Human Molecular Genetics, 2001, Vol. 10, No. 10 **1077–1084**

Trans-ethnic fine mapping of a quantitative trait locus for circulating angiotensin I-converting enzyme (ACE)

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Received 22 January 2001; Revised and Accepted 8 March 2001

Circulating angiotensin I-converting enzyme (ACE) levels are influenced by a major quantitative trait locus (QTL) that maps to the ACE gene. Phylogenetic and measured haplotype analyses have suggested that the ACE-linked QTL lies downstream of a putative ancestral breakpoint located near to position 6435. However, strong linkage disequilibrium between markers in the 3′ portion of the gene has prevented further resolution of the QTL in Caucasian subjects. We have examined 10 ACE gene polymorphisms in Afro-Caribbean families recruited in Jamaica. Variance components analyses showed strong evidence of linkage and association to circulating ACE levels. When the linkage results were contrasted with those from a set of British Caucasian families, there was no evidence for heterogeneity between the samples. However, patterns of allelic association between the markers and circulating ACE levels differed significantly in the two data sets. In the British families, three markers [G2215A, *Alu* insertion/deletion and G2350A] were in complete disequilibrium with the ACE-linked QTL. In the Jamaican families, only marker G2350A showed strong but incomplete disequilibrium with the ACE-linked QTL. These results suggest that additional unobserved polymorphisms have an effect on circulating ACE levels in Jamaican families. Furthermore, our results show that a variance components approach combined with structured, quantitative comparisons between families from different ethnic groups may be a useful strategy for helping to determine which, if any, variants in a small genomic region directly influence a quantitative trait.

INTRODUCTION

There is considerable evidence from physiological and pharmacological studies that angiotensin I-converting enzyme (ACE) plays a critical role in the maintenance of cardiovascular homeostasis (1–3). The important role of ACE is further underscored by evidence from clinical trials showing that the use of ACE inhibitor medication in the treatment of a range of conditions confers benefits that may be independent of blood pressure lowering effects (4–11). More recently, studies of ACE inhibition in experimental animals suggest that ACE activity in early life may be a critical determinant of future blood pressure (12). Thus, there is great interest in defining the mechanisms that influence ACE activity.

Combined segregation and linkage analyses in white European families have shown that ACE activity is strongly influenced by a quantitative trait locus (QTL) either within or very close to the ACE gene (13). This putative QTL is in strong linkage disequilibrium with the *Alu* insertion/deletion (I/D) marker in intron 16. Other polymorphisms in the ACE gene have been detected in sequencing studies (14) and an analysis of British subjects has shown that these polymorphisms define three clades (15); one clade is marked by the *Alu* insertion allele and the other two are marked by the *Alu* deletion allele. One of these clades may have arisen following an ancestral recombination event between members of the other two, more common, clades. These inferences have been confirmed by analyses of the complete genomic sequence of ACE (16) and additional genotyping in white British subjects has facilitated further refinement of the location of the putative ancestral breakpoint to a position 3′ of the 6435 polymorphism (17).

The identification of the putative ancestral breakpoint has enabled more detailed analysis of the genetic determination of circulating ACE levels. Measured haplotype analyses in white British families (15,17) have demonstrated that the major ACE-linked QTL is located 3′ of the putative ancestral breakpoint; the *Alu* insertion clade is associated with low circulating ACE levels while the two *Alu* deletion clades (which share 3′ but not 5′ sequences) are both associated with high ACE levels. Unfortunately, the relatively limited haplotype diversity found in white European populations appears to place restrictions on further refinement of the position of the ACE-linked QTL.

The well-characterized and robust evidence for linkage and association of circulating ACE levels to the ACE locus defines

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a model system for mapping quantitative genetic effects. An exciting strategy for association studies is trans-ethnic mapping, by which differences in linkage disequilibrium patterns between populations can be used to calibrate the resolution of association (18,19). Since the ACE phenotype displays high heritability (>60%) and the ACE locus accounts for much of the genetic variance (15), the prospects for this type of study may be evaluated in relatively modest samples.

In African-descent populations, where greater haplotype diversity is likely (20–22), there have been few studies of the genetic determinants of circulating ACE levels. However, associations between the I/D polymorphism and plasma ACE levels (23) and evidence of significant familial correlations (24) have been demonstrated. In Afro-Caribbean families recruited from Jamaica an ACE-linked QTL for circulating ACE levels, which is in significant disequilibrium with the I/D marker, has also been reported (25). However, the linkage disequilibrium between the I/D marker and the ACE-linked QTL appears to be much weaker than observed in white populations. This observation supports the view that comparisons of Afro-Caribbean and Caucasian families may be useful for fine localization of the putative ACE-linked QTL.

Measured haplotype approaches, although promising, can be difficult to execute, computationally intensive and may depend critically on both inferences related to the topology of maximum-parsimony trees and inferences about the existence of recombinant breakpoints (26). Recently, a flexible, general variance components model of linkage and association has been developed. This approach has the features of explicit modelling of linkage, direct assessment of population substructure, flexible handling of general family structures and also permits direct estimation of the proportion of the major locus additive genetic variance explained by disequilibrium between a marker and a QTL (27,28). These features are important for studying a Jamaican sample, where ~7% European admixture has been noted (29).

In this paper we report the results of tests of association and linkage between 10 polymorphisms at the ACE locus, and circulating ACE levels. Linkage, association and transmission disequilibrium analyses were carried out on African-descent Jamaican families in an attempt to identify the minimal set of polymorphisms associated with ACE. The properties of the unobserved trait locus were also investigated. The novel data from these Jamaican families were then compared and contrasted with data from a set of white British families which have previously been analysed using these methods (30) in order to determine whether the same markers explain the observed linkage effects in both groups. Heterogeneity tests between the samples were constructed to examine the ACE associations in the context of the combined data.

RESULTS

ACE activity measurements were available for 98 men and 138 women in the Jamaican families and 187 men and 218 women in the British families. Table 1 presents allele frequencies and pairwise disequilibrium coefficients for the markers in both sets of families. It is apparent that the allele frequencies are somewhat different in the two sets of families and, in general, the heterozygosity is greater in the British families. It is also evident that there is far less disequilibrium between marker alleles in the Jamaican than in the British population sample (Table 1). Pairwise disequilibrium coefficient (D′) values in the British sample are uniformly greater than 0.7 and are all significantly different from zero ($P < 0.0001$); in contrast, in the Jamaican sample, D′ values show considerable variation and are not significant in several cases.

In the Jamaican families there is significant evidence of linkage between ACE and the genotyped markers. However, the multipoint linkage curve appears to be fairly flat (Fig. 1, top right) with a maximum LOD score of ~4.0 [1 degree of freedom (d.f.), $P < 0.00001$]. There is significant evidence of association between these markers and ACE levels, although there is considerable variation between markers. In these families many markers are only weakly associated with ACE levels. The marker showing strongest association in the Jamaican population is G2350A, both at the population level ($\chi^2 = 25.48$, equivalent to LOD = 5.53; 1 d.f., $P < 0.00001$) and after a transmission disequilibrium test (27) which confirms this association ($\chi^2 = 7.65$, equivalent to LOD = 1.66; 1 d.f., $P = 0.006$). G2350A is also the marker with the lowest χ^2 value for linkage after accounting for association, but significant evidence for additional important variants at this locus can still be found (Fig. 1, bottom); G2350A accounts for only about half of the observed linkage.

We also present the results of our previous analysis of British families (30) for the purpose of examining the differences between the results in these families and our new results in the Jamaican families.

In the British families there is somewhat greater evidence of linkage between circulating ACE and the genotyped markers. The multipoint linkage curve is similarly flat (Fig 1, top left) but the maximum LOD score is ~7.2 (1 d.f., $P < 0.00001$). The evidence for association between these markers and ACE levels appears to be stronger than in the Jamaican families. All markers show significant association ($\chi^2 > 63.6$, equivalent to LOD > 13.8; 1 d.f., $P < 0.00001$) and as in the Jamaican families, the transmission disequilibrium test confirms that these associations are not due to population substructure ($\chi^2 > 41.6$, equivalent to LOD > 9.0; 1 d.f., $P < 0.00001$) (Fig. 1, middle left). When evidence of linkage is assessed after accounting for association, the LOD scores are progressively and substantially diminished as the gene is traversed in the 5′→3′ direction (Fig. 1, bottom left). At markers G2215A, I/D and G2350A, the evidence for linkage is nearly zero (LOD scores of 0.20, <0.01 and <0.01, respectively), which suggests that all of the important functional variants are in very strong linkage disequilibrium with these three markers.

To assess differences between the two data sets, a series of sample heterogeneity tests was conducted (Table 2). The highly significant heritability ($P < 0.00001$) of circulating ACE levels (polygenic variance $\sigma_g^2 = 0.72$ in the Jamaican families and $\sigma_g^2 = 0.67$ in the British families) was not significantly different between the family sets ($\chi^2 = 0.10$, 3 d.f., $P > 0.9$). Moreover, the strength of evidence for linkage between the ACE locus and circulating ACE levels (major locus variance $\sigma_a^2 = 0.64$ in the Jamaican families and $\sigma_a^2 = 0.60$ in the British families) was also not significantly different (tests of heterogeneity for linkage models all give $\chi^2 < 0.28$, 4 d.f., $P > 0.9$). Thus, the differences in the significance of linkage effects (Fig. 1, top) are simply due to differences in statistical power owing to the different cohort sizes. These results indicate that

Table 1. Allele frequencies and pairwise disequilibrium coefficients

Pairwise disequilibrium coefficients (D′) for British families										
	A-5466C	T-3892C	A-240T	T-93C	T1237C	G2215A	ID	G2350A	4656(CT)	Frequency
T-5491C	0.91	0.91	0.92	0.85	0.88	0.78	0.82	0.85	0.75	0.31
A-5466C		0.94	0.89	0.89	0.86	0.77	0.84	0.85	0.75	0.32
T-3892C			0.86	0.91	0.82	0.80	0.79	0.77	0.77	0.42
A-240T				0.91	0.86	0.78	0.85	0.89	0.74	0.31
T-93C					0.88	0.82	0.81	0.83	0.84	0.31
T1237C						0.85	0.86	0.88	0.84	0.45
G2215A							0.93	0.93	0.90	0.48
I/D								0.95	0.88	0.51
G2350A									0.90	0.51
Frequency	0.32	0.42	0.31	0.31	0.45	0.48	0.51	0.51	0.52	
Pairwise disequilibrium coefficients (D′) for Jamaican families										
	A-5466C	T-3892C	A-240T	T-93C	T1237C	G2215A	ID	G2350A	4656(CT)	Frequency
T-5491C	0.89	**0.51**	0.89	0.71	0.63	0.40	0.36	**0.25**	0.26	0.31
A-5466C		1.00	0.89	0.72	0.65	0.38	0.34	**0.09**	0.25	0.33
T-3892C			1.00	**1.00**	0.93	0.74	0.74	**0.67**	0.58	0.14
A-240T				0.71	1.00	0.31	0.27	**0.19**	0.28	0.31
T-93C					**1.00**	**0.42**	**0.40**	0.68	0.60	0.07
T1237C						0.80	0.80	0.85	0.68	0.16
G2215A							0.96	0.95	**0.19**	0.48
ID								1.00	**0.20**	0.47
G2350A									**0.26**	0.17
Frequency	0.33	0.14	0.31	0.07	0.16	0.48	0.47	0.17	0.32	

Allele frequencies and pairwise disequilibrium coefficients estimated from founder haplotypes deduced by Simwalk2 (38).
All the D′ values in the British data set are significantly different from zero ($P < 0.01$).
In the Jamaican data set, D′ values which are not significant ($P > 0.05$) are shown in bold type.

the overall contribution of the ACE locus to circulating ACE levels is similar in the two populations.

In sharp contrast, the results for the association models differ significantly between the two data sets (Table 2). Examination of these χ^2 values demonstrates that there is considerable heterogeneity between the family sets for association between markers and circulating ACE levels, for all of the polymorphic markers (tests of heterogeneity for association models all give $\chi^2 \geq 11.08$, 5 d.f., $P < 0.01$). These results show that patterns of disequilibrium between the ACE-linked QTL and these markers vary significantly between populations.

Although the markers G2215A, I/D and G2350A are in very strong disequilibrium with the putative ACE QTL in the UK families, they can explain only part of the ACE effect in Jamaican families. Now, if a single polymorphism determines ACE levels, it is possible to estimate the properties of the unobserved QTL using asymptotic derivations which have been described previously (28). For the UK data, disequilibrium (D′) between markers G2215A, I/D and G2350A and the unobserved QTL is estimated at >97%. Furthermore, the trait allele is expected to be very common (40–60%). In the Jamaican data, the predicted D′ between the unobserved QTL and G2350A is smaller (>63%) and therefore the boundaries for QTL allele frequencies are wide (10–90%). Alternatively, there may be additional functional polymorphisms in the region [such as the putative 5′ functional variant previously reported in French Caucasian families (14)], at least one of which is in disequilibrium with G2350A (D′ > 63%) and has allele frequencies between 10 and 90%.

DISCUSSION

In the presence of strong disequilibrium, it is difficult to determine which of several markers in a region is actually a functional mutation influencing trait levels. Where families have been sampled from populations of relatively limited haplotype diversity, it has been possible to use measured haplotype analysis to refine the location of the putative ACE-linked QTL to some extent (15,17). Unfortunately, it appears as if the resolution of this approach in such populations will be limited by the rarity of the additional recombinant breakpoints required for further localization. On the other hand, in families drawn from African-descent populations where there is greater haplotype diversity (16,31) the combined requirement for large numbers of

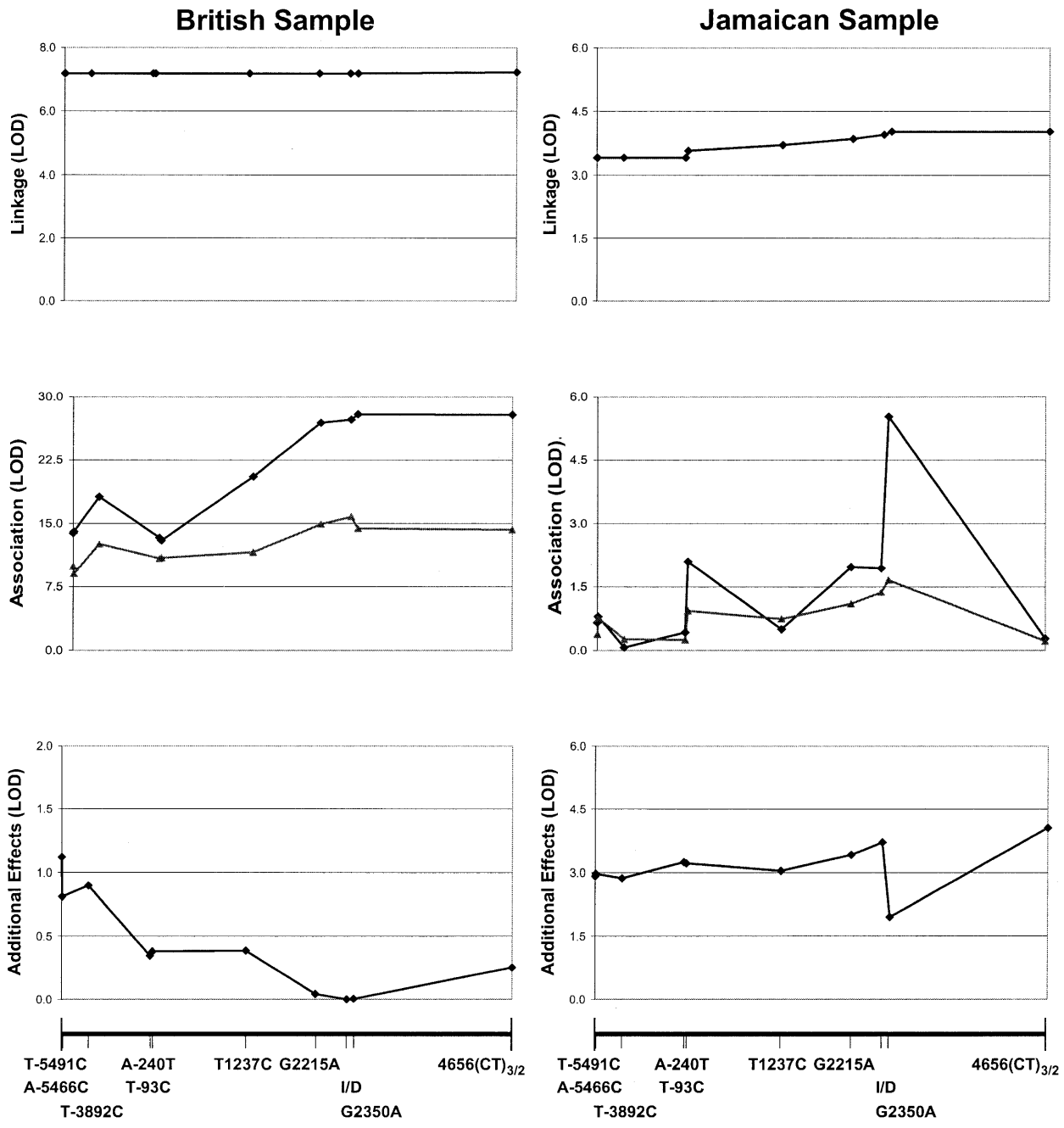

Figure 1. Comparison of linkage and association in British and Jamaican families. The top panels show evidence for linkage without any consideration of allelic association. The middle panels show evidence for global association (diamonds, subject to population substructure) and for robust association (triangles, transmission disequilibrium). The bottom panels show remaining evidence for linkage after accounting for the effects explained by association at each marker.

independent haplotypes as well as suitable numbers of individuals in each haplotype class makes measured haplotype analyses difficult to achieve.

We have used a variance components approach to examine association, linkage and linkage adjusted for association, between 10 polymorphisms in the ACE gene and circulating ACE levels. We have demonstrated similarities between Afro-Caribbean families recruited in Jamaica and Caucasian families recruited in the UK; both the heritability and the overall linkage of the ACE region to circulating ACE levels do not differ between the sets of families ($P > 0.9$). In contrast to these similarities, the associations between markers and ACE levels differ considerably between family sets. Not only is the strength of the association on average far less in the Jamaican families than in the British families but the evidence of association for the marker exhibiting maximum disequilibrium is also clearly different ($P < 0.00001$). In addition, while the linkage statistic adjusted for association approaches zero for

Table 2. Log-likelihoods and χ^2 tests of heterogeneity between British and Jamaican families

	Allowing heterogeneity			Combined sample	
	$\ln(L_{UK})$	$\ln(L_{Jamaica})$	$\ln(L_{UK})+\ln(L_{Jamaica})$	$\ln(L_{combined})$	χ^2
Polygenic model	–544.77	–316.50	–861.27	–861.32	0.10
Linkage					
T-5491C	–528.22	–308.64	–836.86	–836.99	0.26
A-5466C	–528.22	–308.64	–836.86	–837.00	0.28
T-3892C	–528.22	–308.64	–836.86	–837.00	0.28
A-240T	–528.23	–308.64	–836.87	–837.00	0.26
T-93C	–528.22	–308.28	–836.50	–836.60	0.20
T1237C	–528.22	–307.95	–836.17	–836.25	0.16
G2215A	–528.22	–307.65	–835.87	–835.94	0.14
I/D	–528.22	–307.40	–835.62	–835.70	0.16
G2350A	–528.22	–307.22	–835.44	–835.52	0.16
4656(CT)	–528.13	–307.22	–835.35	–835.42	0.14
Association					
T-5491C	–481.40	–303.30	–784.70	–806.16	42.92[a]
A-5466C	–479.50	–300.58	–780.08	–802.76	45.36[a]
T-3892C	–480.55	–304.18	–784.73	–802.67	35.88[a]
A-240T	–493.73	–305.79	–799.52	–818.31	37.58[a]
T-93C	–483.96	–295.53	–779.49	–785.03	11.08[a]
T1237C	–449.86	–301.41	–751.27	–764.13	25.72[a]
G2215A	–428.46	–301.40	–729.86	–746.53	33.34[a]
I/D	–461.56	–302.94	–764.50	–780.95	32.90[a]
G2350A	–422.64	–288.17	–710.81	–730.91	40.20[a]
4656(CT)	–444.82	–300.66	–745.48	–784.03	77.10[a]

The sum of maximum log-likelihoods [ln(L)] computed for each data set independently was compared with the maximum likelihood of a combined Jamaican and British data set. Likelihood ratio χ^2 values are given in the last column.
[a]Significant values at $P < .01$, indicating significant differences between data sets.
For the polygenic model, an overall mean parameter and non-shared and polygenic variances were estimated. The random effects model included the polygenic model and an additional major locus linkage variance. The association model included between- and within-family association parameters and an intercept term.

three markers (G2215A, I/D and G2350A) in the British families, the only marker in Jamaican families that shows a large diminution in the evidence for linkage after adjusting for association is G2350A, where the value is almost halved. Taken together, these results suggest that ACE gene polymorphisms contribute substantially to variation in ACE levels in the two samples.

There are a number of potential explanations for the highly significant differences in the strength of association between the markers and ACE levels in the two cohorts of families. For instance, it is possible that the functional polymorphism was not one of the markers genotyped in this study and, in addition, has different linkage disequilibrium relationships with the markers in the two sets of families. Alternatively, different functional polymorphisms may be responsible for the linkage and association effects in the two populations. Another possibility is that there may be multiple functional variants within the ACE gene which influence ACE levels; in French Caucasian families it has been reported that there may be QTLs in both 5′ and 3′ regions of the ACE gene (14).

With current statistical methods and the present data, it is not formally possible to discriminate between these competing alternatives. However, the hypothesis that suggests that there is a single ACE-linked variant, which has not been typed in either population, appears to be somewhat better supported. In both populations the markers are polymorphic and their major alleles are of comparable frequency. In addition, if the common ACE gene clades diverged ~1.1 million years ago as proposed previously (16), the existence of functionally different clades would predate the separation of humans into present-day ethnic groupings.

For data of the type presented here, trans-ethnic mapping may be a useful tool to complement measured haplotype analyses. In the British families there is strong disequilibrium which allows easy identification of the ACE QTL but with limited resolution. The Jamaican families, on the other hand, show far

less disequilibrium in this region and thus may be more useful for fine localization of the ACE QTL. The greater diversity observed in the Jamaican families might facilitate distinction of the true QTL polymorphism from the other non-functional marker loci, whereas this task might require prohibitively large data sets in white European populations.

In conclusion, our results suggest that the major ACE-linked QTL is not likely to be one of the markers typed although it is likely to lie close to G2350A. Furthermore, our results show that the combination of trans-ethnic comparisons with robust variance components analyses may have utility in efforts to identify a functional polymorphism from among a series of markers in a small genomic region. Extension of this work to polymorphisms which have been identified in the interval between G2215A and 4656$(CT)_{3/2}$ (i.e. both upstream and downstream of G2350A) (16), will be required in order to further refine the minimal set of polymorphisms which need to be examined in biological assays to conclusively determine their functional significance.

MATERIALS AND METHODS

Families and ACE trait

The details of recruitment of the families in this paper have been described previously (25,32). Briefly, 45 extended families in Jamaica and 83 families from the Oxfordshire region were recruited as part of projects to investigate the genetic determinants of blood pressure variability. Both sets of families were ascertained via a single hypertensive proband without regard to ACE activity levels. The University of the West Indies/University Hospital of the West Indies Ethics Committee and The Central Oxford Research Ethics Committee approved the protocols for recruitment of the families.

Circulating ACE activity was measured in both sets of families with the use of comparable techniques employing synthetic substrates. In the Jamaican families a spectrophotometric method was used (25) while in the British families HPLC was used (15). Correlational analyses found no evidence of associations between ACE levels and blood pressure, body mass index or age; consequently, these variables were not used as covariates in the association analyses. ACE levels were standardized using a common mean and standard deviation for males and females in the Jamaican families but were standardized separately in males and females in the British families to account for a small but significant gender difference.

Genotyping

DNA was extracted from whole blood using standard methods (33). The I/D polymorphism was typed using a PCR method (34) and the other nine polymorphisms were typed, according to protocols developed by us, using either PCR alone or PCR followed by restriction digests (15). Experimental details may be found at http://www.well.ox.ac.uk/~mfarrall/ACE_polymorphisms.html.

Statistical methods

Variance components models were used for all assessments of linkage, linkage disequilibrium and heterogeneity. Briefly, the phenotype model allows for fixed association effects, which are partitioned into a between- and within-family effect, as well as random effects, which are partitioned into environmental, polygenic and major locus linkage variances (27). The expectation for the phenotype y of each individual j in family i is:

$$\hat{y}_{ij} = \mu + \beta_b b_{ij} + \beta_w w_{ij}$$

In this model, μ, β_b and β_w are estimated as the population mean and regression coefficients for between- (b_{ij}) and within- (w_{ij}) family effects, respectively. All available genotype data are used in construction of the b_{ij} and w_{ij} values (30). The between-family component of association is specific to each nuclear family and could be influenced by population stratification, but within-family effects are sensitive only to linkage disequilibrium. In the absence of stratification they provide independent and unbiased estimates of the additive genetic effect (28). Deviations from these expectations for each pair of individuals j and k in family i are modelled as:

$$\Omega_{ijk} = \begin{cases} \sigma_a^2 + \sigma_g^2 + \sigma_e^2 & \text{if j = k} \\ \pi_{ijk}\sigma_a^2 + 2\varphi_{ijk}\sigma_g^2 & \text{if j} \neq \text{k} \end{cases}$$

where σ_a^2 is the additive genetic variance of the QTL, σ_g^2 is the variance attributable to polygenes and σ_e^2 is the residual environmental variance. π_{ijk} and φ_{ijk} are the proportion of alleles shared identical-by-descent at the marker locus and the kinship coefficient between individuals j and k in family i, respectively. The likelihood of the data can be expressed in terms of the observed phenotypes $\mathbf{y}_i$, the random effects in Ω_i and the linear model as:

$$L = \prod_i (2\pi)^{-n_i/2} \left|\hat{\Omega}\right|^{-1/2} e^{-1/2[(\mathbf{y}_i - \hat{\mathbf{y}}_i)' \hat{\Omega}_i^{-1} (\mathbf{y}_i - \hat{\mathbf{y}}_i)]}$$

(35). For tests of statistical significance of the parameters, two alternative likelihoods are fitted with the parameter(s) of interest fixed at zero (L_0) and with the parameter(s) left free to vary (L_1). We consider twice the difference in log-likelihoods to be asymptotically distributed as χ^2, with d.f. equal to the number of parameters evaluated in all significance tests. We maximized this likelihood under different sets of constraints to construct the following specific tests: (i) Overall genetic effect (test significance of σ_g^2 while constraining $\sigma_a^2 = \beta_b = \beta_w = 0$); (ii) Linkage (test significance of σ_a^2 while constraining $\beta_b = \beta_w = 0$); (iii) Global association, assuming no stratification (test significance of β_b and β_w while constraining $\beta_b = \beta_w$); (iv) Robust association through a transmission disequilibrium test (test significance of β_w); and (v) Test of population stratification (test whether $\beta_b - \beta_w = 0$).

Note that in all cases, the fixed effects μ, β_b and β_w and the random effects σ_a^2, σ_g^2 and σ_e^2 were left free to vary unless otherwise specified. Tests of significance and parameter estimation were conducted for each population separately, except when testing for heterogeneity. To test for heterogeneity, we fitted one of the models above to each population [leaving the

parameter(s) of interest free to vary] to obtain two likelihoods (L_{UK} and $L_{Jamaica}$). These were compared to the likelihood of the combined sample ($L_{combined}$) under the same model. In this comparison, $2[ln(L_{UK}) + ln(L_{Jamaica}) - ln(L_{combined})]$ is approximately distributed as χ^2 with d.f. equal to the total number of parameters estimated in the combined analysis.

A critical assumption of the variance components approach concerns multivariate normality, as departures from this can yield biased results (36). However, calculation of the Anderson–Darling statistic (37) did not indicate any extreme outliers in the full models, consistent with multivariate normality of the data.

ACKNOWLEDGEMENTS

We are grateful to the Jamaican and British families who participated in these studies. We thank the Oxfordshire general practitioners that contributed index cases, Dr Joy Callender and nurses Hazel Hylton, Valerie Beckford, Hyacinth Gallimore, Sarah Pollitt, Anna Zawadzka and Jane Cadd for expert technical help. We are also grateful to Dr Martin Farrall for helpful discussions and critical reading of the manuscript. This work was supported in part by the Wellcome Trust, by the National Eye Institute, National Institutes of Health, USA (grant EY-12562 to L.R.C.), by the British Heart Foundation and by the Medical Research Council, UK (Training Fellowship to B.K. and grant G108/325 to C.A.M.).

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