27 ± 5
SBP (mm Hg)	119 ± 10	172 ± 27
DBP (mm Hg)	72 ± 7	105 ± 14
Cholesterol (mmol/L)	4.8 ± 0.1	5.1 ± 0.2
Triglycerides (mmol/L)	1.3 ± 0.1	1.9 ± 0.2
HDL (mmol/L)	1.3 ± 0.03	1.1 ± 0.1
LDL (mmol/L)	3.9 ± 0.1	3.9 ± 0.1

Data are mean ± SD or mean ± SE, as appropriate.

BMI = body mass index; DBP = diastolic blood pressure; HDL = high-density lipoprotein; HT = hypertensive subjects; LDL = low-density lipoprotein; NT = normotensive subjects; SBP = systolic blood pressure.

Table 2. Primers and restriction enzyme sites

Polymorphism and primers	Restriction site*
Intron 1 A→G (nt 1075)	*Ava*II: G↓GT/ACC
Sense 5′-TAA CAA GTG CAA GGA TTT AGG-3′	
Antisense 5′-AAG CTG CAA TGA ATC ATG AT-3′	
Intron 3 G→A (nt 8790)	*Alu*I: AG↓CT
Sense 5′-CAT GTG GTC AAA AGG ATA TCT-3′	
Antisense 5′-AAA GTA AGG TTG GCA GAC AT-3′	
Intron 11 C→G (nt 28330)	
Sense 5′-ACG TTG GAT GGG CAG TTT ATT GTA CAT TGT G-3′	
Antisense 5′-ACG TTG GAT GGC TCC AGC AAA TTC AAG GAC-3′	
Intron 16 G→C (nt 36787)	
Sense 5′-ACG TTG GAT GAA TTC CCC AGC ATT TCA GCC-3′	
Antisense 5′-ACG TTG GAT GGA CTT TCT TCA ACC AGC ACC-3′	

* Underlining indicates the location of the single nucleotide polymorphism.

no changes were seen in the normotensive (NT) SBN/y strain.[18] These data are consistent with a protective effect of ACE2 in the salt-resistant strain, with this effect being lost in the HT strain that lacks *ACE2*. Furthermore, *Ace2*-knockout mice have 10 mm Hg lower BP and an enhanced response to Ang II infusion.[13]

In their article in *Nature*, Crackower et al[18] state, "It will be interesting to determine whether single nucleotide polymorphisms (SNPs) in the human *ACE2* locus correlate with changes in blood pressure." To address this question, we therefore conducted a case-control study of *ACE2* polymorphisms in essential HT.

Methods

Subjects

All subjects were Australian individuals of white Anglo-Celtic origin. The subjects with HT (BP >140/90 mm Hg) were the offspring of parents who both had HT. A qualified individual using a cuff sphygmomanometer on three different occasions spanning 2 weeks measured blood pressure. Secondary HT, renal disease, and heart disease were excluded by the patients' general practitioners (GP). All subjects had normal renal function and no evidence of secondary HT by standard clinical evaluation carried out to the satisfaction of the GP. Normotensive subjects (NT) were required to have a BP <130/90 mm Hg and parents who were both normotensive past the age of 50 years. In addition, subjects could not have heart or kidney disease. These individuals were recruited at the Sydney Red Cross Blood Bank. Clinical history was obtained by a standardized questionnaire and examination by a qualified health professional for both groups. The University of Sydney Ethics Committee approved the study and the subjects gave informed consent. Demographic characteristics (mean ± SD or mean ± SE, as appropriate) for the 152 HT and 193 NT subjects are listed in Table 1.

In addition, the subjects participating in the study had been shown to be capable of demonstrating a genetic association with HT,[19,20] should this be present. This has been aided by the enhanced biological power our study design conferred by restricting our choice of subjects to those with a strong family history (two HT parents).

Genotyping

DNA was isolated from whole blood using a QIAamp Blood Mini Kit (Qiagen, Hilden, Germany). he genomic sequence and SNPs for *ACE2* (chromosome Xp22.3)[21] were determined from the Ensembl Human Genome Browser (http://www. ensembl.org/Homo_sapiens/). Nineteen putative SNP have been identified in *ACE2*.

Two of these were amenable to testing by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis, namely, an A→G variant at nt 1075 in intron 1 and a G→A variant at nt 8790 in intron 3. Primers for genotyping of these were synthesized by Sigma-Genosys (Sydney, Australia) (Table 2). The PCR protocol was the same for both SNPs. The 22 μL PCR mixture contained 100 ng genomic DNA, 0.25 pmol each of primer, 0.1 mmol/L each of dNTP, 1 U HotstarTaq DNA polymerase (Qiagen), 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, and 1.5 mmol/L $MgCl_2$. After an initial denaturation step of 94°C for 10 min, 10 cycles were performed at 94°, 65°, and 72°C for 1 min each, then 15 cycles at 94°, 60°, and 72°C for 1 min each, and finally 20

Table 3. Genotype and allele frequencies of *ACE2* polymorphisms in hypertensive (HT) and normotensive (NT) groups

Group	n	Genotype frequencies			χ^2	*P*	Allele frequencies		χ^2	*P*
		MM	*Mm*	*mm*			*M*	*m*		
Intron 1 A→G variant										
Female										
NT	81	55 (0.68)	25 (0.31)	1 (0.01)	2.9	0.23	135 (0.83)	27 (0.17)	1.1	0.31
HT	78	50 (0.64)	23 (0.30)	5 (0.06)			123 (0.79)	33 (0.21)		
Male										
NT	96	—*	—	—			68 (0.71)	28 (0.29)	0.28	0.60
HT	48	—	—	—			36 (0.75)	12 (0.25)		
Intron 3 G→A variant										
Female										
NT	89	63 (0.71)	20 (0.22)	6 (0.07)	1.9	0.39	146 (0.82)	32 (0.18)	0.89	0.35
HT	65	40 (0.62)	21 (0.32)	4 (0.06)			101 (0.78)	29 (0.22)		
Male										
NT	104	—*	—	—			83 (0.80)	21 (0.20)	0.52	0.47
HT	46	—	—	—			39 (0.85)	7 (0.15)		
Intron 11 C→G variant										
Female										
NT	89	23 (0.26)	50 (0.56)	16 (0.18)	2.2	0.33	96 (0.54)	82 (0.46)	1.9	0.17
HT	100	34 (0.34)	54 (0.54)	12 (0.12)			122 (0.61)	78 (0.39)		
Male										
NT	89	—*	—	—			62 (0.70)	27 (0.30)	0.0029	0.96
HT	52	—	—	—			36 (0.69)	16 (0.31)		
Intron 16 G→C variant										
Female										
NT	85	57 (0.67)	24 (0.28)	4 (0.05)	1.5	0.46	138 (0.81)	32 (0.19)	0.13	0.72
HT	91	62 (0.68)	21 (0.23)	8 (0.09)			145 (0.80)	37 (0.20)		
Male										
NT	103	—*	—	—			85 (0.83)	18 (0.17)	0.0017	0.95
HT	47	—	—	—			39 (0.83)	8 (0.17)		

M = major allele, *m* = minor allele, of each polymorphism.
Values in parentheses are fractions.
* As *ACE2* is on the X chromosome (one copy), it is inappropriate to present genotype data.

cycles at 94°, 58°, and 72°C for 1 min each, finishing with a 20-min extension step at 72°C. The PCR products were digested at 37°C for 3 h with 1.9 U of either *Ava*II (MBI Fermentas, Vilnius, Lithuania) for the intron 1 SNP or *Alu*I (MBI Fermentas) for the intron 3 SNP, using the buffers supplied and sterile water (19 μL total volume). The sites where the enzymes cut are listed in Table 2. The intron 1 variant gave two fragments of 305 and 166 bp (*G* allele) when cut, or a single band of 471 bp (*A* allele) for the uncut product. For the intron 3 variant, fragments of 281 and 185 bp (*A* allele) or 466 bp (*G* allele) were generated. Bands were visualized on ethidium bromide–stained agarose gels after electrophoresis.

A further 2 SNPs were tested using the MassARRAY system (Sequenom, San Diego, CA) by the Australian Genome Research Facility (AGRF, Brisbane, Australia). These were a C→G variant at nt 28330 in intron 11, and a G→C variant at nt 36787 in intron 16. The methodology developed used MassArray Assay Design version 2.0.0.1.6 software and primer shown in Table 2. Genotypes were determined using a Bruker (Täby, Sweden) Autoflex MALDI-TOF mass spectrometer by AGRF.

Statistical Analysis

The χ^2 test and one-way analysis of variance (ANOVA) were performed using StatView (Abacus Concepts, Berkeley, CA). Linkage disequilibrium was tested in the largest group (that is, normotensive individuals) by the method of Hill.[22] Haplotype frequencies for NT and HT were estimated by the program Lindeq developed by one of the present investigators (W.Y.S.W.), and they were compared by χ^2 analysis.

Results

Association With Hypertension

Because *ACE2* is on the X chromosome, Hardy-Weinberg equilibrium could be tested only in the female population and was in accordance with expectations for each polymorphism. Genotype and allele frequencies for each polymorphism in female subjects and allele data for male subjects are listed in Table 3. None of the polymorphisms showed an association with HT (Table 3). Our study design provided at least 80% power to detect an existing association with HT in the female subjects with HT, with

Table 4. Haplotype results for two of the *ACE2* polymorphisms

Intron 1 A→G		Intron 3 G→A	χ^2	*P*
M	*v*	*M* *m*	0.11	.74
m	*v*	*M* *m*	3.6	.057
M *m*	*v*	*M*	0.01	.92
M *m*	*v*	*m*	3.6	.059

M = major allele, *m* = minor allele, of each polymorphism.

a two-tailed type 1 error of 0.05 and an allelic odds ratio of 2.0. Thus, if an association with HT were present, we should have been able to detect it. When the analysis was restricted to overweight subjects with HT (BMI >25 kg/m^2), again there was no association with HT in either female subjects (χ^2 = 2.7, 0.14, 2.1, and 2.5; P = .26, .93, .15, and .11 for the intron 1, 3, 11, and 16 variants, respectively) or male subjects (χ^2 = 0.24, 0.16, 0.30, and 1.6; P = .62, .69, .58 and .20, respectively). Similarly, for lean subjects with HT (BMI ≤25 kg/m^2), results were negative for female subjects (χ^2 = 0.29, 2.9, 0.41, and 0.01; P = .86, .24, .52, and .91, respectively) and male subjects (χ^2 = 0.47, 2.9, 0.05, and 2.6; P = .49, .087, .82, and .11, respectively).

Linkage Disequilibrium and Haplotype Analysis

Each polymorphism was in linkage disequilibrium (LD) (D' = 54% to 100%, P = .05 to .0001), that is, all were part of the same LD block. None of the haplotypes showed an association with HT. Table 4 illustrates this for haplotypes from SNPs in introns 1 and 3. The entire data set for combinations arising from all four SNP is not shown.

Genotype–Phenotype Interactions

Diastolic BP was higher in lean male subjects with HT having the minor (*G*) allele of the intron 1 variant (mean ± SD, 99 ± 7 *v* 120 ± 10 for *A v G*, respectively; F = 9.8, P = .0088), noting that there is only one copy (either *A* or *G*) in each individual because male subjects have only one X chromosome. Tracking with genotype was not seen in female subjects with HT. Body mass index in male subjects with HT was marginally higher in those with the minor (*A*) allele of the intron 3 variant (26 ± 4 *v* 30 ± 6 [mean ± SD] for *G v A*, respectively; F = 4.2, P = .048). Systolic BP was slightly higher in overweight male subjects with HT (160 ± 16 *v* 183 ± 22 [mean ± SD] for *G v A*, respectively; F = 6.5, P = .020). Body mass index was also higher in male subjects with HT having the intron 16 variant (27 ± 4 *v* 23 ± 2 [mean ± SD] for *G v C*, respectively; F = 9.4, P = .0036). However, Bonferroni correction for multiple comparisons rendered all of these associations nonsignificant.

Discussion

The present study found that an A→G SNP in intron 1, a G→A SNP in intron 3, a C→G SNP in intron 11 and a G→C SNP in intron 16 of *ACE2* were in linkage disequilibrium, but none were associated with HT. Our study design (using the offspring of two HT parents) meant that the subjects with HT used were drawn from a general hypertensive population (~1520 individuals) that was 10 times greater than the number of subjects studied.[6] Such a study design also leads to a considerable reduction in sample size requirements for detection of the same population effect of genetic variants (W.Y.S. Wang, unpublished observation). For example, assuming a multiplicative effect of alleles and the absence of gene–gene and gene–environment interactions, in the case of an autosomal disease allele with population frequency of 0.20 that contributes to HT with a disease odds ratio of 1.5, sampling based on disease status alone would require at least 267 case subjects and control subjects to provide 80% power for a significant threshold of P = .05; however, when considering the disease status of both parents as being affected, only 64 samples might be required in each group to detect the same effect. The reason for this is the disease allele frequency of the specially sampled case subjects would be higher than that of their affected parents. The opposite is true for the control subjects. Therefore, despite having the same underlying effect in the population, the disease odds ratio between the two selected groups is increased. As a practical demonstration of increased power when sampling is based on family history of HT, we reanalyzed data in one of our reports[20] using a test for trend that allows the expectation for increased risks in different groups, that is, subjects with HT with two HT parents should have more genetic risk than subjects with HT with only one HT parent. This gave a P value of .0066, as can be seen below:

```
ptrend var2 var3 var1
+----------------------------+
| Allele1 Allele2  _Freq  Status
|----------------------------|
1. |   84       7   0.077 1 | HT with 2 HT parents
2. |   77       3   0.038 2 | HT with 1 HT parent
3. |  198       4   0.020 3 | HT with 1 HT sibling
4. |   89       1   0.011 4 | Normotensive (NT)
+----------------------------+
Trend analysis for proportions
------------------------------
Regression of p = Allele1/(Allele1+Allele2) on Status:
Slope = –.022, std. error = .0082, Z = 2.72
Overall χ²(3) = 8.1, pr > χ² = 0.043 (independent of trend)
χ²(1) for trend = 7.4, pr > χ² = 0.0066
```

Despite finding no association of *ACE2* variants with HT, we did, however, note higher diastolic BP for the *G* allele of the intron 1 SNP in lean male subjects with HT

and higher systolic BP for the *A* allele of the intron 3 SNP in obese male subjects with HT. These were nonetheless weak and became nonsignificant after correction for multiple comparisons by the Bonferroni method. The latter is regarded as overly severe, thus risking the elimination of a true positive finding.[23]

The physiologic function of ACE2 is still being unraveled. As well as the effects described earlier, *Ace2* knockout mice exhibit cardiac abnormalities[18] resembling cardiac stunning (reversible decline in cardiac contractility under ischemic conditions) in humans.[24] Loss of *Ace2* is also associated with upregulation of hypoxia-inducible genes,[18] suggesting a possible role for ACE2 in response to cardiac ischemia.[24] As well, Ang II is increased and could contribute to cardiac dysfunction via induction of oxidative stress unrelated to BP. Elevated Ang II could be blunted by compensatory changes in Ang 1–7, kinin metabolites, the apelin system,[17] or nitric oxide.[25]

In conclusion, the present study provides no evidence for an association of *ACE2* polymorphisms with essential HT in an Anglo-Celtic Australian population. However, this does not totally exclude a role for *ACE2* in the causation of essential HT. The testing of additional polymorphisms in *ACE2* and association analyses in other settings and ethnic groups is needed before eliminating *ACE2* completely. A role in heart failure and coronary artery disease also remain to be examined.

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