

ORIGINAL ARTICLE

Angiotensin-converting enzyme gene and retinal arteriolar narrowing: The Funagata Study

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The purpose of this study is to determine whether the angiotensin-converting enzyme (ACE) gene polymorphism is associated with retinal arteriolar narrowing, a subclinical marker of chronic hypertension. The Funagata Study examined a population-based sample of Japanese aged 35+ years; 368 participants had both retinal vessel diameter measurements and ACE insertion/deletion (ACE I/D) polymorphism analyses performed. Assessment of retinal vessel diameter and retinal vessel wall signs followed the protocols used in the Blue Mountains Eye Study. ACE gene polymorphisms D/D, I/D and I/I were present in 34 (9.2%), 170

(46.2%) and 164 (44.5%) participants, respectively, distributed in Hardy–Weinberg equilibrium. After multi-variable adjustment, retinal arteriolar diameter was significantly narrower in subjects with the D/D genotype compared to subjects with I/D and I/I genotypes (mean difference $-6.49\mu\text{m}$, 95% confidence interval (CI): $-12.86\mu\text{m}$, $-0.11\mu\text{m}$). Our study suggests that the ACE I/D polymorphism may be associated with sub-clinical structural arteriolar changes related to chronic hypertension.

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Introduction

Retinal arteriolar narrowing is a structural microvascular sign associated with chronic hypertension¹ that predicts the risk of stroke² and coronary heart disease.³ Recent studies suggest that a substantial proportion of the variation in retinal arteriolar diameter might be genetically determined, independent of concomitant risk factors.⁴ However, no definite candidate genes associated with retinal arteriolar diameter have yet been consistently identified.

Angiotensin-converting enzyme (ACE) is a key component of the renin–angiotensin system and can promote vasoconstriction, inflammation, thrombosis and vascular remodeling. It was reported that

there was a high level of ACE expression in the regions of atherosclerotic lesions in human vasculature.⁵ Retinal arteriolar narrowing, which reflects intimal thickening, medial hyperplasia and hyalinization and sclerosis of retinal arterioles has also been associated with atherosclerosis.

Polymorphisms in the ACE gene, absence (deletion, D allele) rather than presence (insertion, I allele) of the 287-bp Alu insert in intron 16, has been found associated with hypertension,⁶ carotid wall thickening⁷ and coronary heart disease.⁸ The D allele of this polymorphism was also reportedly associated with two-fold higher circulating levels of ACE.⁹

It was reported that ACE gene and renin mRNA (messenger ribonucleic acid) expressed in the retinal pigment epithelium, the choroid and neural retina of rats.¹⁰ This suggested that the local renin–angiotensin system (RAS) is likely involved in the regulation of the retinal vasculature. Furthermore, one study suggested that patients with hypertensive retinopathy were more likely to have the D/D

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polymorphism than the I/D or I/I polymorphisms.¹¹ However, there were no studies that had examined the association of the *ACE* gene with quantitatively measured retinal vessel diameter.

The purpose of this study is to determine the independent association of *ACE* I/D polymorphisms and retinal arteriolar narrowing, and whether this association is independent of measured blood pressure and other cardiovascular risk factors.

Materials and methods

Study population

The Funagata study is a population-based epidemiologic study examining diabetes and other vascular disease in adult Japanese persons aged 35 years or older. Details of study participants and research methodology were described elsewhere.¹² In this study, systemic and ophthalmologic data were obtained between June 2000 and June 2002. Of 3676 eligible residents in Funagata community, 743 (20.2%) agreed to participate in the genetic part of the study. Participants included in this current study had a lower frequency of pre-diabetes and diabetes than those excluded (22.8 vs 30.7%; $P=0.003$). There were no significant differences between included and excluded participants in other demographic characteristics, such as age, gender, smoking, body mass index and systolic and diastolic blood pressure. Of the 743, 651 (87.6%) had fundus photographs with sufficient quality for assessment of retinal microvascular structural signs. Only 368 (49.5%) participants with retina–optic disc photographs had adequately captured a sufficient number of retinal vessels within a zone 0.5 disc diameter away from the optic disc margin, to grade retinal vessel diameter using a standardized computer-assisted method.¹³ Written consent was obtained from all study participants, the study was conducted according to the recommendation of the Declaration of Helsinki and was approved by the Ethics Committee of the Yamagata University Faculty of Medicine, Yamagata, Japan.

Assessment of retinal microvascular changes

Fundus photographs were taken using non-stereoscopic 45° non-mydratic fundus camera (CR5-NM45, Canon Inc., Tokyo, Japan; and TRC, Topcon Inc., Tokyo, Japan); a single field centered between the macula and optic disc was taken. Fundus photographs were graded for retinal microvascular signs at the Centre for Vision Research, University of Sydney, Australia. Grading was performed by a trained grader following a standard protocol; details of image preparation and grading protocols have been described previously.^{13,14} In brief, retinal photographs on 35-mm film were converted to digital images using a high-resolution scanner (LS2000; Nikon, Tokyo, Japan). Digital images were

centered on the optic disc, and all vessels passing through the entire zone between 0.5 and 1 disc diameter away from the disc margin were measured using image analysis software (Retinal Analysis, Department of Ophthalmology Visual Science, University of Wisconsin, WI, USA). A trained grader identified each vessel either as an arteriole or venule. The computer program measured and calculated the average width from five equidistant measures of each vessel. The average diameter of retinal vessels was calculated using the Parr–Hubbard formula, and summarized as the central retinal artery equivalent (CRAE) and the central retinal vein equivalent (CRVE), representing the average arteriolar and venular diameter, respectively.^{15,16}

Retinal arteriolar wall signs (focal arteriolar narrowing, arterio-venous nicking and enhanced arteriolar wall reflex) and retinopathy signs (microaneurysms, retinal hemorrhages and exudates) were also graded using a light box method following the standard photographs selected by a retinal specialist (PM) from the standard photographic sets developed for the Modified Airlie House Classification of Diabetic Retinopathy¹⁷ and the Wisconsin Age-Related Maculopathy Grading System.¹⁸ All grading was performed by a trained grader and adjudicated by a senior researcher (JJW) and a retinal specialist (PM).¹²

Assessment of systemic characteristics

Blood pressure was measured after rest for 5 min, and using a mercury sphygmomanometer. Hypertension status was defined for systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg, or if persons had a previous diagnosis of hypertension and were using anti-hypertensive medications. Diabetes or pre-diabetes was defined as having fasting plasma glucose ≥ 110 mg dl⁻¹ or 2 h post-load glucose ≥ 140 mg dl⁻¹. Smoking status was assessed during an interview. Body mass index was calculated as weight (kg) divided by the square of height (m).

Assessment of insertion/deletion polymorphism of 287 bp Alu insert in intron 16 of ACE gene

Genomic DNA was isolated from peripheral blood leukocytes by proteinase K and the phenol/chloroform extraction procedure. Insertion (I) or deletion (D) of the 287-bp Alu insert in intron 16 of the *ACE* gene was determined by polymerase chain reaction fragment length polymorphism analysis.¹⁹ The frequency of the D and I allele were 32.3% ($n=238$) and 67.6% ($n=498$), respectively. The frequency of D/D, I/D and I/I were 9.2% ($n=34$), 46.2% ($n=170$) and 44.6% ($n=164$), respectively. The genotypes frequency was found to be in Hardy–Weinberg equilibrium.

Statistical analysis

Data analysis was performed by statistical analysis software (Stata 10, StataCorp, TX, USA and SPSS 14.0, SPSS Inc., Chicago, USA). Demographic characteristics among three genotype groups were compared using analysis of variance. To examine whether the *ACE* I/D polymorphism was associated with retinal vessel diameter, linear regression analysis was used, with retinal vessel diameter as the dependent variable, adjusting for cardiovascular risk factors selected based on our previous analysis.¹² Associations between the *ACE* I/D polymorphism and presence of retinal arteriolar wall signs/retinopathy were examined using logistic regression models while adjusting for cardiovascular risk factors. Crude, age-gender adjusted and multivariable (age, gender, systolic blood pressure, smoking status and body mass index) adjusted estimates for the associations are presented. We used models following additive models (I/I vs I/D vs D/D), dominant models (I/I vs I/D or D/D) or recessive models (I/I or I/D vs D/D). Models for CRAE additionally adjusted for CRVE and vice versa.²⁰ We also analyzed if the interaction between *ACE* I/D polymorphism (D/D vs I/D or I/I) and hypertension status (hypertension vs normotension) affected retinal vessel diameters. We further examined if the *ACE* I/D polymorphism was associated with systolic and diastolic pressure; linear regression was used to estimate the mean differences in systolic and diastolic blood pressure by *ACE* I/D polymorphism, adjusting for other cardiovascular risk factors.

Results

Demographic characteristics by *ACE* I/D polymorphism are presented in Table 1. There were no significant differences in demographic characteristics among *ACE* I/D polymorphism groups.

Difference in systolic and diastolic blood pressure by *ACE* I/D polymorphisms

Mean systolic and diastolic blood pressure was highest in subjects with I/I polymorphism, although these were not statistically significant (Table 1).

Table 2 shows relationships between *ACE* I/D polymorphism and blood pressure status. Mean diastolic pressure was significantly lower in subjects with D allele (D/D or I/D) compared with that in subjects with I/I (−2.50 mmHg 95% CI: −4.78 mmHg, −0.23 mmHg) after adjusting for age, gender, body mass index and smoking status. However, there was no significant difference in systolic blood pressure (1.43 mmHg, 95% CI: −4.82 mmHg, 7.68 mmHg) and no significant association between the presence of hypertension and the *ACE* I/D polymorphisms (odds ratio for D/D compared with I/I: 1.02, 95% CI: 0.38–73).

Difference in retinal vessel diameters by *ACE* I/D polymorphisms

Table 3 shows relationships between *ACE* I/D polymorphism and CRAE. Mean CRAE (\pm s.d.) for subjects with D/D, I/D and I/I polymorphism was $173.77 \pm 19.73 \mu\text{m}$, $179.46 \pm 20.54 \mu\text{m}$ and $179.50 \pm 20.39 \mu\text{m}$, respectively. After adjusting for CRVE, CRAE was significantly smaller in subjects with D/D compared with that in subjects with I/I by $-6.69 \mu\text{m}$ (95% confidence interval (CI) for the β coefficient: $-12.88 \mu\text{m}$, $-0.51 \mu\text{m}$). This remained significant after age-gender ($-6.60 \mu\text{m}$, 95% CI: $-12.78 \mu\text{m}$, $-0.42 \mu\text{m}$) and multivariable adjustment ($-6.86 \mu\text{m}$, 95% CI: $-13.58 \mu\text{m}$, $-0.13 \mu\text{m}$). Similar findings are obtained when comparing subjects with D/D with those with the I allele (I/D and I/I) after adjusting for CRVE, age-gender or multiple variables (Table 3). There were no significant differences in mean CRAE between subjects with I/I and I/D polymorphism, or between subjects with I/I and those with D allele (I/D and D/D polymorphisms) (Table 3).

Table 3 shows CRVE by *ACE* I/D polymorphism. Mean CRVE for the subjects with D/D, I/D and I/I polymorphisms was $217.37 \pm 20.07 \mu\text{m}$, $216.12 \pm 20.37 \mu\text{m}$ and $215.61 \pm 21.52 \mu\text{m}$, respectively. Mean CRVE was non-significantly larger in subjects with D/D compared with that in subjects with I/I or I/D.

After adjusting for CRVE, subjects with hypertension had significantly smaller CRAE than those who were normotensive by $-3.63 \mu\text{m}$ (95% CI: $-7.11 \mu\text{m}$,

Table 1 Demographic characteristics by polymorphism of insertion (I) or deletion (D) of the 287 bp Alu insert in intron 16 of the angiotensin-converting enzyme (ACE) gene, The Funagata Study, Japan, 2000–02

	D/D polymorphism N = 34	I/D polymorphism N = 170	I/I polymorphism N = 164	P-value
Male gender (%)	17 (50)	67 (39.4)	67 (40.9)	0.518
Hypertension (%)	13 (38.2)	70 (41.2)	64 (39.0)	0.915
Pre-diabetes or diabetes (%)	6 (17.6)	39 (22.9)	39 (23.7)	0.739
Current smoker (%)	3 (8.8)	32 (18.8)	25 (15.2)	0.313
	<i>Mean (s.d.)</i>			
Age, years	58.9 (11.6)	60.1 (11.6)	60.5 (10.9)	0.776
Body mass index, kg m ⁻²	24.5 (3.2)	23.4 (3.1)	24.0 (3.5)	0.159
Systolic blood pressure, mm Hg	127.8 (17.0)	125.9 (15.5)	128.0 (15.5)	0.447
Diastolic blood pressure, mm Hg	75.3 (8.8)	75.1 (9.7)	77.2 (10.6)	0.169

Table 2 Difference in systolic and diastolic blood pressure (mm Hg) and odds ratio in hypertension, by angiotensin-converting enzyme insertion/deletion polymorphisms, the Funagata Study, Japan, 2000–02

	N	Mean blood pressure (mm Hg)	Crude difference (95%CI) (mm Hg)	Age-gender-adjusted (95%CI) (mm Hg)	Multivariable adjusted (95%CI) (mm Hg) ^a
Systolic blood pressure					
D)I/I polymorphism	164	128.0 ± 15.5	(reference)	(reference)	(reference)
I/D or D/D	204	126.2 ± 15.8	-2.67 (-6.27, 0.93)	-2.32 (-5.70, 1.07)	-1.76 (-5.00, 1.48)
R)I/D or I/I polymorphism	334	126.9 ± 15.5	(reference)	(reference)	(reference)
D/D	34	127.8 ± 17.0	2.06 (-4.09, 8.21)	3.06 (-2.73, 8.85)	2.16 (-3.46, 7.79)
Diastolic blood pressure					
D)I/I polymorphism	164	77.2 ± 10.6	(reference)	(reference)	(reference)
I/D or D/D	204	75.2 ± 9.6	-2.98 (-5.36, -0.59)*	-2.88 (-5.24, -0.51)*	-2.50 (-4.78, -0.23)*
R)I/D or I/I polymorphism	334	76.1 ± 10.2	(reference)	(reference)	(reference)
D/D	34	75.3 ± 8.8	-0.06 (-4.17, 4.04)	0.16 (-3.93, 4.24)	-0.45 (-4.43, 3.53)
	N	Prevalence (%)	Crude (95%CI)	Age-gender-adjusted (95% CI)	Multivariable adjusted (95% CI) ^a
Hypertension					
A)I/I polymorphism	164	64 (39.0)	(reference)	(reference)	(reference)
I/D	170	70 (41.2)	1.09 (0.71, 1.70)	1.07 (0.48, 2.42)	0.91 (0.37, 2.58)
D/D	34	13 (38.2)	0.97 (0.45, 2.07)	1.12 (0.70, 1.79)	0.98 (0.52, 1.60)
D)I/I polymorphism	164	64 (39.0)	(reference)	(reference)	(reference)
I/D or D/D	204	83 (40.7)	1.07 (0.70, 1.63)	1.11 (0.71, 1.74)	0.92 (0.54, 1.58)
R)I/D or I/I polymorphism	334	134 (40.1)	(reference)	(reference)	(reference)
D/D	34	13 (38.2)	0.92 (0.45, 1.91)	1.01 (0.46, 2.20)	1.03 (0.41, 2.59)

Abbreviations: D, deletion polymorphism; I, insertion polymorphism.

^aAdjusted for age, gender, systolic blood pressure, body mass index and smoking status.**P* < 0.05.

-0.14 μm). However, the interaction between D/D polymorphism and hypertension was not significantly associated with CRAE after adjusting for CRVE (-8.71 μm, 95% CI; -20.76 μm, 3.34 μm).

Association of retinal arteriolar wall signs/retinopathy and ACE I/D polymorphism

There was no significant association between focal retinal arteriolar wall signs (focal arteriolar narrowing, arterio-venous nicking and enhanced arteriolar wall reflex) or retinopathy and the ACE I/D polymorphisms (data not shown).

Discussion

In this population-based study of adult Japanese, we reported associations between the ACE I/D polymorphism and retinal arteriolar narrowing, a sub-clinical structural microvascular sign associated with chronic hypertension. We found that retinal arteriolar diameter was smaller in subjects with D/D than those with the I allele after adjusting for systolic blood pressure and other cardiovascular risk factors. We also found that diastolic blood pressure was significantly lower in subjects with D allele polymorphism compared with I/I polymorphism, but there was no difference in systolic blood pressure and no association for the presence of hypertension by the ACE I/D polymorphism.

Retinal arteriolar narrowing is a structural microvascular sign associated with chronic hypertension;¹

it has been shown to predict the incidence of stroke² and coronary heart disease.³ On the other hand, other studies suggested that retinal arteriolar narrowing might be antecedent of the hypertension, and involved in the pathogenesis of hypertension itself. If retinal arteriolar narrowing is associated with genetic disposition, such as ACE I/D polymorphism, which we have examined assessing the retinal vessel diameters will enable us to stratify those who are vulnerable to developing hypertension or further cardiovascular diseases. The Beaver Dam Eye Study showed that genetic factors affected retinal vessel diameters in a genome-wide linkage analysis.⁴ Within or near the linkage regions with retinal vessel diameter, there were genes associated with endothelial function, vasculogenesis, hypertension and coronary heart disease.⁴ However, to date, exact genes associated with retinal vessel diameter have not been identified.

The RAS and endothelial cells are intimately involved in atherosclerosis.⁵ It is known that local RAS exists in the eye,¹⁰ and retinal vascular endothelial cells can express angiotensin type 1 receptors.²¹ Previous experimental reports in studies of streptozocin-induced diabetic rats revealed that RAS inhibition ameliorates endothelial dysfunction.²² Retinal vessel diameter has also been linked with endothelial dysfunction, with studies showing association of retinal arteriolar narrowing with von Willebrand factor and factor VIII, which are systemic markers of endothelial dysfunction.²³ Therefore, our findings that D/D polymorphism is associated with narrower retinal arteriolar diameters

Table 3 Difference in central retinal artery and vein equivalent (μm), by angiotensin converting enzyme insertion/deletion polymorphisms, the Funagata study, Japan, 2000–02

	N	Mean vessel diameter (s.d.) (μm)	Crude difference (95% CI) (μm) ^a	Age-gender-adjusted (95% CI) (μm)	Multivariate adjusted ^b (95% CI) (μm)
<i>Central retinal artery equivalent</i>					
(A) I/I polymorphism	164	179.50 (20.39)	(reference)	(reference)	(reference)
I/D	170	179.46 (20.54)	-0.33 (-3.92, 3.26)	-0.43 (-3.98, 3.13)	-0.32 (-4.15, 3.51)
D/D	34	173.77 (19.73)	-6.69 (-12.88, -0.51)*	-6.60 (-12.78, -0.42)*	-6.86 (-13.58, -0.13)*
(D) I/I polymorphism	164	179.50 (20.39)	(reference)	(reference)	(reference)
I/D or D/D	204	178.51 (20.47)	-1.39 (-4.84, 2.06)	-1.50 (-4.90, 1.91)	-1.51 (-5.21, 2.19)
(R) I/I or I/D polymorphisms	334	179.48 (20.44)	(reference)	(reference)	(reference)
D/D	34	173.77 (19.73)	-6.56 (-12.45, -0.67)*	-6.61 (-12.43, -0.79)*	-6.49 (-12.86, -0.11)*
<i>Central retinal vein equivalent</i>					
(A) I/I polymorphism	164	215.61 (21.52)	(reference)	(reference)	(reference)
I/D	170	216.12 (20.37)	0.54 (-3.14, 4.21)	0.60 (-3.05, 4.26)	-0.07 (-3.87, 3.73)
D/D	34	217.37 (20.07)	5.22 (-1.31, 11.74)	4.75 (-1.78, 11.28)	3.93 (-3.01, 10.88)
(D) I/I polymorphism	164	215.61 (21.52)	(reference)	(reference)	(reference)
I/D or D/D	204	216.33 (20.28)	1.30 (-2.22, 4.82)	1.31 (-2.19, 4.81)	0.69 (-2.78, 4.35)
(R) I/I or I/D polymorphisms	334	215.87 (20.92)	(reference)	(reference)	(reference)
D/D	34	217.37 (20.07)	4.88 (-1.16, 10.92)	4.55 (-1.48, 10.58)	3.84 (-2.51, 10.20)

Abbreviations: D, deletion polymorphism; I, insertion polymorphism.

Estimated β coefficient, using multiple linear regression models, represents a mean difference in retinal arteriolar diameter and venular diameter by each unit change in genotype or allele, in additive (A), dominant (D) and recessive (R) models.

^aAdjusted for central retinal vein equivalent in models of central retinal artery equivalent, and for central retinal artery equivalent in models of central retinal vein equivalent, respectively.

^bAdjusted for age, gender, systolic blood pressure, body mass index and smoking status.

* $P < 0.05$.

might be explained by the activation of RAS in retinal vasculature.

Our study shows both *ACE* I/D polymorphism and hypertension-influenced retinal arteriolar diameters. However, we could not confirm whether an interaction between the D/D polymorphism and the presence of hypertension affecting retinal arteriolar diameters exists. Because there were only 13 subjects (38.2%) with D/D polymorphism who have hypertension in this study, a statistical power may be weak to prove this association.

In this older Japanese population, presence of the I allele is associated with essential hypertension.²⁴ In contrast, the D allele of the *ACE* I/D polymorphism has been associated with atherosclerosis.⁷ Retinal arteriolar narrowing, which reflects intimal thickening, medial hyperplasia and hyalinization and sclerosis of retinal arterioles has also been associated with atherosclerosis.²⁵ Our findings that the *ACE* I/D polymorphism is linked to retinal arteriolar narrowing may also be explained by its association with atherosclerotic processes.

Limitations and potential biases of this study should be mentioned. First, only 20.2% of total eligible subjects were included in this genetic analysis. Low participant rate of genetic analysis may affect the statistical power to prove associations. Furthermore, only 49.5% of this subsample had fundus photographs with sufficient quality for computer-assisted measurement of retinal vessels. Thus, unknown selection biases could have altered these results. Second, we did not have a detailed history of medications, including use of anti-hypertensive agents. Current use of ACE inhibitors

to lower blood pressure could have influenced our findings of an association between the *ACE* I/D polymorphism and retinal vessel caliber. Also, we were not able to measure plasma ACE levels in this study.

In conclusion, we found that subjects with D/D of the *ACE* I/D polymorphism had significant retinal arteriolar narrowing, a subclinical structural marker of chronic hypertension in this adult Japanese population. This association was stronger in subjects with a known history of hypertension. Our finding suggests that the *ACE* I/D polymorphism may influence the microvasculature, thereby possibly contributing to genetic susceptibility for the development of hypertension. Definitely, further research is needed to confirm this finding in other population-based samples.

What is known about this topic

- Retinal arteriolar narrowing is a structural sign associated with chronic hypertension.¹
- A substantial proportion of the variation in retinal arteriolar diameter might be genetically determined, independent of concomitant risk factors.⁴
- *ACE* I/D polymorphism is associated with hypertension⁵ and atherosclerosis, such as carotid wall thickness⁶ and coronary heart disease.⁷

What this study adds

- Subjects with D/D of the *ACE* I/D polymorphism had significantly narrower retinal arteriolar caliber in this adult Japanese population.
- *ACE* I/D polymorphism may influence the peripheral microcirculation, thereby possibly contributing to genetic susceptibility for the development of hypertension.

Conflict of interest

The authors declare no conflict of interest

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