

Promoter Polymorphisms in ACE (Angiotensin I–Converting Enzyme) Associated With Clinical Outcomes in Hypertension

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Genetic variants of *ACE* are suspected risk factors in cardiovascular disease, but the alleles responsible for the variations remain unidentified. To search for regulatory polymorphisms, allelic angiotensin I–converting enzyme (ACE) mRNA expression was measured in 65 heart tissues, followed by genotype scanning of the *ACE* locus. Marked allelic expression imbalance (AEI) detected in five African-American subjects was associated with single-nucleotide polymorphisms (SNPs) (rs7213516, rs7214530, and rs4290) residing in conserved regions 2–3 kb upstream of *ACE*. Moreover, each of the SNPs affected transcription in reporter gene assays. SNPs rs4290 and rs7213516 were tested for associations with adverse cardiovascular outcomes in hypertensive patients with coronary disease (International Verapamil SR Trandolapril Study Genetic Substudy (INVEST-GENES), $n = 1,032$). Both SNPs were associated with adverse cardiovascular outcomes, largely attributable to nonfatal myocardial infarction in African Americans, showing an odds ratio of 6.16 (2.43–15.60) ($P < 0.0001$) for rs7213516. The high allele frequency in African Americans (16%) compared to Hispanics (4%) and Caucasians (<1%) suggests that these alleles contribute to variation between populations in cardiovascular risk and treatment outcomes.

Angiotensin I–converting enzyme (ACE) plays a key role in cardiovascular biology. Its functions are formation of angiotensin II and inactivation of bradykinin, resulting in vasoconstriction and increased blood pressure. ACE inhibitors are recommended as first-line agents in the treatment of hypertension and heart failure. Expressed in many tissues, ACE further affects a broad spectrum of physiological processes. As a result, the *ACE* gene has been implicated in susceptibility to hypertension, myocardial infarction (MI), renal pathophysiology, diabetes, and Alzheimer's disease. The suggestion of a heritable component to serum ACE activity¹ led to extensive phenotype–genotype studies with ACE-related pathophysiology and response to ACE inhibitors.² Numerous studies have focused on an insertion/deletion (I/D) polymorphism in intron 15. However, meta-analyses of phenotypic associations largely failed to confirm a role for I/D,³ and *in vitro* experiments did not reveal any effect on transcription⁴ or splicing.⁵ Therefore, genetic factors contributing to

differential *ACE* expression remain unidentified. As ACE is one of the main therapeutic targets of antihypertensive therapies, a better understanding of genetic differences is required to assess disease risk and response to therapy.

Genetic family studies map the heritable contribution to ACE activity and blood pressure to the region of the *ACE* gene, particularly in subjects of African ancestry.^{6,7} Moreover, allele frequencies at the *ACE* locus vary greatly between African Americans and European Americans.⁸ African Americans are at higher risk of hypertension⁹ and its target organ sequelae¹⁰ and less responsive to ACE inhibitors.^{11,12} In addition, African Americans are more likely to experience adverse drug effects.¹³ In this study, we searched for functional polymorphism in the *ACE* gene locus that might account for differences in disease susceptibility or response to therapy with ACE inhibitors.

The *ACE* gene consists of 25 exons spanning ~25 kb and encoding a soluble or a membrane-bound protein variant with

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two peptidase domains (**Figure 1**). *ACE* harbors a number of polymorphisms; however, frequent nonsynonymous single-nucleotide polymorphisms (SNPs) that affect the protein sequence are lacking, suggesting that regulatory polymorphisms that are yet to be discovered may contribute to genetic susceptibility in cardiovascular diseases involving *ACE*. To search for regulatory polymorphisms, we measured allelic mRNA expression of *ACE* in human cardiac tissues. In contrast to total mRNA levels, allelic mRNA ratios cancel out *trans*-acting factors, so that any detectable allelic expression imbalance (AEI) is a strong indicator of *cis*-acting regulatory factors.^{14–19} Allelic mRNA ratios, as the most proximate and accurate phenotype for SNP scanning, can then be exploited in a search for regulatory polymorphisms, followed by molecular genetic studies to address underlying mechanisms.^{14–16,19}

In this study, we report regulatory alleles affecting *ACE* expression that are common among African Americans, discovered in a screen of human myocardial tissues. To assess the clinical relevance of these alleles, we conducted a clinical genetic association study in the International Verapamil SR Trandolapril Study Genetic Substudy (INVEST-GENES).²⁰

RESULTS

Allelic mRNA expression of *ACE* and association with promoter SNPs in heart tissues

We selected two marker SNPs (rs4309 and rs4343)—located in exon 8 and exon 16, respectively, of *ACE* (**Figure 1**)—to measure allelic ratios of genomic DNA (gDNA) and mRNA in heart tissues using the SNaPshot method (Applied Biosciences,

Foster City, CA). Standard curves performed with mixtures of DNA alleles were linear over the observed range ($r^2 = 0.996–0.999$). As gDNA ratios varied within a small range (less than ± 2 SD), no variable copy number polymorphisms were detectable (although the SNaPshot method used would have missed hemizygous subjects). Therefore, the mean allelic gDNA ratios were normalized to 1. Allelic *ACE* mRNA expression in heart tissues varied up to fourfold compared to gDNA ratios, indicating the presence of strong *cis*-acting regulatory factors (**Figure 2**; using a \log_2 scale). Allelic expression ratios obtained with the two marker SNPs in compound heterozygotes ($n = 20$) indicate that the results are reproducible. Allelic mRNA ratios deviated significantly from unity in 5 of 33 subjects. Strikingly, each of the five tissues showing strong AEI was obtained from African-American subjects, even though only 8 African Americans were heterozygous for a marker SNP. In contrast, none of the Caucasian displayed significant AEI (**Figure 2**), showing a significant difference between ethnic groups.

To ascertain the responsible regulatory polymorphisms, we sequenced the *ACE* locus in gDNA from the eight African-American subjects. No polymorphisms within the transcribed mRNA region (untranslated regions or protein-coding regions) matched the pattern of allelic expression. On the other hand, three polymorphisms (rs7213516, rs7214530, and rs4290) (**Table 1**) in a region 2–3 kb upstream of the *ACE* transcription start site were strongly associated with allelic mRNA expression imbalance in African Americans ($P < 10^{-7}$). Moreover, these three SNPs were absent in Caucasian cardiac tissues, which also failed to show detectable AEI (**Figure 2b**). In the cardiac

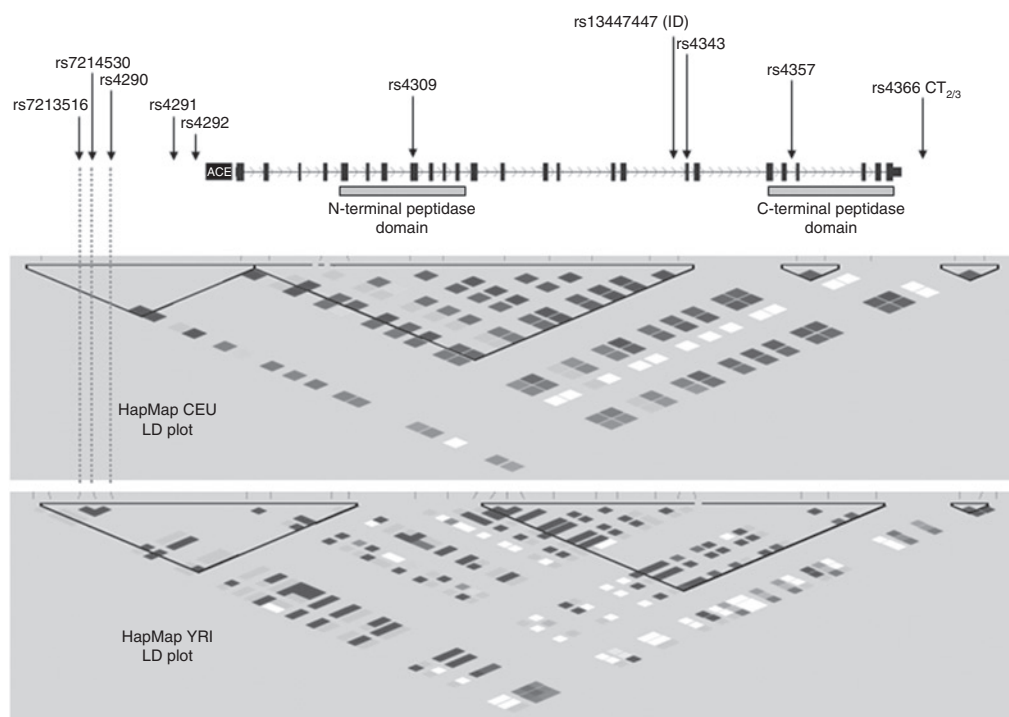


Figure 1 *ACE* gene structure (University of California–Santa Cruz genome browser) and location of polymorphisms tested in this study. Overviews of HapMap linkage disequilibrium (LD) in the gene region for individuals from Utah of northern-European ancestry (CEU) and individuals from Yoruba, Nigeria (YRI), are shown at bottom (Haploview). ACE, angiotensin I-converting enzyme.

tissues surveyed, rs7213516, rs7214530, and rs4290 were in extensive but incomplete linkage disequilibrium (LD), so we cannot exclude any of the three SNPs from contributing to the AEI ratios. In the HapMap data for the Yuruba population in Ibadan, Nigeria, rs4290 was in complete LD with rs7214530 ($D' = 1.0$, $r^2 = 1.0$) but not with rs7213516 ($D' = 1.0$, $r^2 = 0.55$) (Figure 1). The incomplete LD in HapMap between rs4290 and rs7213516 motivated later selection of these two markers for the clinical association study.

We next genotyped additional ACE markers (rs4291, rs4292, rs4357, rs4363, rs13447447, and rs4366) for all samples with allelic mRNA data (Table 1). The only additional SNP showing significant association with AEI, rs4357 ($P < 10^{-7}$) located in intron 21 (Figure 1), was in partial LD with the upstream SNPs

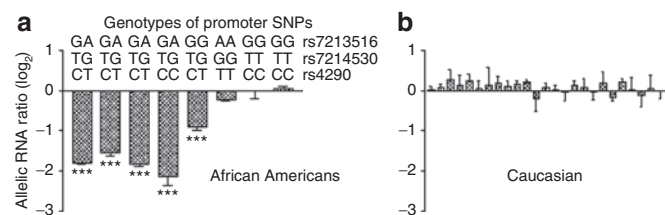


Figure 2 ACE allelic mRNA expression in left ventricular heart tissues from (a) African Americans and (b) Caucasians. Allelic mRNA expression ratios (major/minor allele for marker single-nucleotide polymorphisms (SNPs) rs4309 (C/T), rs4343 (A/G)) are averages of results using both markers. Allelic expression imbalance was prevalent in (a) African-American but not (b) Caucasian heart tissues. Genotypes for the promoter SNPs are indicated above the African-American samples. Data are mean \pm SD, *** $P < 0.001$ vs. pooled DNA ratios. ACE, angiotensin I-converting enzyme.

Table 1 Polymorphisms analyzed in this study and minor allele frequencies observed in heart tissues, sorted by race/ethnicity

SNP location	Minor allele frequencies, human heart tissues	Minor allele frequencies, human heart tissues	
		Caucasians (%)	African Americans (%)
rs7213516 (G>A)	-2883 from TSS	0.0	27.3 (n = 12)
rs7214530 (T>G)	-2828 from TSS	n.d.	29.0 (n = 12)
rs4290 (C>T)	-2305 from TSS	0.0	25.0 (n = 12)
rs4291 (A>T)	-240 from TSS	34.2	31.3 (n = 8)
rs4292 (T>C)	-93 from TSS	31.6	6.3 (n = 8)
rs4309 (C>T) AEI marker SNP	Exon 8 (Pro>Pro)	44.4	37.5 (n = 12)
rs13447447 (I/D, insertion/deletion)	Intron15	49.1 ^a	45.5 (n = 11)
rs4343 (A>G) AEI marker SNP	Exon 16 (Thr>Thr)	48.1 ^a	20.8 (n = 12)
rs4357 (C>T)	Intron 21	0.0	22.7 (n = 11)
rs4363 (A>G)	Intron 24	47.2	36.4 (n = 11)
rs4366 (22/33)	3' Downstream	49.1	27.3 (n = 11) ^b

AEI, allelic expression imbalance; n.d., not detected; SNP, single-nucleotide polymorphism.

^ars13447447 (I) and rs4343 (A) were the minor alleles in the Caucasian heart tissue samples. ^b22 is the minor allele in Hispanics and African Americans; 33 is the minor allele in Caucasians.

(with rs4290: $D' = 1.0$, $r^2 = 0.77$; with rs7213516: $D' = 0.71$, $r^2 = 0.38$). Because several subjects with AEI were homozygous for rs4357, this argues against a functional role. The commonly studied I/D variant (rs13447447) had a P value of 0.09 for association with AEI, again owing to LD with the promoter SNPs, but it was also ruled out because many I/D heterozygotes failed to show AEI.

The value of allelic mRNA ratios < 1 in the five African-American subjects showing AEI indicated that the less frequent allele had reduced mRNA expression (considering the inferred phasing between the marker SNP alleles and those of the promoter SNPs). To test this further, we measured overall ACE mRNA levels using reverse transcriptase-PCR. Although no association with mRNA levels was observed with the I/D variant (Figure 3a), carrying the minor allele of the promoter SNPs was associated with decreased ACE mRNA expression (rs4290 T; $P < 0.02$ (Figure 3b), rs7213516 A; $P < 0.04$). This result indicates that the minor alleles of the promoter SNPs reduce expression.

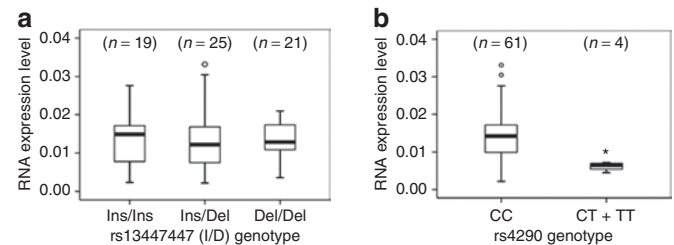


Figure 3 Total mRNA expression levels of angiotensin I-converting enzyme (ACE) in 65 heart tissues. Box plots display median expression \pm one quartile. Results are grouped by (a) genotype of the insertion/deletion (I/D) ($P = 0.93$) and (b) carriers of the promoter rs4290 T allele. ACE mRNA levels are relative to β -actin. * $P < 0.05$ vs. CC genotype.

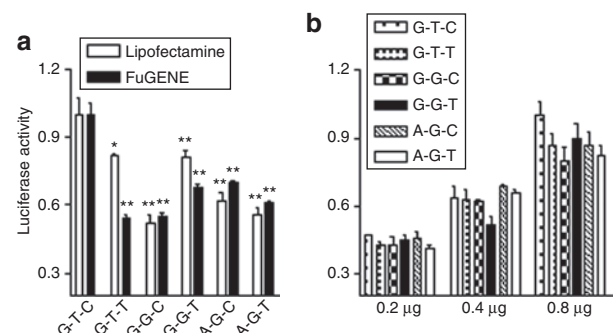


Figure 4 Luciferase reporter gene assay of the ACE promoter in bovine aortic endothelial cells (BAEC) (a) and HEK293 cells (b). An ACE promoter fragment from -4,335 to +1 was cloned into pGL3.basic vector containing the promoter single-nucleotide polymorphisms (SNPs) rs7213516 (G/A), rs7214530 (T/G), and rs4290 (C/T). The reference haplotype was G-T-C, whereas the variant constructs contained one to three minor alleles. In BAEC, 0.8 μ g plasmids were cotransfected with 40 ng *Renilla* luciferase plasmid using either Lipofectamine or FuGENE reagent. Luciferase activities from fused-pGL3 vector were normalized using *Renilla* luciferase activity as an internal control. * $P < 0.05$; ** $P < 0.001$ compared to reference haplotype G-T-C. In HEK293 cells, various amounts of plasmid were transfected using Lipofectamine, with no differences observed between any conditions. ACE, angiotensin I-converting enzyme.

Table 2 Polymorphisms analyzed in this study and minor allele frequencies observed in the INVEST-GENES cohort, sorted by race/ethnicity

	Minor allele	Minor allele frequencies INVEST-GENES				P value ^a
		Overall (%)	Caucasians (%)	Hispanics (%)	African Americans (%)	
rs7213516 (G>A)	A	3.37	0.17	4.33	16.00	<0.0001
rs4290 (C>T)	T	3.60	0.57	4.21	16.03	<0.0001
rs4291 (A>T)	T	37.12	38.78	34.68	32.92	0.11
rs13447447 (I/D)	I	41.16	41.67	39.62	40.57	0.38
rs4366 (22/33)	22 ^b	47.91	55.89	49.79	40.16	0.0004

The P values indicate the level of significance for interethnic differences in minor allele frequencies.

INVEST-GENES, International Verapamil SR Trandolapril Study Genetic Substudy.

^aP value for χ^2 -tests or Fisher's exact test for the genotype frequencies by race/ethnicity, as appropriate. ^b22 is the minor allele in Hispanics and African Americans; 33 is the minor allele in Caucasians.

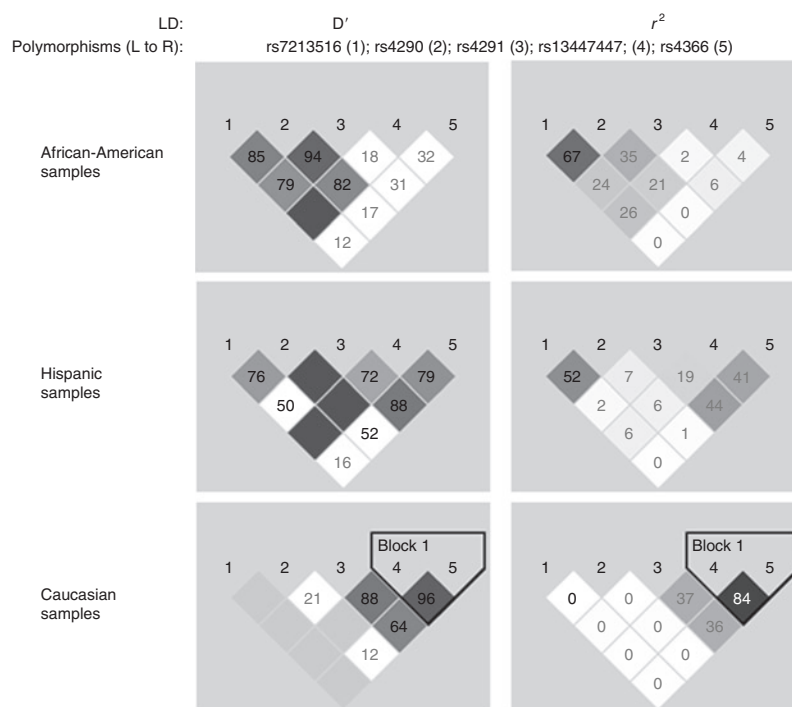


Figure 5 Linkage disequilibrium (LD) structure of polymorphisms in the International Verapamil SR Trandolapril Study Genetic Substudy clinical genetic association study. Values for D' and r^2 are shaded on a gradient based on the strength of correlation. Boxes without number values and shaded dark gray indicate perfect correlation. Boxes without number values and shaded light gray indicate that low allele frequency prevented calculation of D' .

Reporter gene analysis of three ACE promoter SNPs

To determine whether the promoter SNPs rs7213516, rs7214530, and rs4290 affect transcription, we compared the activities of a 4.3-kb fragment from the ACE promoter region, which contained either the reference sequence (G-T-C) or different combinations of the three SNPs, using a reporter gene assay in HEK293 and bovine aortic endothelial cells (BAEC). As shown in Figure 4a, the expression constructs containing any of the minor alleles of the three promoter SNPs significantly reduced reporter gene expression in BAEC, using two different transfection reagents. Although there were differences in the degree of reduction between the various constructs, no single SNP could account for all results. To test for cell context-dependent effects, we also measured promoter activity in HEK293 cells. In contrast to the results with BAEC, none of the SNPs had an effect

on promoter activity in HEK293 cells regardless of plasmid amounts used for transfection (Figure 4b). As the experiments in BAEC and HEK293 cells were performed side by side with the same plasmid preparations, the negative results in HEK293 cells further indicate that the plasmid preparations had similar transfection efficiencies, which can be a source of error if not controlled for. Taken together with the mRNA analysis in heart tissues (Figures 2 and 3), we conclude that each of the three promoter SNPs appears to reduce ACE gene expression, although any effects appear tissue dependent.

Genetic association of ACE with adverse cardiovascular outcomes in INVEST-GENES

We genotyped rs7213516, rs4290, and three additional polymorphisms (Table 2) in 258 subjects who experienced a

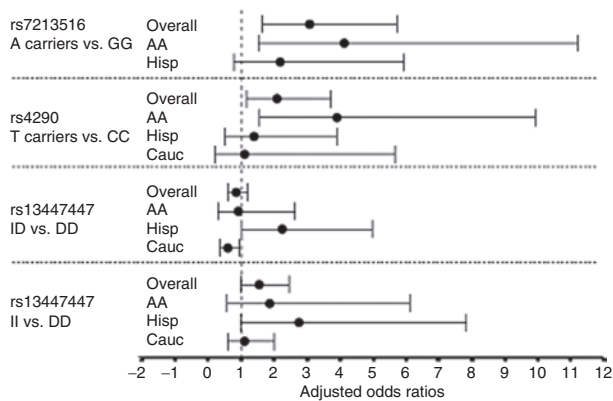


Figure 6 Odds ratios of three polymorphisms for primary outcome in the overall population and within each group. Odds ratios were adjusted for age, sex, race/ethnicity, body mass index, smoking, International Verapamil SR Trandolapril Study treatment strategy, previous myocardial infarction, previous stroke, heart failure, diabetes, renal insufficiency, baseline systolic blood pressure, diuretic use, and angiotensin I-converting enzyme inhibitor use. AA, African American; Cauc, Caucasian; Hisp, Hispanic.

Table 3 Unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for secondary outcomes by genotype

	Unadjusted OR (95% CI)	Adjusted OR ^a (95% CI)
All-cause death (n = 90)		
rs7213516 (A carriers vs. GG)	1.04 (0.44–2.49)	1.68 (0.62–4.54)
rs4290 (T carriers vs. CC)	1.41 (0.68–2.94)	1.92 (0.83–4.46)
rs13447447 (ID vs. DD)	1.49 (0.87–2.55)	1.45 (0.83–2.52)
rs13447447 (II vs. DD)	1.34 (0.65–2.77)	1.34 (0.63–2.82)
Nonfatal MI (n = 75)		
rs7213516 (A carriers vs. GG)	2.81 (1.40–5.64)*	6.16 (2.43–15.60)**
rs4290 (T carriers vs. CC)	1.78 (0.85–3.73)	2.37 (0.99–5.69)
rs13447447 (ID vs. DD)	0.59 (0.34–1.02)	0.61 (0.34–1.07)
rs13447447 (II vs. DD)	1.41 (0.76–2.65)	1.44 (0.75–2.79)
Nonfatal stroke (n = 81)		
rs7213516 (A carriers vs. GG)	1.68 (0.77–3.65)	1.72 (0.70–4.24)
rs4290 (T carriers vs. CC)	1.43 (0.66–3.09)	1.27 (0.54–3.02)
rs13447447 (ID vs. DD)	0.69 (0.40–1.20)	0.66 (0.37–1.16)
rs13447447 (II vs. DD)	1.34 (0.70–2.59)	1.41 (0.72–2.76)

MI, myocardial infarction.

^aAdjusted for: age, sex, race/ethnicity, body mass index, smoking, previous myocardial infarction, heart failure, diabetes, and ancestry. * $P = 0.004$; ** $P = 0.0001$.

primary outcome event (first occurrence of all-cause death, nonfatal MI, or nonfatal stroke) and 774 hypertensive controls who lacked primary outcome events in the genetic substudy (INVEST-GENES) of the randomized controlled clinical trial INVEST. All genotype frequencies were in Hardy–Weinberg equilibrium in all three race/ethnicity groups and displayed substantial differences between ethnic groups. LD is shown in **Figure 5**, illustrating the relationships between the genotyped SNPs. For the five polymorphisms tested in the INVEST-GENES, allele frequencies in Hispanics were intermediate

between those of Caucasians and African Americans. Minor allele frequencies of both rs7213516 and rs4290 differed significantly between African Americans (16%), Hispanics (4%), and Caucasians (<1%). Genotyping quality-control checks showed >99.5% concordance between different assays for the same polymorphisms (see also **Supplementary Materials and Methods** online).

Consistent with our finding that the rs7213516 A and rs4290 T alleles are associated with ACE expression differences, these alleles were also robustly associated in the INVEST-GENES cohort with increased risk of a primary outcome event (**Figure 6**). The main effect was strongest in African Americans for both SNPs, with similar trends in Hispanics and Caucasians, despite limited power in these latter groups because of low allele frequency. In African Americans, rs7213516 A and rs4290 T carriers had four times higher odds of experiencing a primary outcome event (odds ratio (OR): 4.13, 95% confidence interval (CI): 1.52–11.21 ($P = 0.0054$), and OR: 3.91, 95% CI: 1.54–9.90 ($P = 0.0041$), respectively). Subgroup analysis by treatment category indicated that the A allele of rs7213516 was associated with higher risk (i.e., OR > 1) in all drug combinations, although this reached statistical significance only in patients receiving both atenolol and trandolapril (OR: 4.31, 95% CI: 1.56–11.89 ($P < 0.005$)) (**Supplementary Table S1** online).

In secondary outcomes analysis, rs7213516 conferred the highest risk for nonfatal MI (OR: 6.16, 95% CI: 2.43–15.60 ($P = 0.0001$)), whereas there was no significantly higher risk for all-cause mortality ($P = 0.92$) or nonfatal stroke ($P = 0.19$) (**Table 3**). Similarly, the association with rs4290 is also largely driven by nonfatal MI (OR 2.37); however, it reached only marginal significance.

The ACE I/D polymorphism (rs13447447) was inconsistently associated with outcomes (**Figure 6**). Associations were not directionally similar in the different racial/ethnic groups, nor was there a linear trend between I/D heterozygotes and I/I homozygotes. Finally, the I/D was not associated with any of the individual components of the composite outcome (**Table 3**). We therefore conclude that there is no meaningful association with clinical outcomes analyzed here, consistent with a lack of effects on ACE mRNA level in heart tissue (**Figure 3**). There was also no evidence for association of the primary outcome with polymorphisms rs4291 and rs4366.

DISCUSSION

This study used allelic mRNA expression analysis of ACE in human heart tissues, followed by SNP scanning, to identify regulatory polymorphisms in the ACE locus, long suspected of conferring genetic risk for cardiovascular disease and possibly affecting treatment outcomes. This approach revealed strong effects on ACE mRNA expression attributable to three promoter SNPs—rs7213516, rs7214530, and rs4290—located in conserved regions 2–3 kb upstream of the transcription start site. The excellent congruence between AEI ratios and clearly identifiable polymorphisms, and a significant association between genotypes and total ACE mRNA expression in human heart tissues, support the notion that these promoter SNPs reduce



Figure 7 Promoter sequence alignments and transcription factor-binding sites. The three promoter single-nucleotide polymorphisms rs7213516, rs7214530, and rs4290 are located –2883, –2828, and –2306 bp upstream of the transcription start site (+1). Predicted MEF2A-binding sites (JASPAR position-weight matrices) are shown. Sequence alignments (made with CLUSTALW) are based on Basic Local Alignment Search Tool of the human promoter region. *1-bp insert in rhesus, dog, elephant, and armadillo; †9-bp insert in dog and armadillo.

ACE mRNA expression. This conclusion is further buttressed by results from reporter gene assays. The three *ACE* promoter SNPs are common in individuals of African-American ancestry (Table 1) but rare in Caucasians and intermediate in Hispanics. As the promoter SNP effects on ACE mRNA expression were observed only in tissues from heart failure patients, we cannot exclude the possibility that AEI is a result of the disease state. However, consistent with our gene expression results, a clinical association study in a non-heart failure group of hypertensive patients revealed a robust genetic effect on outcomes, suggesting that the heart failure state was not an important determinant of the findings.

Three promoter SNPs linked to ACE expression

Allelic mRNA expression ratios were strongly linked with the three promoter SNPs ($P \leq 0.0001$), but because of the extensive LD among them, a conclusion cannot be drawn regarding which polymorphism is functional. All three SNPs reside in conserved regions (Figure 7). Moreover, rs7214530 is part of a predicted recognition site for MEF2, a cardiac transcription factor previously implicated in cardiovascular disease and MI.^{21–23} It is therefore possible that all three SNPs have co-evolved as part of a haplotype block prevalent in subjects of African origin, each contributing to gene regulation, possibly to different extents in different tissues. Reporter gene assays with an *ACE* promoter fragment containing various combinations of the three suspected SNPs demonstrated decreased promoter activity for each combination of variant alleles, compared to the reference sequence in endothelial cells (BAEC) but not in HEK293 cells, indicating that these effects can be tissue specific. It is therefore likely that these *ACE* promoter polymorphisms have different effects in different target tissues and therefore could be associated with different pathophysiologies. The genetic effects on expression observed here in cardiac ventricular tissues and endothelial cells are interesting because prior evidence from animal models supports tissue-specific effects of ACE. ACE is expressed in endothelial cells as a membrane-bound enzyme on the luminal surface of the vasculature and cleaved to derive an active, soluble form in the plasma. The specific regulation of ACE in endothelial cells plays a key role in blood pressure regulation.²⁴ Furthermore, animal models of ACE overexpression in cardiac tissues and experiments in cell lines suggest that cardiac-specific gene expression of ACE may contribute to cardiac hypertrophy.²⁵

Together these results suggest that further experiments to elucidate the mechanisms and contribution of functional alleles in specific tissues may provide valuable insights with clinical relevance.

Other polymorphisms in ACE

We found no evidence for a functional effect of the *ACE* I/D polymorphism in intron 15 on mRNA expression in human heart tissue, consistent with previous negative *in vitro* studies.^{4,5} In addition, our clinical association data did not support an effect of I/D on outcomes across the various ethnic populations, despite an allele frequency and power that was substantially higher than for the promoter SNPs. Yet countless genetic association studies are based on the I/D polymorphism, even though evidence for a physiological function is lacking and clinical associations are borderline at best.³

Association of ACE promoter SNPs with clinical outcomes

We tested several *ACE* polymorphisms for association with clinical outcomes in hypertensive patients with coronary artery disease (INVEST-GENES). The promoter SNPs identified in our mechanism-based screen (rs7213516 and rs4290; rs7214530 was not genotyped because of strong LD with rs4290) were highly associated with cardiovascular disease outcomes ($P < 0.001$) (Figure 6) and in particular with MI (Table 4). The ORs ranging from 4 to 6 suggest an unexpectedly strong genetic effect, which must be further assessed in replication studies.

We also assessed relative risk of primary outcome as a function of drug treatment, showing association with the *ACE* promoter SNPs in individuals receiving ACE inhibitor and/or β -blocker therapy (Supplementary Materials and Methods online). However, the INVEST-GENES design was not optimal for assessing the effects of genetic factors on drug treatment outcomes, and therefore these results can be taken only as a guide for future experiments. Nevertheless, this association may have a biological basis, given that trandolopril and atenolol target overlapping systems of blood pressure control, of which ACE is a critical component. Results from the Valsartan Heart Failure (Val-HeFT) trial raise the possibility that excessive neurohormonal inhibition may contribute to adverse outcomes in heart failure treatment.²⁶ Because the promoter alleles identified here are associated with decreased ACE expression, we hypothesize that they may potentiate pharmacological ACE inhibition plus

Table 4 Baseline characteristics for INVEST-GENES case and control patients

Characteristic (N, % unless otherwise noted)	Cases (N = 258)	Controls (N = 774)
Age, mean (SD), years	71.5 (9.9)	70.2 (9.3)
Women	131 (50.8)	393 (50.8)
BP, mean (SD), mm Hg		
Systolic	150.6 (19.0)	147.4 (19.0)
Diastolic	83.6 (11.1)	83.3 (11.1)
Race/ethnicity		
White	158 (61.2)	472 (61.0)
Black	36 (14.0)	101 (13.1)
Hispanic	63 (24.4)	198 (25.6)
Other/multiracial	1 (0.4)	3 (0.4)
BMI, mean (SD), kg/m ²	27.4 (4.8)	29.0 (5.5)
Medical history		
Prior myocardial infarction	96 (37.2)	230 (29.7)
Angina pectoris	153 (59.3)	483 (62.4)
Prior stroke/TIA	36 (14.0)	71 (9.2)
Left ventricular hypertrophy	46 (17.8)	136 (17.6)
Heart failure (classes I–III)	28 (10.9)	29 (3.8)
Peripheral vascular disease	43 (16.7)	88 (11.4)
Smoking		
Past	133 (51.6)	355 (45.9)
Within 30 days	34 (13.2)	83 (10.7)
Diabetes ^a	102 (39.5)	224 (28.9)
Hypercholesterolemia ^a	161 (62.4)	485 (62.7)
Renal impairment ^b	14 (5.4)	18 (2.3)
Cancer	20 (7.8)	46 (5.9)
Medication at enrollment		
Aspirin/other antiplatelet agent	162 (62.8)	451 (58.3)
Antidiabetic medication	86 (33.3)	188 (24.3)
Any lipid-lowering agent	106 (41.1)	331 (42.8)
Nitrates	92 (35.7)	232 (30.0)

Percentages may not add up to 100 due to rounding.

BMI, body mass index; BP, blood pressure; INVEST-GENES, International Verapamil SR Trandolapril Study Genetic Substudy; TIA, transient ischemic attack.

^aHistory of or currently taking antidiabetic or lipid-lowering medications. ^bHistory of or current serum creatinine level that is elevated but <4 mg/dl (<354 μmol/l).

β-blockade, resulting in higher event rates via excessive neurohormonal inhibition. A prospective study of the *ACE* promoter SNP effect on drug treatment outcomes is required to resolve these issues.

Given that the promoter alleles are common in African Americans, they may partially account for phenotypic variation in *ACE* levels, blood pressure (e.g., ref. 6), and response to *ACE* inhibitors^{11–13} in individuals of African ancestry. Although these alleles were found at lower frequencies in Hispanics and Caucasians, they could be clinically relevant in the population at large, but we had limited statistical power to address this question.

Further investigation will determine whether these alleles may have clinical utility as biomarkers in the selection of therapeutic options for individual patients. The use of race in guiding treatment is controversial but does play a role in clinical practice.²⁷ Ultimately, therapy may be best optimized for individual patients with tests for functional biomarkers instead of relying on assumptions related to apparent, or self-identified, race or ethnicity.²⁸

Physiological roles for *ACE* include blood pressure regulation, kidney function, processing of kinins and other peptides, and degradation of amyloid β-protein,^{29,30} suggesting that the new *ACE* promoter alleles may be relevant in other human pathologies. Among the heart tissues obtained from 12 African-American heart transplant patients, only 8 were eligible for AEI analysis. Among the 12 samples, the minor allele frequencies of rs7213516 and rs4290 (25–27%) were higher than expected (16.0% in INVEST-GENES), with one patient homozygous for the minor alleles, arguing for a larger study of heart failure patients.

In conclusion, the discovery of regulatory alleles in key genes through allelic mRNA expression analysis, followed by clinical association studies, has broad potential to lead to viable biomarkers guiding an individual's therapy.³¹

METHODS

Analysis of *ACE* mRNA expression in heart tissues. Approval for use of human subjects was obtained from the Ohio State University Institutional Review Board. Left-ventricle tissue from 65 heart transplant patients was obtained through the Cooperative Human Tissue Network, Midwestern Division, at Ohio State University and stored at –80°C until extraction. gDNA and RNA were isolated, and cDNA was prepared from 1.0 μg RNA in three independent preparations, using oligo dT and gene-specific primers close to the two marker SNPs to minimize the effects of mRNA decay in postextract tissues (see **Supplementary Materials and Methods** online for details).

Total mRNA expression levels. Overall *ACE* mRNA expression was measured using reverse transcriptase–PCR for each sample (details in **Supplementary Materials and Methods** online). Gene expression results by genotype were analyzed by *t*-test for mean differences using SPSS 14 (Chicago, IL).

Measurement of allelic *ACE* mRNA expression. We measured allelic mRNA expression, as described previously,^{14–19} amplifying short regions of gDNA and cDNA around *ACE* exonic marker SNPs from heart tissues of heterozygous individuals (rs4309, located in exon 8, *n* = 28; rs4343, located in exon 16, *n* = 24). Primer extension with fluorescent dideoxynucleotides by SNaPshot (Applied Biosciences, Foster City, CA) allowed quantitation of relative amounts of each allele by capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosciences, Foster City, CA). Corrected allelic mRNA expression ratios for individual cDNAs were calculated by normalizing to the mean ratio of gDNA peaks (SD for gDNA: rs4309 ± 12.4%, rs4343 ± 8.6%). Each sample was assayed from three independent cDNA syntheses, each performed at least in duplicate (details in **Supplementary Materials and Methods** online).

Scanning the *ACE* locus for functional polymorphisms. To link SNPs with allelic mRNA expression ratios, we genotyped SNPs selected to represent the major haplotype blocks (**Table 1**) in all 65 heart tissues. SNPs were genotyped as described in **Supplementary Materials and Methods** online. In addition, we sequenced full-length cDNAs and the 5′-upstream region over 3 kb in eight African Americans, detecting five SNPs in the upstream region (**Table 1**). The presence of AEI was set at allelic mRNA ratios >1.5 or <1/1.5 as a cutoff. Association between genotype status (heterozygous or homozygous) and AEI was

determined using HelixTree (Golden Helix, Bozeman, MT). LD between SNPs (expressed as D') and haplotypes was calculated using HelixTree (Golden Helix).

ACE reporter gene assay. A promoter fragment ranging from $-4,335$ to $+1$ (the major transcription start site) in PGL3.basic vector (Promega, Madison, WI) was provided by Melanie Eyries.³² Various combinations of rs7213516/rs7214530/rs4290 haplotypes were obtained using site-directed mutagenesis or restriction digest of amplified gDNA with *MscI* and *BstEII* and subsequent cloning (see **Supplementary Materials and Methods** online for details). All inserts were fully sequenced to verify the intended sequence. The constructs were transfected into HEK293 and BAEC, cultured in Dulbecco's modified Eagle's medium/F12 media containing 10% fetal bovine serum, penicillin (10 U/ml), and streptomycin (10 μ g/ml), at 37°C with 5% CO₂. Twenty-four hours before transfection, 1×10^5 to 2×10^5 cells were plated into 24-well plates and transiently transfected with FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, IN) or Lipofectamine (Invitrogen, Carlsbad, CA) in serum-free medium for 5 h. As a control, *Renilla* luciferase constructs were cotransfected with PGL3.basic-fused constructs at a 1:20 ratio. Cells were harvested after 48 h and transferred to 96-well plates, and luciferase activity was detected using Dual-Glo luciferase assays (Promega, Madison, WI) on a fluorescence plate reader (PerkinElmer, Waltham, MA). Two independent transfections and triplicate luciferase assays were performed for each construct and cell line. Results were analyzed using Prism (GraphPad, La Jolla, CA).

Clinical genetic association study: INVEST and INVEST-GENES. The INVEST trial evaluated adverse cardiovascular outcomes in patients randomized to atenolol or verapamil SR hypertension treatment strategy in 22,576 patients with documented coronary artery disease and hypertension²⁰ (details in **Supplementary Materials and Methods** online). The primary outcome was the first occurrence of death (all cause), nonfatal MI, or nonfatal stroke. These events were taken separately as secondary outcomes. In the genetic substudy (INVEST-GENES), gDNA was collected from 5,979 patients using buccal cells from mouthwash samples.³³ All patients provided written informed consent, as approved by the University of Florida Institutional Review Board. The present case-control study focused on the 258 INVEST-GENES patients who experienced a primary outcome event during study follow-up (cases), frequency matched 3:1 for age, sex, and race/ethnicity with 774 individuals who were event-free during study follow-up (controls) (baseline characteristics in **Table 4**). The patients had a mean age of 71 years, 50% were female, 25% were of Hispanic ethnicity, and, 13% were African Americans. Previous analyses showed that case-control analysis in this group matched findings from the entire INVEST cohort, the inclusion of which increases only the number of controls.³⁴

We genotyped promoter SNPs rs7213516 and rs4290 and the tagging markers (rs4291, rs13447447, and rs4366) (genotyping details in **Supplementary Materials and Methods** online) to sample major haplotype blocks. Quality-control procedures included blind duplicate genotyping of 5% of samples using the same or an alternative method, assessment of Hardy-Weinberg equilibrium, and assay validation using Coriell samples previously genotyped as part of HapMap. To address potential population stratification, we genotyped 87 autosomal ancestry informative markers interspaced with large interlocus distances across the genome to give independent association with the disease and genetic background (see **Supplementary Materials and Methods** online).^{35,36}

Statistical analysis of clinical genetic associations. Baseline characteristics between cases and controls in INVEST-GENES were compared using t -test for continuous variables and χ^2 -test for categorical variables. Hardy-Weinberg equilibrium of genotype frequencies within each racial/ethnic group was tested using χ^2 -test with one degree of freedom. Because of the low minor allele frequencies for rs7213516 and rs4290 in the entire INVEST cohort, we decided *a priori* to combine heterozygous

patients with those homozygous for the variant alleles for all analyses. Logistic regression was performed to assess the association of genotypes/haplotypes with the primary and secondary outcomes after adjusting for ancestry and prespecified confounding factors, namely age (by decades), gender, race/ethnicity, history of MI and heart failure, and drug treatments.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/cpt>

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CONFLICT OF INTEREST

A.D.J., D.W., and W.S. are all coinventors on a submitted patent application pertaining to the ACE polymorphisms reported here. The other authors declared no conflict of interest.

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