Disease Markers 24 (2008) 11–17
IOS Press

Homogeneous assay of rs4343, an *ACE* I/D proxy, and an analysis in the British Women's Heart and Health Study (BWHHS)

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Abstract. Current literature suggests that *ACE* SNP rs4343, *ACE* 2350A>G in exon 17, T202T, may be the best proxy for the *ACE Alu* I/D whereas rs4363 and rs4362 may be slightly stronger predictors of ACE levels. Considering reported difficulties in genotyping *ACE* I/D and stronger associations of rs4343 than *ACE* I/D with plasma ACE levels in Africans, and suitability of rs4343 for allelic mRNA (cDNA) studies, we developed and validated a liquid phase assay for rs4343, which has advantage on both functional and technical grounds. We confirmed that rs4343, is in near perfect linkage disequilibrium (D' = 1, $r^2 = 0.88$, $n = 64$) with *ACE* I/D in Europeans (A and G alleles of rs4343 marking insertion and deletion alleles of *ACE* I/D respectively). We then studied its association with metabolic and cardiovascular traits in 3253 British women (60–79 years old).
Apart from a nominal trend of association with diastolic blood pressure (p anova = 0.08; p trend = 0.05), no other associations were observed. A *post-hoc* vascular and general phenome scan revealed no further associations.
We conclude that *ACE* I/D is not a major determinant of metabolic and cardiovascular traits in this population. Liquid phase genotyping of SNP rs4343 may be preferable to gel based *ACE* I/D genotyping both for technical and functional reasons.

Keywords: Angiotensin converting enzyme, insertion deletion polymorphism, metabolic syndrome trait, *Alu* element, single nucleotide polymorphism

1. Introduction

Genetic variants in the renin angiotensin system (RAS), which affect cardiovascular function and fluid balance, have been widely studied. Among them, there is an insertion deletion (I/D) polymorphism representing the presence or absence of a 287 base pair (bp) *Alu* element situated in intron 16 of *ACE* on chromosome 17.

ACE D (deletion) allele has a strong and well replicated association with higher plasma ACE levels [1] and the major role of ACE in the RAS and in the function of many tissues, has invited continued study in diverse clinical phenotypes, with over 1759 papers identified in PubMed (http://www.ncbi.nlm.nih.gov/) using the search phrase "ACE AND genotype". Large scale studies or meta-analyses have not found *ACE* I/D to be associated with hypertension [2–4] or predictive of

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ISSN 0278-0240/08/$17.00

antihypertensive drug response [5]. For myocardial infarction, some have not found significant association [2], while others have reported modest positive associations [6,7]. With stroke, association with DD genotype has been claimed [8], although negative in another study [9]. DD genotype has been associated with reduced risk of Alzheimer's disease (AD) [10], and it has been suggested that decreased ACE activity may influence AD susceptibility through beta-amyloid metabolism [11]. DD genotype has also been reported to be associated with diabetic nephropathy but not retinopathy [12], and subjects with II genotype have reduced risk [13]. With systemic lupus erythematous (SLE), there was no association with *ACE* I/D in meta-analysis [14]. Generally, it seems that some reported associations might be due to small-study bias, and when very large studies are undertaken, some associations disappear, such as earlier claims concerning *ACE* I/D association with pre-eclampsia [15]. However, other interesting associations such as with endurance performance and positive energy balance [16,17] and abdominal aortic aneurysm [18] and endothelial-dependent vasodilatation with exercise [19] have not been subjected to extensive replication studies and meta-analyses. However, *ACE* I/D genotyping has presented difficulties due to failures of amplification of the larger allele in heterozygotes [20]. More complex amplification protocols [21] using flanking primers plus a primer internal to the *Alu* insertion element have reduced this problem, but gel-based resolution of products is still necessary, which is laborious and time-consuming, although faster or higher throughput electrophoresis systems such as microplate array diagonal gel electrophoresis (MADGE) [22], PhastSystem [23] and capillary electrophoresis [24] offer improvements. In Europeans, rs4343, *ACE* 2350A>G (≡A2350G) a silent substitution in exon 17, is in complete linkage disequilibrium (LD) with *ACE* I/D [25]. Here, we report development of a simple liquid phase fluorescence assay of the rs4343, its validation as a proxy for *ACE* I/D and association study with metabolic syndrome traits and phenome scan in 3253 British women.

2. Materials and methods

The British Women's Heart and Health study (BWHHS) DNA bank consisted of 3253 DNA samples from British women aged 60–79 years old recruited in BWHHS [26]. DNA was extracted using a salting out procedure [27]. All the women included in this study were described as white by the examining nurse. A priori we examined the association of rs4343, *ACE* 2350A>G, with blood pressure and other metabolic traits (see Table 1) to see if we could replicate earlier findings with these outcomes. We then undertook a phenome scan in which dense phenotypic information in human cohorts is scanned for associations with individual genetic variants [28]. We examined the association of this SNP with 87 socioeconomic, lifestyle and health related characteristics of the women. Please find the list of variables in the supplement 1. Associations with these variables were examined at two alpha levels: 0.01 and 0.05.

Oligonucleotide primers and probes were:

ACE 2350A>G F 5' – CCCCTTACAAGCAGAGGTGA

ACE 2350A>G R 5' – CCATGCCCATAACAGGTCTT

ACE 2350A>G P 5' – ATGGCCAC**G**TCCCGG

ACE 2350A>G Q 5' – ATTCAAACCCCTACCAGATCTGACGAATG

ACE 2350A>G P (probe) was derivatised with fluorescein at 5' and phosphate at 3' ends, position of the SNP is in bold, and ACE 2350A>G Q (quencher) was derivatised with Dabcyl at 3'.

PCR conditions: templates were 20 ng of genomic DNA, plated on 384-well PCR plates (Cat No. TF-0384/W, www.abgene.com) in 2 μl of water, and dried at 80°C for ten minutes for storage. PCR mixture contained: 0.5 μl of 10× PCR buffer, 0.2 mM dNTPs, 0.02 μM of the forward and 0.1 μM of the reverse primers, 0.04 μM of each probe (MWG-Biotech, Ebersberg, Germany), 2 mM $MgCl_2$, 0.01 U/μl of *Taq* DNA Polymerase (Promega, Madison, WI, USA) and H_2O to 5 μl. Thermal cycling was on an MJ Tetrad (Bio-Rad, Hercules, CA): 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds 72°C for 30 seconds, last three steps were repeated for 99 cycles, and 72°C for 2 minutes. PCR products were loaded with 5 μl of chillout 14 Liquid wax^{TM} (MJ Research Inc, www.mjr.com), then centrifuged at 3000 rpm for 3 minutes before loading the 384 well plate into a LightTyper instrument (Cat No. 03 357414001, Roche Diagnostics GmbH, USA). LightTyper is a high-throughput genotyping technology using fluorescent melting curves [29, 30]. In brief, an oligonucleotide pair (a quencher and a fluorescent probe) was present during PCR. The single-base variation binding probe was derivatized with fluorescein at the 5' end and phosphate at the 3' end and was complementary in its middle third to one of the alleles represented, and the quencher oligonucleotide

Table 1
Associations of genotype with components of the metabolic syndrome

	Mean or % (95% CI) of phenotype by genotype			P anova (2df)[b]	P trend (1df)[b]
	AA ($n = 806$)	GA ($n = 1671$)	GG ($n = 776$)		
Insulin	6.89	6.88	7.08	0.57	0.41
(μU/l)*	(6.55, 7.24)	(6.66, 7.10)	(6.76, 7.42)		
Glucose	5.95	5.91	5.92	0.73	0.67
(mmol/l)*	(5.87, 6.03)	(5.86, 5.97)	(5.85, 6.00)		
Triglyceride	1.65	1.65	1.70	0.30	0.20
(mmol/l)*	(1.60, 1.70)	(1.61, 1.69)	(1.65, 1.75)		
HDL-C	1.65	1.66	1.65	0.71	0.95
(mmol/l)	(1.62, 1.68)	(1.64, 1.69)	(1.62, 1.68)		
CRP	1.88	1.85	1.83	0.94	0.64
(mg/l)*	(1.74, 2.03)	(1.75, 1.95)	(1.69, 1.98)		
Fibrinogen	3.41	3.46	3.42	0.19	0.80
(g/l)	(3.37, 3.46)	(3.43, 3.50)	(3.37, 3.47)		
Systolic BP	147.2	146.8	147.8	0.36	0.67
(mmHg)	(145.4, 149.0)	(145.6, 148.1)	(146.0, 149.6)		
Diastolic BP	79.1	79.3	80.3	0.08	0.05
(mmHg)	(78.3, 79.9)	(78.7, 79.8)	(79.5, 81.1)		
Hypertensive	48.7	48.4	50.1	0.72	0.57
(%)[a]	(45.2, 52.2)	(46.0, 50.8)	(46.6, 53.7)		
BMI	27.4	27.7	27.6	0.55	0.52
(kg/m^2)	(27.1, 27.8)	(27.4, 27.9)	(27.2, 27.9)		
Waist/hip	0.821	0.817	0.817	0.52	0.21
	(0.817, 0.826)	(0.814, 0.820)	(0.813, 0.822)		
Height	1590	1587	1589	0.44	0.76
(mm)	(1586, 1594)	(1584, 1590)	(1585, 1593)		

*Geometric means and 95% CI of geometric mean.
[a]hypertension defined as systolic blood pressure >=160 mmHg or diastolic blood pressure >=100 mmHg or taking antihypertensive medication.
[b]2df is test for genotype and 1df is regression on allele.

was derivatized with Dabcyl at its 3' end located adjacent to the probe for the single-base variation. The anchor quencher oligonucleotide quenches the probe for the single-base variation while in its vicinity. With increasing temperature, the probe dissociates from its target strand, thus releasing its fluorescein from the vicinity of the Dabcyl quencher with a consequent increase in fluorescence. The first derivative of the fluorescence curve thus shows peaks at two separate melting temperatures when the single-base variations is in the heterozygous state.

64 random DNA samples of the BWHHS were also genotyped for *ACE* I/D [22] in order to perform LD analysis with the SNP. Statistical analyses including ANOVA (for genotype associations with phenotypes) and regression (for allelic association with phenotypes) were performed using Stata 9.0 (http://www.stata.com). D' and r^2 were calculated according to Lewontin RC (1964) and Zapata C et al. (2001) [31,32].

3. Results

Figure 1 shows the pattern of genotypes done blinded to sample and other genotyping (e.g. *ACE* I/D) data. Three completely distinctive patterns were obtained. Asymmetric locations of blank wells in microplates ensured no possibility of plate swap errors or rotations. Genotype frequencies were AA = 806, AG = 1671 and GG = 776, which are in Hardy-Weinberg equilibrium ($\chi^2 = 2.5$, $p = 0.12$). The SNP was in complete LD with the *ACE* I/D (D' = 1, $\chi^2 = 112.90$, $p = 2 \times 10^{-26}$) with an r^2 (correlation) of 0.88. It should be noted that any significant genotyping error rate would 'create' a fourth haplotype, i.e. unity value for D', implying fewer than four haplotypes, would not occur. The I allele corresponds with A2350, and the D allele corresponds with 2350G, consistent with a previous report in a similar population [33].

Apart from a borderline association with diastolic blood pressure (p anova = 0.08; p trend = 0.05) with GG (corresponds with DD) genotype averaging 1 mmHg higher level, there was no statistically significant association with any metabolic syndrome trait (Table 1) nor with any of the characteristics included in the phenome scan (all p-values >0.2). Analysis of a core set of 16 variables is shown in Table 1. Adjustment of the genotype-diastolic blood pressure association for age, use of antihypertensive medication and body mass

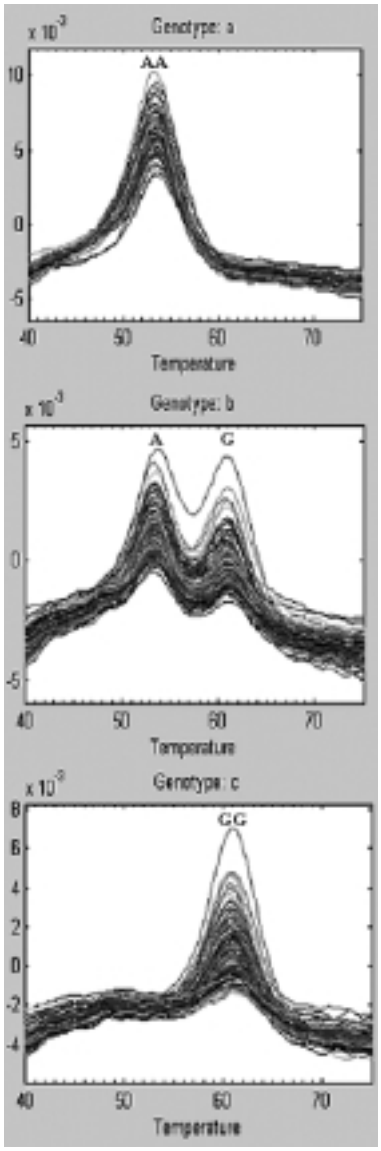

Fig. 1. The pattern of 2350A>G genotypes. Genotypes a, b and c show genotypes AA, AG, and GG respectively. X axis represents temperature in °C, Y axis represents the first derivative of the fluorescence curve against temperature.

index produced results identical to those without these adjustments (presented in Table 1), since genotype was not associated with any of these characteristics.

4. Discussion

Here, we describe a liquid phase genotyping assay of rs4343 using 384 well endpoint PCR and 384 well microplate LightTyper reader. We also validated the strong LD ($r^2 = 0.88$) of this SNP with *ACE* I/D in comparison with ($r^2 = 0.91$) [11]. In Europeans, this assay can substitute gel based *ACE* I/D typing which is susceptible to size dependent preferential amplification of alleles [20,34]. Further, this assay has benefits with respect to cost, convenience, time and automation which are of particular importance to large scale epidemiological studies. This assay could also facilitate the implementation of genotype-specific reference ranges for serum ACE level in diagnostic contexts.

As the I/D is located in an intron, there is uncertainty about its possible causal role or the mechanism of its effects. The D allele of *ACE* I/D is associated with higher mRNA levels [35,36] and with higher ACE activity [1], with almost two fold difference between opposite homozygotes. The *ACE* I/D does not seem to affect splicing [37], but fine mapping does not exclude a causal role for another SNP in very strong LD with *ACE* I/D. There appears to be stronger association with ACE levels for rs4343 than for the *ACE* I/D itself, in Africans [38]. However, we note that splice score predictions (http://www.fruitfly.org/seq_tools/splice.html) are essentially identical for the alleles of rs4343 and Zhu et al. (2000) [39] demonstrated highest SNP association with ACE levels for rs4363 in an interval from intron 25 to 3' noncoding region, slightly 3' to *ACE* I/D and rs4343. SNP rs4343, by contrast, most closely tags *ACE* I/D and is exonic.

Our study was of a female population and did not identify any positive associations with metabolic traits or with a large number of health and lifestyle related characteristics. Per allele effects (based on known trend of ACE levels across the three genotype groups) and hence the use of 1 degree of freedom (d.f) regression tests for phenotypes, might be considered the most appropriate genetic model, but 2d.f ANOVA tests of genotypes were also negative for all traits studied. There is significant evidence in the literature that various associations of *ACE* I/D (hypertension, diastolic blood pressure, overweight, and abdominal obesity) may be male specific [40–42]. However, not all studies have confirmed a male specific effect [43–45], and caution is required in interpreting such sub-group analyses since they are often performed *post-hoc* when no overall effect has been found. In mice also, the blood pressure effect of *ACE* I/D predominates in males [46,47], although the mechanism remains obscure.

Prince JA et al. (2001) [48], genotyped rs4343 as a surrogate to *ACE* I/D, using Dynamic Allele Specific Hybridization. The DASH system utilises allele specific hybridization and fluorescence monitoring of

oligonucleotide dissociation from target, but requires post PCR steps of solid phase binding, double strand denaturation, neutralisation and oligonucleotide annealing prior to the read step.

TaqMan assays represent a more integrated one step liquid phase assay design and TaqMan assay of *ACE* SNPs has been reported in a Japanese population [49]. Among seven studied SNPs, which were in LD with *ACE* I/D, rs4341 (in intron 16) was shown to give good resolution in a TaqMan assay. However, rs4343, which is presently the most plausible causal site for effect on ACE level [38] was not studied. Capital and consumables costs are much higher for TaqMan than for the approach described here. rs4343 is an exonic SNP with the potential also of being studied at the mRNA level.

In summary, we have developed and validated an economical high throughput SNP assay for rs4343, a SNP tag for the *ACE* I/D which is also a silent coding SNP expressed at the mRNA level. We have applied this assay to a phenome scan in a large cardiovascular risk survey of British women. We found no strong evidence that this SNP was associated with blood pressure, metabolic traits or other characteristics in these women.

Acknowledgements

BGEL laboratory start up was supported by University of Bristol. We thank those who participated in data collection and analysis and the women who have participated in this study.

Funding

BWHHS is funded by the UK Department of Health. Genetic studies in BWHHS have been supported by the British Heart Foundation (BHF). DAL is funded by a UK Department of Health career scientist award. TRG is a BHF Intermediate Fellow (FS/05/065/19497). The work of INMD, SH, SR and TRG has been supported by the British Heart Foundation, MRC UK.

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