

α -Adducin and Angiotensin I–Converting Enzyme Polymorphisms in Essential Hypertension

Catherine J. Clark, Eleanor Davies, Niall H. Anderson, Rosemary Farmer, Elaine C. Friel, Robert Fraser, John M.C. Connell

Abstract—This study focused on two genes that have previously been implicated in hypertension and may influence renal sodium handling, adducin, and angiotensin I–converting enzyme (ACE). We compared their polymorphic frequencies and interaction in patients with essential hypertension (n=128) and individually age- and gender-matched normotensive control subjects. The α -adducin *G460W* polymorphism was genotyped by DNA amplification and restriction digestion. The *ACE I/D* polymorphism was assayed by a triple-primer method, with a “nested” polymerase chain reaction primer situated completely within the insertion sequence of the *I* allele. The distributions of genotypes and alleles for the two polymorphisms were not significantly different between the case and control populations, and the cross-classification of cases by α -adducin and *ACE* genotype gave a distribution similar to that of control subjects. We have previously reported that the distributions of genotypes for two linked polymorphisms in the aldosterone synthase gene (one in the steroidogenic factor-1 [*SF-1*] binding site and the other an intronic conversion [*IC*]) were significantly different between this cohort of essential hypertensives and matched control subjects. The cross-classification of cases by α -adducin and *SF-1*, α -adducin and *IC*, *ACE* and *SF-1*, and *ACE* and *IC* genotype gave a distribution similar to that of control subjects. Hence, no evidence was found to suggest an association between either the α -adducin *G460W* or the *ACE I/D* polymorphism and hypertension in a careful case-control study. Furthermore, the α -adducin *G460W*, *ACE I/D*, and aldosterone synthase *SF-1* and *IC* polymorphisms do not appear to interact in our hypertensive population. (*Hypertension*. 2000;36:990-994.)

Key Words: angiotensin I ■ angiotensin-converting enzyme ■ hypertension, essential ■ polymorphism ■ sodium

The cause of essential hypertension is multifactorial; part is due to genetic factors. For example, young offspring of hypertensive parents themselves have a significant predisposition to hypertension.¹ A number of candidate genes present themselves. Those that influence renal sodium handling, including components of the cytoskeleton,² the renin-angiotensin system (RAS),³ and factors that affect the regulation of secretion and action of aldosterone⁴ are obvious candidates.

Studies of the Milan hypertensive rat and of humans with essential hypertension suggest that genetic alterations in adducin may contribute to hypertension.^{5,6} Adducin is an α/β heterodimeric protein thought to regulate cell-to-cell contact,⁷ cell membrane ion transport,⁵ and signal transduction.⁸ A specific mutation (*G460W*) in the human α -adducin gene has recently been described that results in the substitution of tryptophan (*W*) for glycine (*G*) at amino acid number 460.² From initial case-control and linkage analyses, this locus was implicated in the genetic component of hypertension in Italian and French populations.² Additionally, Cusi et al² reported that a group of Italian hypertensive subjects with the *W* allele had lower plasma renin and showed a significantly

greater fall in blood pressure with sodium restriction or diuretic treatment. In contrast, studies in Japanese and Scottish populations have not been able to confirm this association.^{9,10} Indeed, no association was discernible in a later study of a different Italian population by Glorioso and colleagues.¹¹ Nevertheless, the findings of Cusi et al² suggest that adducin affects blood pressure through the control of renal sodium excretion, a conclusion strengthened by their later finding of an association of hypotensive response to diuretic therapy with α -adducin genotype.¹¹ It is therefore possible that genetic variation in other systems controlling sodium homeostasis may interact, which explains some of this population variability.

The RAS produces angiotensin II (Ang II), which plays both an autocrine and paracrine role in maintaining cardiovascular homeostasis.¹² Ang II is produced from angiotensin I (Ang I) mainly but not exclusively by the metalloproteinase enzyme angiotensin I–converting enzyme (ACE).¹³ In 1990, Rigat et al¹⁴ identified a biallelic polymorphism in the *ACE* gene that is characterized by either the absence (deletion *D*) or presence (insertion *I*) of a 287-bp *Alu* repeat sequence.

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From the MRC Blood Pressure Group (E.D., R.F., J.M.C.C.), Department of Medicine and Therapeutics (C.J.C., N.H.A., R.F., E.C.F.), Western Infirmary, Glasgow, Scotland.

Correspondence to Prof Robert Fraser, MRC Blood Pressure Group, Department of Medicine and Therapeutics, Gardiner Institute, Western Infirmary, Glasgow, G11 6NT, Scotland. E-mail rfraser@clinmed.gla.ac.uk

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TABLE 1. Primers and PCR Conditions

Primer	Sequence	PCR Conditions
<i>ADD</i> sense	5' GACAAGATGGCTGAACCTCTGGCCGG 3'	5 min(s), 94°C 1 min(s) 94°C, 1 min(s) 67°C, 1 min(s) 72°C; 35 cycles
<i>ADD</i> antisense	5' GACTTGGGACTGCTTCCATTCTGGC 3'	5 min(s), 72°C
<i>ACE</i> sense	5' CCCATCCTTTCTCCATTTC 3'	3 min(s), 94°C
<i>ACE</i> sense; "nested"	5' GGTTCACCGTTTTAGCCGGGA 3'	1 min(s) 94°C, 1 min(s) 62°C, 1.5 min(s) 72°C; 30 cycles
<i>ACE</i> antisense	5' CCATGCCATAACAGGTCTTCA 3'	5 min(s), 72°C

Healthy male homozygotes for the *D* allele have higher serum ACE levels and higher circulating endogenous Ang II levels than those with the *I*I genotype.¹⁵ In addition, they have an enhanced pressor response during infusion of Ang I caused by increased Ang II generation.¹⁵ Furthermore, healthy subjects with the *DD* genotype displayed an impaired blunting of pressor responsiveness to Ang I after a small dose of intravenous enalaprilat compared with the *I*I genotype.¹⁶ That is, *DD* homozygotes may be resistant to ACE inhibitor therapy. However, the results of studies assessing the role of the *ACE I/D* polymorphism in essential hypertension have been conflicting.¹⁷

Blood pressure is reported to correlate positively with body sodium and negatively with body potassium, suggesting a role for the sodium-retaining hormone aldosterone in essential hypertension.¹⁸ We have previously reported that the distributions of genotypes for the two linked polymorphisms in the gene encoding aldosterone synthase (one in the steroidogenic factor-1 [*SF-1*] binding site and the other an intronic conversion [*IC*]) were significantly different between the same groups of essential hypertensives and control subjects used in this study.¹⁹

In the present study, the distributions and interaction of the α -adducin *G460W* and *ACE I/D* polymorphisms have been compared in a group of essential hypertensives and a group of normotensive control subjects with the use of a new, more rapid, and simple method for generating genotypic data for the α -adducin *G460W* polymorphism. The interactions of the aldosterone synthase *SF-1* and *IC* polymorphisms with the α -adducin *G460W* and *ACE I/D* polymorphisms have also been compared between the two populations, as the full impact of a particular genetic variant on phenotype may depend on an epistatic interaction with another (other) polymorphism(s).

Methods

Cases

Approval for this study was obtained from the appropriate hospital and community medicine ethics committees, and all subjects gave informed consent. White patients with essential hypertension (n=128) were recruited from the Blood Pressure Clinic of the Western Infirmary, Glasgow. All were <64 years of age. Secondary hypertension was excluded by physical examination and biochemical and radiological investigations where appropriate. All subjects had a positive family history of hypertension; high blood pressure was diagnosed before the age of 60 years. Subjects with a history of alcohol excess (>21 U per week) and obesity (body mass index [BMI] >33 kg/m²) were excluded. Blood pressure was measured in the clinic by a trained observer with a mercury sphygmomanometer. The diagnosis of hypertension was based on a minimum of 3 blood

pressure readings of >160/90 mm Hg before initiation of treatment, although most patients were receiving treatment at the time of the study.

Control Subjects

Control subjects were drawn from the North Glasgow coronary risk survey, which had \approx 200 randomly selected members of the North Glasgow population in each 10-year age/gender band from 25 to 64 years. They were normotensive (<140/90 mm Hg), and none were receiving antihypertensive therapy, treatment for heart disease, or hormone replacement therapy. They were individually age- and gender-matched with the cases by random selection from all control subjects who matched the criteria of the cases. Blood pressure was measured on 2 occasions with the Hawksley random-zero sphygmomanometer, with results averaged.

Molecular Genetic Analyses

Blood was taken into EDTA-containing receptacles and DNA extracted by means of a standard phenol-chloroform method.²⁰

The α -adducin *G460W* polymorphism, which is characterized by the substitution of guanine (*G*) for thymine (*T*) at nucleotide position 614 of exon 10,² was genotyped by DNA amplification by PCR followed by digestion with *Bsa*M I (Promega Ltd). This novel protocol was validated as a more rapid and simple method by comparison with the previously described allele-specific oligonucleotide hybridization protocol.² Fifteen human genomic DNA samples were kindly supplied by Prof Daniele Cusi and Dr Cristina Barlassina, Postgraduate School of Nephrology, University of Milan (Italy), for comparison. Genomic DNA (50 ng) from each subject was added to a well of a microtiter plate and evaporated to dryness at 60°C for 25 minutes. Reaction mix (25 μ L/well) was then added, containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 100 μ mol/L of each deoxynucleotide triphosphate (Promega Ltd), 10 pmol of each primer (Oswel DNA Service, University of Southampton [UK]; see Table 1), 0.25 U AmpliTaq Gold (Perkin Elmer), 5% dimethyl sulfoxide, and 0.05% W-1 (Gibco BRL). The *G*→*T* transition does not alter a restriction site but produces a "half-site" for *Bsa*M I. Introduction of the corresponding half-site is achieved by a PCR primer with 1 mismatch (located at position 2 from its 3' end) and does not interfere with elongation (*ADD* antisense, mismatch underlined). The cycling conditions are shown in Table 1. The PCR products were digested by *Bsa*M I (1 U enzyme/2 μ L PCR product) at 65°C for 3 hours. The amplification yielded a product of 72 bp. In the presence of *T* at nucleotide position 614, cleavage by *Bsa*M I generated fragments of 50 bp and 22 bp. The digestion products were efficiently resolved on prestrained 10% acrylamide/bisacrylamide (19:1) gels by microplate array diagonal gel electrophoresis ([MADGE]; MadgeBio Ltd), avoiding the need for expensive high-percentage metaphor agarose gels. These polyacrylamide gels have the capacity to accommodate samples from a whole microtiter plate in a single run and require smaller sample volumes, which can be loaded rapidly with multichannel pipettes.

The *ACE I/D* polymorphism, located in intron 16, was assayed by a triple-primer method with a "nested" PCR primer situated completely within the insertion sequence of the *I* allele. The inclusion of a third internal PCR primer is the most reliable PCR strategy for *ACE I/D* genotyping.²¹ Genomic DNA was evaporated to dryness as described above, then amplified in a reaction mix similar to the one

TABLE 2. Clinical Details of Subjects Studied

Variable	Men (n=59)		Women (n=69)	
	Cases	Control Subjects	Cases	Control Subjects
Age, y	49.1±10.7	49.8±10.9	49.2±11.3	49.7±11.2
Weight, kg	86.7±11.3	76.3±13.2	72.5±16.3	64.9±8.6
BMI, kg/m ²	28.7±3.3	25.6±3.4	27.8±6.1	25.5±3.1
SBP, mm Hg	154.5±22.9*	128.8±13.5	154.3±22.5*	120.1±15.2
DBP, mm Hg	98.0±11.2*	80.5±8.5	94.3±10.9*	73.9±7.8
Cholesterol, mmol/L	6.0±1.1	5.8±1.1	6.1±1.2	6.1±1.2

SBP indicates systolic blood pressure; DBP, diastolic blood pressure.

Study included 128 cases and 128 control subjects.

Data are mean±SD.

* $P<0.00005$ (paired t test, comparing cases with control subjects).

for the α -adducin variant, except for the following modifications: 10 mmol/L Tris-HCl (pH 9.0), 1.5 mmol/L MgCl₂, ACE primers (see Table 1), 1 U Taq DNA polymerase (Promega Ltd), 0.1% Triton X-100, and W-1 was absent. The cycling conditions are listed in Table 1. The PCR products were detected on prestained 7.5% polyacrylamide gels with MADGE. The banding patterns of the 3 possible genotypes were as follows: *DD*, 210-bp fragment; *II*, 498- and 264-bp fragments; *ID*, 498-, 264-, and 210-bp fragments.

The genotypic data and methods of analysis for the polymorphisms associated with the aldosterone synthase gene have been reported in an earlier communication.¹⁹

Statistical Methods

Comparisons between cases and control subjects of demographic variables and genotype frequencies were carried out by paired t test and McNemar's test, respectively. In particular, a variation of McNemar's test appropriate for case-control comparisons involving a 3×3 contingency table was used with analyses by genotype.²² Hardy-Weinberg equilibrium was checked by a χ^2 test, and the strength of genotypic interaction among the α -adducin, ACE, and aldosterone synthase polymorphisms was estimated by fitting and testing the appropriate pairwise interaction terms in hierarchical logistic regression models for disease status (case-control). Each model consisted of 1 main effect term for each of 2 polymorphisms included, plus an interaction term representing departures from a

simple, additive relation, as well as a continuous covariate term to correct for BMI.

Results

Demographic Data

The details of cases and control subjects are given in Table 2. It shows that the age and gender matching of the groups was accurate. The patient blood pressures were those at the time of the study when many were receiving treatment. Despite this, blood pressure in the patient group was significantly higher than in the control group ($P<0.00005$).

Genetic Analysis

The distributions of genotypes and alleles for the two polymorphisms in the case and control populations are shown in Table 3. It can be seen that the frequencies of the α -adducin *G460W* and ACE *ID* polymorphisms were not significantly different between the two groups. The control group and the case group were in Hardy-Weinberg equilibrium for both polymorphisms (α -adducin: cases, $P=0.894$; control subjects, $P=0.346$; ACE: cases, $P=0.821$; control subjects, $P=0.821$).

TABLE 3. Distributions of Genotypes and Alleles for α -Adducin *G460W* and ACE *ID* Polymorphisms in Case and Control Populations

Polymorphism	Cases (n=128)	Control Subjects (n=128)	McNemar's Test (CI)
α -Adducin <i>G460W</i>			
<i>GG</i>	88 (0.69)	74 (0.58)	$P=0.091$
<i>GW</i>	36 (0.28)	44 (0.34)	
<i>WW</i>	4 (0.03)	10 (0.08)	
% <i>G</i>	0.83	0.75	(-22.2, 0.7)
% <i>W</i>	0.17	0.25	
ACE <i>ID</i>			
<i>DD</i>	40 (0.31)	40 (0.31)	$P=0.924$
<i>ID</i>	62 (0.48)	62 (0.48)	
<i>II</i>	26 (0.20)	26 (0.20)	
% <i>D</i>	0.55	0.55	(-10.2, 10.2)
% <i>I</i>	0.45	0.45	

Confidence interval is for difference in proportions between cases and control subjects discordant for *W* allele (top table) or *D* allele (lower table), expressed as percentage.

TABLE 4. Interaction Analyses for α -Adducin, ACE, and Aldosterone Synthase Polymorphisms Comparing Case and Control Populations, Controlling for BMI

Loci	Interaction χ^2 Test
α -Adducin and ACE	$P=0.397$
α -Adducin and SF-1	$P=0.105$
α -Adducin and IC	$P=0.125$
ACE and SF-1	$P=0.305$
ACE and IC	$P=0.481$

The cross-classification of cases by α -adducin and ACE genotype gave a distribution similar to that of control subjects (Table 4), suggesting that a significant interaction between these two genes does not exist in the hypertensive population. This was also true when the cross-classification of cases by α -adducin and SF-1 genotype, α -adducin and IC genotype, ACE and SF-1 genotype, and ACE and IC genotype were compared with that of control subjects.

Discussion

We have described a new method for generating genotypic data for the α -adducin G460W polymorphism, combining DNA amplification by PCR and digestion with a restriction endonuclease. This method is advantageous in comparison with the allele-specific oligonucleotide hybridization protocol² because it avoids the use of radiochemicals, reduces the likelihood of false results, and enables high throughput.

There is close homology ($\approx 94\%$) for the α -adducin gene between rats and humans.²³ Known point mutations, 1 each in the α - and β -adducin subunits, account for up to 50% of the difference in blood pressure between the Milan hypertensive and normotensive rat strains.²³ Furthermore, transfection of hypertensive and normotensive α -adducin variants into rat renal epithelial cells showed that the former variant increased the surface expression and maximum velocity of the sodium-potassium pump compared with the latter variant, resulting in increased renal tubular sodium reabsorption.⁵

Cusi et al,² studying Italian and French hypertensive populations, reported a significant linkage of the α -adducin locus to essential hypertension and greater sensitivity to changes in sodium balance among patients with the mutant (*W*) allele, suggesting that α -adducin is associated with a salt-sensitive form of essential hypertension. Subsequent studies of sodium depletion and sodium loading revealed that as in Milan hypertensive strain rats, humans bearing 1 *W* α -adducin variant displayed an increased renal tubular sodium reabsorption.⁶ However, results of studies investigating the association between the α -adducin G460W polymorphism and hypertension in Japanese subjects have proved conflicting. As in Italian and French populations, Tamaki et al²⁴ found that the GG genotype of the α -adducin polymorphism was more common in the normotensive group than in the hypertensive group and that the *W* allele was significantly associated with lower plasma renin activity. However, although Kato et al⁹ were unable to confirm this significant association, they found that the *W* allele appeared to be relatively common in the Japanese (54% to 60%) compared

with a reported prevalence of 13% to 23% in whites. Furthermore, in a Scottish population, a study involving parents and offspring with blood pressures in either the upper or bottom 30% of the population distribution revealed that the α -adducin *W* allele was not related to blood pressure and did not affect whole-body or cellular sodium metabolism.¹⁰ However, it might be argued that this study examined only subjects who had blood pressures at the upper and lower sections of the young population and did not assess the frequency of this polymorphism in hypertension. Glorioso et al¹¹ examined the possible cause(s) of these discrepancies between populations. In a case-control study, they found no association of the α -adducin *W* allele with hypertension in a large population from Sassari, Italy, but confirmed a positive association in a large population from Milan, Italy. The authors suggested 2 reasons for this. First, the detection of a positive association may be heavily dependent on mild case-control differences in confounding factors such as population stratification, environment, lifestyle, age, BMI, and gender. Second, different frequencies of another genetic variant may exist between Sassari hypertensives and normotensives, affecting either the constitutive effect of the *W* allele on tubular reabsorption or the sequence of events linking the rate of tubular reabsorption to arterial hypertension.

In the present study, we found no evidence of a significant difference in the frequency of the α -adducin G460W polymorphism between our Scottish hypertensive and normotensive populations. The overall frequency of the *W* allele was within the same range as that reported in other studies of white populations.^{2,10} Thus, our failure to repeat the finding of Cusi et al² is consistent with the negative report from the young Scottish population.¹⁰ Although case-control studies can be criticized because of the risk of false-positive findings, particularly where populations are not homogeneous, we were careful to ensure that the matching of cases and control subjects was exact. Furthermore, all subjects were drawn from an ethnically and geographically limited catchment area.

Studies in genetically hypertensive rats and their normotensive controls revealed a linkage of a chromosomal region containing the ACE gene with blood pressure. This led to the hypothesis that ACE is a possible candidate gene for primary hypertension in humans. However, Schmidt et al,²⁵ studying Dutch parental couples who both had either high or low blood pressure and their offspring, found that allele frequencies were similar in parents with high and low blood pressure and in their offspring. Also, Harrap et al²⁶ could find no evidence that in a group of whites selected from the general population, the ACE gene was associated with genetic predisposition to high blood pressure. Interestingly, in Japanese patients with essential hypertension, the *D* allele was associated with early onset of hypertension and left ventricular hypertrophy, although blood pressure levels and the severity of damage to other organs were unaltered.²⁷ The Framingham Heart Study, which consisted of a large, population-based sample of men and women (3095 participants in the association analysis and 1044 pairs of siblings in the linkage analysis), found evidence of an association and genetic linkage of the ACE locus with hypertension and with diastolic blood pressure in men but not women, supporting the hypothesis that ACE, or a nearby

gene, is a gender-specific candidate gene for hypertension.²⁸ However, in this study, we found no evidence of a significant difference in the distribution of the *ACE I/D* polymorphism between our cases and control subjects.

Blood pressure is reported to correlate positively with body sodium and negatively with body potassium, suggesting a role for the sodium-retaining hormone aldosterone in essential hypertension.¹⁸ Therefore, genes that influence the regulation of secretion and action of aldosterone are of particular interest. We have previously reported that the distributions of genotypes for the *SF-1* and *IC* polymorphisms in the gene encoding aldosterone synthase were significantly different between the same groups of essential hypertensives and control subjects in the present study.¹⁹ Previous studies have examined the α -adducin, *ACE*, and aldosterone synthase polymorphisms individually in hypertensive populations; however, it is of interest to study their interaction because all 3 genes play key roles in the regulation of renal sodium handling. We found no evidence to suggest an interaction among these loci in our hypertensive population.

The α -adducin and *ACE* genes are not the first to show apparent variability in their relation with blood pressure in different studies. Inconsistency among populations has been noted for a number of other proposed candidate genes for hypertension. It is important, therefore, to identify within specific groups the local relevance of particular genetic markers. In our Scottish population, the α -adducin *G460W* and *ACE I/D* polymorphisms appear to exert no influence on blood pressure either individually or in combination.

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