Cardiovascular Topics

Association study of eight candidate genes with renin status in mild-to-moderate hypertension in patients of African ancestry

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Summary

Aim. We evaluated whether any one variant of genes that encode for substances that could modulate renin-angiotensin-aldosterone (RAA) system activity can account for a substantial proportion of the variability of plasma RAA system profiles in black South African hypertensives (HTs).

Methods. Plasma renin activity (PRA) and aldosterone concentrations (ALD) were determined in 59 black subjects with mild-to-moderate HT off therapy on an *ad libitum* diet. Patients were genotyped for the angiotensin-converting enzyme (ACE) gene insertion/deletion, angiotensinogen (AGT) gene M235T, A-20C and G-6A, aldosterone synthase (*CYP11B2*) gene C-344T, G protein β3-subunit (GNB3) gene C825T, G_s protein gene C131T, atrial natriuretic peptide (ANP) gene exon 3 stop codon and intron 2, α-adducin gene Gly460Trp, and epithelial Na^+ channel (eNa^+_c) gene T594M polymorphisms.

Results. Risk genotype frequencies for the G_s (7%), ANP intron 2 (0%), and eNa^+_c (7%) variants were too low for each to account for a substantial portion of the variability of plasma RAA profiles in the group studied. Moreover, assuming either recessive or dominant inheritance models, neither ACE, AGT, GNB3, *CYP11B2*, ANP exon 3 nor α-adducin polymorphisms were significantly associated with the variance of PRA, ALD or ALD/PRA.

Conclusions. These results do not support a substantial individual role for the gene candidates studied in contributing to plasma RAA system profiles in black South African HTs. However, a potential small role for some loci may exist, and epistasis or genotype-phenotype interactions as well as alternative inheritance models and variants still need to be evaluated.

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Although not routinely used in clinical practice, measures of the activity of the renin-angiotensin-aldosterone (RAA) system have for decades been considered key indicators of specific pathophysiological mechanisms involved in the development of both primary and secondary forms of hypertension (HT).[1] Importantly, the predominant RAA system characteristics in black subjects of sub-Saharan ancestry differ from other population groups.[2] Black hypertensives (HTs),[3] including those of South African ancestry,[4] have a higher prevalence of low-renin (LR) HT (LRHT). LRHT represents a unique phenotype in that it is not associated with a concomitant reduction in plasma aldosterone concentrations (ALD) in patients consuming an *ad libitum* salt diet,[4,5] but is associated with a reduced adrenal response to angiotensin II infusions on a low-salt diet.[6] Moreover, LRHT is associated with salt-sensitivity[6] and an increased blood pressure (BP) response to angiotensin infusions.[6] Therefore LRHT may represent a marker of a subgroup of patients with specific pathophysiological changes contributing to the development of HT.

The mechanisms responsible for LRHT have not been elucidated, but a genetic basis has been proposed.[3] RAA system status in HT has been shown to be associated with a number of variants of genes that encode for substances that could either directly or indirectly (through alterations in Na^+ homeostasis) modulate the renal release of renin or the synthesis/release of aldosterone from the adrenal cortex.[7-12] The aim of the present study was therefore to assess whether variants of these[7-12] and other genes that encode for substances that could either directly or indirectly influence RAA system activity can account for RAA system variance in black HTs.

Methods

This study was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand (approval numbers M940520 and M951122).

Study group and blood pressures

In order to avoid population stratification, only HT patients of the Nguni and Sotho chiefdoms of South Africa were selected. Fifty-nine HTs initially screened at the Johannesburg Hospital HT clinic were recruited if they had mean daytime ambulatory diastolic BPs (DBP) > 90 mmHg (Spacelabs model 90207) after 4 weeks off medication. Patients with type I diabetes mellitus, uncontrolled type II diabetes mellitus (defined as an HbA_{1c} level of > 10%), secondary HT, severe HT (BP > 220/115 mmHg), hypertensive retinopathy, or diseases which could have interfered with BP and RAA system hormonal measurements (renal, endocrine and cardiac diseases including heart failure) were excluded. Ambulatory BP measurements were performed at least half-hourly during the day and hourly during the night from 22h00 to 06h00. Ambulatory monitors were calibrated against a mercury sphygmomanometer before use in each patient with the calibration being considered acceptable if the monitor readings were within 3 mmHg or 2% of the manometer readings. A 24-hour reading was considered successful if > 85% of all intended recordings were obtained. Patients in whom unsuccessful recordings were obtained had recordings repeated the following day. All patients were advised not to smoke, imbibe alcohol or ingest caffeine during this period.

Hormonal measurements

Venous blood samples were obtained between 08h00 and 13h00 hours for hormone assessments after 20 minutes of rest in the supine position at the end of the 4-week period off therapy. No dietary modifications were imposed before collecting blood samples. Plasma samples were stored at −70°C until the time of shipment to BCO Analytical Services, The Netherlands, where assays were performed according to good laboratory practice. Plasma renin activity (PRA) in ng/ml/h and plasma ALD in pmol/l were determined using standard radio-immunoassay kits from DuPont (Billercia, Mass.) and Diagnostic Products Corporation (Los Angeles), respectively.

Genotyping

Deoxyribonucleic acid was extracted from whole blood by lysing red blood cells and digesting the remaining white cell pellet with proteinase K. The insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene was detected by the polymerase chain reaction (PCR) technique using oligonucleotide primers flanking the insertion sequence as well as insertion-specific primer pairs.[13] Genotyping of the conservative threonine-to-cytosine transition at nucleotide 704 in exon 2 of the angiotensinogen (AGT) gene, where cytosine corresponds to the 235T polymorphism and threonine to the M235 polymorphism;[14] the A-to-C transition at nucleotide -20 of the 5′ upstream promoter region of the AGT gene;[15] the C-344T promoter region polymorphism of the aldosterone synthase (*CYP11B2*) gene;[16] an exon 3 variant in a region of a stop codon of the atrial natriuretic peptide (ANP) gene which results in the extension of ANP by two additional arginines;[17] a conservative intron 2 variant of the ANP gene;[17] a C825T polymorphism in exon 10 of the G protein β3-subunit (GNB3) gene;[12] a T-to-C transition at codon 131 of exon 5 of the stimulatory G ($G_s\alpha$) protein gene;[18] and the C-to-T transition of the epithelial Na^+ channel (eNa^+_c) gene which encodes for a T594M eNa^+_c β-subunit protein)[11] was undertaken using mismatch PCR-restriction fragment length polymorphism (RFLP)-based techniques employing the appropriate primer pairs and restriction enzymes. Moreover, the Gly460Trp variant of the α-adducin gene was determined using an allele-specific oligonucleotide hybridisation technique,[19] and the G-6A variant of the promoter region of the AGT gene was determined by sequencing using an ABI Dye Terminator Cycle Sequencing kit (Perkin Elmer) and an ABI 373 automated DNA sequencer.

Data analysis

To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers was determined using a χ^2 test. To evaluate whether genotype was associated with renin status, ALD (pmol/l)-to-PRA (ng/ml/h) ratios were determined and the frequency of patients with the risk genotype in the upper quartile of ALD-to-PRA ratios was compared with the frequency of patients with the risk genotype in the total group. To make these comparisons a 2 × 2 contingency table was constructed and Fisher's exact test performed. To evaluate whether individual gene loci are associated with hormonal measurements, PRA, plasma ALD and ALD-to-PRA ratios were compared between patients grouped according to genotype for individual loci using ANCOVA techniques adjusting for age, gender, body mass index (BMI), duration of hypertension, the presence or absence of type II diabetes mellitus, mean 24-hour BP measurements, and other genotypes as covariates. Because of the study sample size and the inequitable distribution of alleles, analysis was performed assuming a limited number of inheritance models only (see Table II for summary). Risk genotypes were identified from either published data (references given in Table II) showing an association with the development of HT, an intermediate phenotype, or RAA system status, or from data obtained in our laboratory showing an association with the development of HT in the population sampled in the present study. If the frequency of one of the alleles was low for specific variants, data from patients homozygous for this allele (if patients homozygous for this allele were identified) was added to data obtained for patients heterozygous for this allele. Only unadjusted values are provided and data are expressed as mean ± standard error of the mean (SEM).

Results

General characteristics of the study group

In the total group studied a preponderance of females and individuals with an increased BMI was noted (Table I). BP measurements reflect recruitment of patients with mild-to-moderate HT. Mean PRA values for the group reflect a high prevalence of LRHT (Table I). In contrast, ALD-to-PRA ratio values in the group are not consistent with some published definitions of LRHT.[8,9] However, because normal values for PRA, ALD and ALD-to-PRA have not been adequately defined in the population sampled we cannot comment on the frequency of LRHT in the HT group.

TABLE I. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF HYPERTENSIVE PATIENTS OF AFRICAN ANCESTRY STUDIED

Number studied	59
Age (years)	50.9 ± 1.1
Gender (female/male) (% female)	38/21 (64)
Body mass index (kg/m²)	30.1 ± 0.9
Type II diabetes mellitus (%)	3.4
Duration of hypertension (yrs)	2.64 ± 0.44
Auscultatory blood pressure (mmHg)	164 ± 2/103 ± 1
Ambulatory blood pressure (mmHg)	
24-hour	153 ± 2/99 ± 1
Day	156 ± 2/103 ± 1
Night	147 ± 2/94 ± 1
PRA (ng/ml/h)	0.93 ± 0.13
ALD (pmol/l)	249 ± 20
ALD/PRA	39 ± 34

PRA = plasma renin activity; ALD = plasma aldosterone concentrations.

TABLE II. RISK GENOTYPE NUMBERS AND FREQUENCIES (*N* AND %) IN THE TOTAL GROUP (*N* = 59) AND IN A GROUP COMPRISED OF PATIENTS WITH ALDOSTERONE-TO-RENIN (ALD-TO-PRA) RATIOS IN THE UPPER QUARTILE (*N* = 13).

				Risk genotype			
				Total group		*Upper quartile ALD-to-PRA*	
Gene (variant)		*Assumed risk genotype**	*Assumed model of inheritance*	*N*	%	*N*	%
AGT	(M235T)	TT[14]	Recessive	41	70	9	69
	(G-6A)	AA[10]	Recessive	51	86	12	92
	(A-20C)	AA[15]	Recessive	50	85	12	92
ACE	(I/D)	DD[22]	Recessive	25	42	6	46
CYP11B2	(C-344T)	TC[8,9]	Dominant	19	32	4	31
ANP	(exon 3)	WM + MM	Dominant	37	63	8	62
	(intron 2)	WM[17†]	-	0	0	0	0
GNB3	(C825T)	CT + CC[unpublished‡]	Dominant	14	24	2	15
G_s	(T131C)	CT[unpublished‡]	Dominant	4	7	0	0
eNa^+_c	(T594M)	CT[11]	Dominant	4	7	2	15
α-adducin	(Gly460Trp)	Gly460Trp[7]	Dominant	8	14	3	23

* Superscripts in this column indicate reference numbers.
† No risk genotype was detected.
‡Data obtained from our own group in a large case-control study involving over 500 patients and 500 controls assessing the development of HT.
N = number of patients in the sample with the risk genotype; AGT = angiotensinogen; ACE = angiotensin-converting enzyme; *CYP11B2* = aldosterone synthase; ANP = atrial natriuretic peptide; GNB3 = G protein β3 subunit; G_s = stimulatory G protein; eNa^+_c = epithelial Na^+ channel.

Genotype frequencies

All genetic variants examined were in Hardy-Weinberg equilibrium. The assumed risk genotypes for each of the variants examined are summarised in Table II. The frequencies of the risk genotypes for the eNa^+_c, G_s protein, and ANP intron 2 variants were too low (Table II) to be compatible with a hypothesis that these variants could individually account for a significant proportion of the variability of plasma RAA system profiles in the group. In contrast, the risk genotype frequencies of the other variants studied were in proportions that may account for a significant portion of plasma RAA system variability in the study group (Table II).

Genotype frequencies in patients with ALD-to-PRA ratios in the upper quartile

The frequency of patients with the risk genotype for each of the variants examined was not greater in patients with ALD-to-PRA ratios in the upper quartile of the group in comparison to the risk genotype frequencies noted in all 59 patients (Table II).

Effects of genotype on RAA system activity

The inheritance models assumed for each of the risk alleles in the analysis performed on data given in Table III are summarised in Table II. The frequencies of the risk genotypes for the eNa^+c, G_s protein and ANP intron 2 variants were too low to determine with confidence whether or not a relationship exists between these variants and RAA system activity. Assuming specific inheritance models (either recessive or dominant effects), none of the other gene polymorphisms examined were significantly associated with either PRA, ALD, or ALD-to-PRA ratios (Table III). However, PRA values tended to be lower in patients homozygous for the G-6A variant (Table III), and ALD values tended to be lower in patients with the C825 allele of the GNB3 gene variant (Table III).

Discussion

The main findings of the present study are as follows: The risk genotypes for the eNa^+_c, Gs protein and ANP intron 2 gene variants examined were at too low a frequency (0 -

TABLE III. RELATIONSHIP BETWEEN GENOTYPE AND RENIN-ALDOSTERONE ACTIVITY IN HYPERTENSIVE PATEINTS OF AFRICAN ANCESTRY. GENE VARIANTS WITH GENOTYPE FREQUENCIES TOO LOW TO ALLOW FOR MEANINGFUL COMPARISONS (G PROTEIN, ANP INTRON 2 AND eNa^+_C) HAVE BEEN EXCLUDED

Genotype	*N*	*PRA (ng/ml/h)*	*ALD (pmol/l)*	*ALD/PRA*
		Angiotensinogen gene M235T polymorphism		
TT	41	1.06 ± 0.18	260 ± 21	397 ± 43
MT	18	0.66 ± 0.08	231 ± 43	392 ± 58
		Angiotensinogen gene G-6A polymorphism		
AA	51	0.99 ± 0.13	263 ± 23	406 ± 39
GA	8	0.40 ± 0.33*	220 ± 90	511 ± 78
		Angiotensinogen gene A-20C polymorphism		
AA	50	0.94 ± 0.14	262 ± 23	410 ± 40
GA	9	1.08 ± 0.51	231 ± 50	341 ± 70
		Angiotensin-converting enzyme gene I/D polymorphism		
ID + II	29 + 5	1.00 ± 0.19	227 ± 22	372 ± 49
DD	25	0.84 ± 0.17	279 ± 36	416 ± 45
		CYP11B2 gene C-344T polymorphism		
CT + CC	18 + 1	0.94 ± 0.21	273 ± 30	396 ± 54
TT	40	0.95 ± 0.18	247 ± 27	404 ± 46
		Atrial natriuretic peptide gene exon 3 polymorphism		
WW	22	1.16 ± 0.26	250 ± 31	382 ± 64
WM + MM	30 + 7	0.80 ± 0.13	248 ± 26	396 ± 39
		G protein β3 subunit gene C825T polymorphism		
CT + CC	12 + 2	0.91 ± 0.30	187 ± 28*	345 ± 76
TT	45	0.95 ± 0.15	269 ± 24	410 ± 39
		α-adducin Gly460Trp polymorphism		
GlyGly	51	0.90 ± 0.14	243 ± 20	371 ± 38
GlyTrp	8	0.91 ± 0.3	267± 50	464 ± 103

*P = 0.09 versus TT and AA groups.
PRA = plasma renin activity; ALD = plasma aldosterone concentrations; *CYP11B2* = aldosterone synthase; W = wild type; M = mutation. No significant differences in PRA, ALD, or ALD-to-PRA ratios between genotype groups were noted.

7%) to account for a significant proportion of RAA system variability in the group studied. Moreover, assuming specific inheritance models, neither ACE, AGT, *CYP11B2*, ANP (exon 3), α-adducin, nor GNB3 protein gene variants examined were significantly associated with RAA system activity in the group studied.

In the present study, only urban black HTs were recruited. We deliberately excluded normotensive urban blacks and rural black subjects for the following reasons. An inverse relationship between plasma renin status and BP has been noted in urban but not in rural blacks,[20] and the relationship that normally exists between plasma renin and aldosterone concentrations is lost in hypertensive but not in normotensive or rural black South Africans.[20] Therefore, the RAA system hormonal characteristics of urban black hypertensives, but not urban black normotensives or rural black subjects, are consistent with a low-renin phenotype. Although the reduction in PRA that occurs with urbanisation in black South Africans[20] suggests that environmental factors contribute to RAA system activity, this finding explains neither the lower PRA values in urban hypertensives compared with normotensives[20] nor the normal plasma aldosterone concentrations in the presence of low PRA values,[20] changes which suggest a genetic effect also contributing to renin phenotype.

In the present study we were unable to distinguish between patients with LRHT and normal-to-high renin hypertension, because normal values for ALD, PRA and ALD-to-PRA ratios have not been adequately defined for the population sampled. In comparison with previous definitions of LRHT provided for other populations, our study group might have had either a high or a low prevalence of LRHT. A PRA value of < 1 ng/ml/h has been used to define LRHT in a population with a high prevalence of LRHT (Chileans).[21] Using this definition, 75% of our study group had LRHT. However, when using other definitions of LRHT as defined for Chilean and Japanese (who are also thought to have a high frequency of LRHT) populations (ALD-to-PRA ratios of > 830,[9] or PRA values < 0.2 ng/ml/h[8]), only 9 - 14% of our study group had LRHT. However, irrespective of whether our study group had a high prevalence of LRHT, the gene variants studied could not account for a substantial proportion of the variability of RAA system profiles.

The reasons for selection of the gene candidates examined in the present study were based on their potential direct or indirect effects on either renin release or aldosterone synthesis/release, as LRHT is characterised by a low PRA, normal ALD and hence an inappropriate relationship between ALD and PRA.[4,5] ACE, AGT and *CYP11B2* gene polymorphisms were examined as they could explain an inappropriately 'normal' ALD in the presence of reduced PRA values. ANP, eNa^+_c and α-adducin gene variants were examined as they could induce a salt-sensitive state and hence a reduced PRA. G-protein polymorphisms were examined as they could influence renin release through direct (renin release being mediated by a β_1-adrenoceptor-G protein coupled-cAMP second messenger system) or indirect (sodium retention mediated through a G protein coupled Na^+ exchanger in renal tubules) mechanisms.

The small sample size employed in this study could have prevented us from detecting a small contribution of each of these genes to RAA system status. Indeed, a number of the gene variants examined in the present study have been shown in other population groups to be associated with RAA system activity. These include the Gly460Trp variant of the α-adducin gene,[7] the C-344T variant of the *CYP11B2* gene,[8,9] the G-6A variant of the AGT gene,[10] the T594M variant of the eNa^+_c gene,[11] and the C825T polymorphism of the GNB3 gene.[12] Although we were unable to detect a significant association between the RAA system profiles and either the AGT gene G-6A variant or the GNB3 polymorphism examined, there was a trend to differences in PRA values between patients with different G-6A genotypes of the AGT gene and for differences in ALD values between patients with different GNB3 gene C825T genotypes. The relevance of these potential associations is questionable, since in contrast to previously published data,[10,12] patients homozygous for the -6A variant had higher rather than lower PRA values, and patients homozygous for the 825T allele had higher rather than lower ALD values.

In contrast to data obtained in a previous study,[17] in the sample of patients recruited for the present study the ANP gene intron 2 variant was not detected. However, these results do not indicate that this variant is absent in the population sampled, as our group have shown that the variant exists in black South Africans with a frequency of 3 - 5% (unpublished data).

In the present study we have assumed that the risk allele for the GNB3 protein C825T variant to be the C825 allele and the risk allele for the G_s protein T131C variant the 131C allele. In contrast, previous studies have indicated that the 825T and T131 alleles are risk alleles for the development of hypertension.[12,18] However, our choice of potential risk allele for the present study is based on our own data which demonstrate a strong association between both the C825 and the 131C alleles for the development of hypertension in a large case-control study in black South Africans (over 500 cases and 500 control subjects) (Nkeh *et al.* — unpublished data).

Our inability to detect an association between the ACE, AGT, *CYP11B2*, ANP (exon 3), GNB3 and α-adducin gene variants examined and RAA system activity in black South Africans could also be attributed to the fact that the frequency of either risk or non-risk alleles of the variants examined were too low for us to perform statistical procedures assuming all potential inheritance models. Only a much larger study sample (obtainable only if multiple study sites were involved) would have allowed us to assess all inheritance models.

Not only did the relatively small study sample size and the low allele frequency of some of the variants examined prevent us from assessing a potential impact of all inheritance models on RAA system activity, it also precluded us from evaluating potential epistasis (genotype-genotype interactions)[22] or genotype-phenotype interactions[23] moderating the actions of a single locus on RAA system activity. These interactions could potentially disguise important effects of single loci and are likely to explain many of the discrepancies currently evident in the field. Further work is

required to assess these potential interactions in black South Africans.

Although the data in the present study indicate that the gene variants examined were not associated with RAA system activity in black South Africans, the data do not exclude a role for the loci examined. Alternative variants not assessed in the present study may exist at each locus.

In summary, the results of the present study do not support a clinically meaningful role for any single gene candidate examined in contributing to RAA system profiles in black South African HTs. However, the results obtained in this study, which involved a small study sample, do not preclude a minor role for any single locus. Moreover, alternative variants in the same loci, epistasis (in a larger study sample), genotype-phenotype effects (in a larger study sample), and alternative inheritance models need to be considered before excluding a potential role for these loci. Importantly however, alternative loci should also be assessed in order to attempt to identify a gene which could account for a clinically meaningful proportion of the variance of RAA system profiles in black South Africans.

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