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Risk factor–gene interaction in carotid atherosclerosis: effect of gene polymorphisms of renin-angiotensin system

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Abstract The risk factor–gene interaction in carotid atherosclerosis was investigated in 205 community-dwelling healthy subjects aged 50 years or more in Japan. The intima–media thickness (IMT) of the common carotid artery was evaluated by ultrasonography with a 7.5-MHz probe. Gene polymorphisms were determined for each subject with angiotensin-converting enzyme (ACE) insertion/deletion (I/D), angiotensinogen (AGT) M235T, angiotensin II type 1 receptor (AT1R) A1166C, and apolipoprotein E (apoE) genotypes. There was no genotype-specific difference in carotid IMT among any genes examined. Combinations of genotypes did not increase carotid IMT compared with subjects without these genotypes. In the total population, multiple regression analysis showed that age, systolic blood pressure (SBP), sex, and body mass index (BMI) were significantly associated with carotid IMT. However, the association between risk factors and IMT was genotype-specific. Age was significantly associated with IMT in ACE D carriers, but not in subjects with the ACE II genotype. Analysis of covariance adjusted with other risk factors showed that the age-dependent change in IMT was significantly different between subjects with the ACE II genotype and the ACE D carriers ($F[1.196] = 4.97$; $P = 0.027$). Similarly, the regression of IMT on SBP was significantly different between AGT TT and AGT MT + MM ($F[1.196] = 7.20$; $P = 0.0079$). The regression of IMT on BMI was also significantly different between apo E4 carriers and noncarriers ($F[1.196] = 6.78$; $P = 0.0099$). Furthermore, general linear model analysis with risk factors, genotype, and risk factor-genotype interactions revealed that the age*ACE genotype interaction, the SBP*AGT genotype interaction, and the BMI*apoE genotype interaction were significantly associated with IMT. These findings further support the role of risk factor-gene interaction in carotid atherosclerosis.

Key words Carotid atherosclerosis · ACE · Angiotensinogen · AT1 receptor · apoE · Polymorphism

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Introduction

Carotid atherosclerosis has been shown to be a risk factor for future cardiovascular events (O'Leary et al. 1999). The genetic predisposition to carotid atherosclerosis has been studied with respect to several candidate genes. Because the renin-angiotensin system (RAS) plays a pivotal role in the regulation of blood pressure, as well as in cardiovascular remodeling, the genes encoding the components of RAS have been the focus of much research (Hegele et al. 1998; Nakauchi et al. 1996; Takami et al. 1998; Yoshida et al. 2000). An insertion/deletion (I/D) polymorphism of the gene encoding angiotensin-converting enzyme (ACE) was shown to have a possible association with intima–media thickness (IMT) in several studies (Castellano et al. 1995; Kogawa et al. 1997; Pujia et al. 1996). Other polymorphisms of genes, such as angiotensinogen (AGT) M235T and angiotensin II type 1 receptor (AT1R) A1166C, have also been investigated in relation to carotid atherosclerosis (Castellano et al. 1996; Jeng 1999). However, conflicting results have been reported regarding the association of the genetic background of RAS with carotid atherosclerosis (Barley et al. 1995; Girerd et al. 1998; Zannad et al. 1998).

One of the possible reasons for the lack of replicable results is the ignoring of the genetic and environmental context of the studied populations (Kardia 2000). It is likely that the underlying multi-environmental distribution is significantly different across studies. Because atherosclerosis is a multifactorial disorder, with many environmental and genetic factors, risk factor-dependent evaluation of genetic effect could provide new information on the disorder (Kardia 2000; Turner et al. 1999).

Bearing this background in mind, we investigated the association between gene polymorphisms of the RAS and risk-factor-dependent augmentation of carotid atherosclerosis in community-dwelling healthy subjects, including an elderly population. Because isoforms of apolipoprotein E (apoE) have also been shown to be related to cardiovascular disease and atherosclerosis (Cattin et al. 1997), we also investigated the association of apoE genotype with carotid IMT, as an independent genetic factor of the RAS.

Subjects and methods

Subjects

All participants were recruited from healthy residents of a community located in the southern part of Japan. The total population of the community was 944, of whom 205 healthy individuals aged 50 years or more who were free of any history or symptoms of cardiovascular disease and not taking any medications were enrolled in the study. Informed consent for the procedures to be used was obtained from each subject. All procedures were approved by the Ethics Committee of Ehime University School of Medicine. Two hundred and five subjects participated.

Measurement of intima–media thickness (IMT)

The right carotid artery was evaluated with a SSD-900 apparatus (Aloka, Tokyo, Japan), using a 7.5-MHz probe (Jiang et al. 2000). After having the subject rest for at least 10 min in the supine position with the neck in slight hyperextension, we evaluated the optimal visualization of the right common carotid artery, carotid bulb, and extracranial internal and external carotid arteries. From multiple approaches, we detected plaque as the presence of wall thickening at least 50% greater than the thickness of the surrounding wall. From the anterior, lateral, and posterior approaches, the IMT of the far wall was measured in the right common carotid artery, 1 cm proximal to the bulb, and averaged to obtain mean IMT. Measurements were never taken at the level of a discrete plaque.

Evaluation of risk factors

Systolic and diastolic brachial blood pressures were measured twice, at a 5-min interval, with the subject in the supine position, with an automatic oscillometric blood pressure recorder (HEM-705CP; Omron, Tokyo, Japan) during the carotid echo examination. The mean value of two measurements was obtained. The validity and reproducibility of the device have been reported (O'Brien et al. 1996). Serum levels of total cholesterol, high-density (HDL) cholesterol, and glucose were determined by conventional methods.

Detection of gene polymorphisms

Genomic DNA was extracted from peripheral blood samples, using an extraction kit (Qiagen, Hilden, Germany). Gene polymorphisms of ACE I/D, AGT M235T, AT1R A1166C, and apoE E2/E3/E4 were determined by standard methods.

In brief, the I/D polymorphism of the *ACE* gene was identified by polymerase chain reaction (PCR), using a set of oligonucleotide primers flanking the polymorphic site in intron 16 (sense primer, 5′-GCCCTGCAGGTGTCTGCA GCATGT-3′ and antisense primer, 5′-GGATGGCTCTC CCCGCCTTGTTCTC-3′) (Rigat et al. 1992). To avoid mistyping, each sample found to have the DD genotype was subjected to a second, independent, PCR amplification with a primer pair that recognizes an insertion-specific sequence (5′-TGG GAC CAC AGC GCC CGC CAC TAC-3′ and 5′TCG CCA GCC CTC CCA TGC CCA TAA-3′) (Shanmugam et al. 1993).

To identify the AGT M235T polymorphism, a sense primer, 5′-TGACAGGATGGAAGACTGGCTGCTCCC TGC-3′, and an antisense primer, 5′-AGCAGAGAGGTT TGCCTTACCTTG-3′, were used (Russ et al. 1993). The PCR product (5 μl) was digested with 5 units *Msp*I for 1 h, and cleaved products were separated by electrophoresis.

The AT1R A1166C polymorphism was determined using a sense primer, 5′-TCCTCTGCAGCACTTCACTA CCAAATGGGC-3′, and an antisense primer, 5′-TTCAT CGAGTTTCTGACATT-3′ (Nakauchi et al. 1996). The PCR product (5 μl) was digested with 5 units *Hae*III for 1 h, and cleaved products were separated by electrophoresis.

The common apoE genotype was determined using a sense primer, 5′-ACAGAATTCGCCCCG-GCCTGGTC AC-3′, and an antisense primer, 5′-TAAGCTTGGCACG GCTGTCCAAGGA-3′ (Emi et al. 1988). The PCR product (5 μl) was digested with 5 units *Hha*I for 1 h, and cleaved products were separated by electrophoresis.

Statistical analysis

All values are expressed as means ± SD if not specified. Statistical comparisons among genotypes were performed by analysis of variation (ANOVA). The prevalence of genotypes and Hardy-Weinberg equilibrium were analyzed by the χ^2 method. The inheritance models of dominant (DD + ID versus II) and additive (DD versus ID versus II) were considered, because of the small number of subjects with ACE DD, AGT MM, AT1R CC, and apoE4. Analysis of covariance was employed to evaluate the differences in risk factor-genotype interactions in IMT. As risk factors, we evaluated age, sex, SBP, body mass index (BMI), total cholesterol, and blood glucose. A general linear model was employed to evaluate the significant contribution of risk factor-gene interactions for IMT. All analyses were performed with software packages (JMP; SAS Institute, Cary, NC, USA; and SPSS; SPSS, Chicago, IL, USA). A probability value of less than 0.05 was considered statistically significant.

Results

Distributions of genotypes in study population

Table 1 summarizes the demographic profiles of all participants. The distributions of the ACE, AGT, AT1R, and apoE genotypes observed in the study population were in agreement with Hardy-Weinberg equilibrium ($P = 0.988$, 0.935, 0.352, and 0.471, respectively). The distribution of each genotype was consistent with that published in other reports of Japanese subjects (Kato et al. 1999; Nakata et al. 1996; Nakauchi et al. 1996; Takami et al. 1998).

Table 1. Clinical characteristics of study population

Variables	$n = 205$
Age (years)	70 ± 9
Sex (male/female) (%)	66(32)/139(68)
Body height (cm)	153 ± 9
Body weight (kg)	53 ± 10
Body mass index (kg/m^2)	22.8 ± 3.0
Current smoker/ex-smoker/ never smoked (%)	28/29/148 (14/14/72)
Systolic blood pressure (mmHg)	135 ± 22
Diastolic blood pressure (mmHg)	74 ± 10
Total cholesterol (mg/dl)	183 ± 43
HDL cholesterol (mg/dl)	49 ± 15
LDL cholesterol (mg/dl)	108 ± 33
Plasma glucose (mg/dl)	104 ± 34

Values are means ± SD
HDL, High-density lipoprotein; LDL, law-density lipoprotein

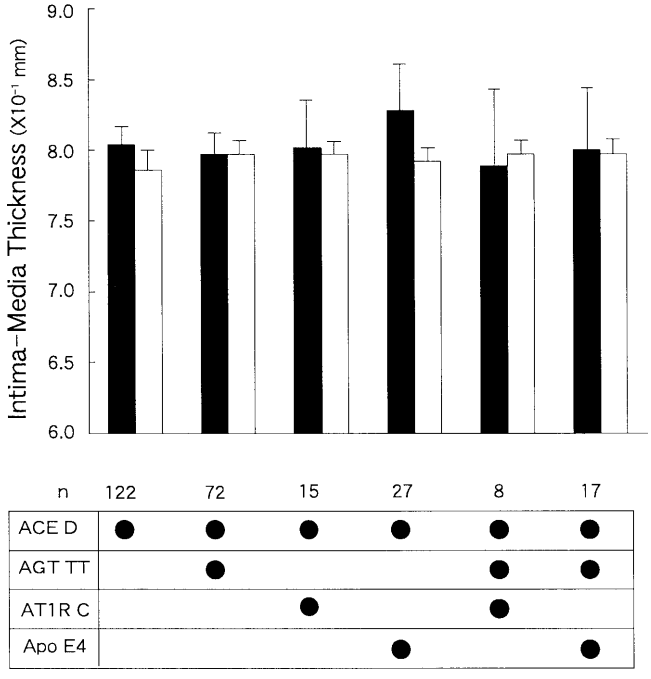

Fig. 1. Combinations of genotype and carotid intima–media thickness (IMT). Any combination of angiotensin-converting enzyme (*ACE*) ID + DD, angiotensinogen (*AGT*) TT, angiotensin II type 1 receptor (*AT1R*) AC + CC, and apolipoprotein (*Apo*) E2/E4 + E3/E4 did not increase carotid IMT compared with findings in subjects without these combinations of genotypes. Other combinations were omitted because the number of subjects was small. Values are means ± SEM. *Black bars*, Gene combination; *white bars*, control

Gene and carotid atherosclerosis

Table 2 summarizes the parameters of carotid atherosclerosis in all participants, classified by polymorphisms of the four genes. Carotid internal diameters were significantly different among *AT1R* gene polymorphisms. However, no significant differences were observed in IMT and numbers of plaques for any polymorphisms of the RAS genes or of *apoE*. Because a single gene, per se, did not influence IMT, the genotype-genotype interactions were studied. Any combination of ACE D, AGT TT, AT1R C, and Apo E4 did not increase IMT significantly, compared with findings in subjects without these combinations (Fig. 1).

Gene and risk factors for atherosclerosis

To find possible gene and risk factor interactions for IMT, multiple regression analysis for IMT was performed with

Table 2. Parameters of carotid atherosclerosis classified by gene polymorphisms

	II	ID	DD	
ACE				
n	83	95	27	
Age (years)	70.1 ± 9.3	70.4 ± 9.6	68.7 ± 8.2	
IMT ($\times 10^{-1}$mm)	7.9 ± 1.2	8.0 ± 1.4	8.1 ± 1.4	
Diameter (mm)	6.6 ± 0.9	6.5 ± 0.9	6.8 ± 0.9	
Plaque (*n*)	0.12 ± 0.4	0.20 ± 0.5	0.15 ± 0.4	
	MM	MT	TT	
AGT				
n	10	69	126	
Age (years)	67.7 ± 8.1	71.0 ± 9.9	69.7 ± 9.0	
IMT ($\times 10^{-1}$mm)	7.7 ± 1.5	8.0 ± 1.3	8.0 ± 1.3	
Diameter (mm)	6.5 ± 0.9	6.7 ± 0.8	6.5 ± 0.9	
Plaque (*n*)	0.40 ± 1.0	0.13 ± 0.4	0.16 ± 0.5	
	AA	AC	CC	
AT1R				
n	183	19	3	
Age (years)	69.8 ± 9.1	72.4 ± 10.2	70.3 ± 13.7	
IMT ($\times 10^{-1}$mm)	8.0 ± 1.3	8.0 ± 1.4	8.0 ± 1.5	
Diameter (mm)	6.5 ± 0.9	6.9 ± 1.0	7.3 ± 0.9*	
Plaque (*n*)	0.15 ± 0.5	0.21 ± 0.5	0.33 ± 0.6	
	E3/E3	E3/E2	E3/E4	E2/E4
ApoE				
n	137	27	38	3
Age (years)	70.2 ± 9.6	71.5 ± 8.6	68.9 ± 8.4	65.0 ± 7.0
IMT ($\times 10^{-1}$mm)	7.9 ± 1.3	7.9 ± 1.2	8.3 ± 1.6	8.6 ± 1.7
Diameter (mm)	6.6 ± 0.9	6.3 ± 0.9	6.7 ± 1.0	7.1 ± 0.5
Plaque (*n*)	0.15 ± 0.4	0.22 ± 0.5	0.16 ± 0.6	0.3 ± 0.6

* $P < 0.05$
Values are means ± SD
ACE, angiotensin-converting enzyme; *AGT*, angiotensinogen; *AT1R*, angiotensin II type 1 receptor; *ApoE*, apolipoprotein E; *IMT*, intima–media thickness

Table 3. Multiple regression analysis for IMT in subjects with specific genotypes

Subjects with	*n*		Total	Age	Sex	SBP	BMI	T.chol	BS
Total no. of subjects	205	β	0.54	0.41	0.11	0.22	0.18	−0.04	0.07
		P	<0.0001	<0.0001	0.099	0.0008	0.005	0.55	0.26
ACE II	83	β	0.49	0.22	0.08	0.342	0.12	−0.02	0.07
		P	0.0016	0.07	0.44	0.003	0.31	0.83	0.52
ID + DD	122	β	0.60	0.52	0.11	0.15	0.20	−0.07	0.07
		P	<0.0001	<0.0001	0.18	0.06	0.01	0.41	0.36
AGT TT	126	β	0.58	0.37	0.08	0.34	0.17	−0.12	0.08
		P	<0.0001	<0.0001	0.32	<0.0001	0.04	0.15	0.31
MT + MM	79	β	0.56	0.48	0.20	0.04	0.18	0.06	0.09
		P	0.0001	<0.0001	0.08	0.71	0.09	0.57	0.42
AT1R AA	183	β	0.52	0.41	0.11	0.20	0.19	−0.02	0.09
		P	<0.0001	<0.0001	0.13	0.0043	0.005	0.83	0.21
AC + CC	22	β	0.77	−0.04	0.02	0.56	0.02	−0.44	−0.14
		P	0.021	0.91	0.74	0.024	0.92	0.08	0.48
Apo E3/E2 + E2/E3	164	β	0.52	0.43	0.09	0.20	0.10	0.004	0.04
		P	<0.0001	<0.0001	0.20	0.007	0.20	0.96	0.61
E2/E4 + E3/E4	41	β	0.73	0.36	0.09	0.21	0.45	−0.23	0.13
		P	0.0001	0.0097	0.52	0.12	0.001	0.12	0.37

Multiple regression analyses were performed with age, sex, systolic blood pressure (SBP), body mass index (BMI), total cholesterol (T.chol), and blood sugar (BS). Standard correlation coefficient (β) and *P* values are shown

risk factors in subjects with a specific genotype (Table 3). It was shown that age was significantly associated with IMT in ACE D carriers (ID + DD), but not in subjects with the ACE II genotype. Age itself was not significantly different between ACE D carriers and subjects with the ACE II genotype (70 ± 9 and 70 ± 9 years, respectively; $P = 0.98$). Analysis of covariance adjusted with other risk factors showed that the regression line between age and IMT was significantly different between subjects with the ACE II genotype and ACE D carriers (age*ACE F[1.196] = 4.97; $P = 0.027$) (Fig. 2). Similarly, SBP was significantly associated with IMT in subjects with AGT TT, but not in subjects with AGT MT + MM (Table 3). There was no significant difference in SBP between subjects with the AGT TT genotype and AGT M carriers (134.7 ± 20.0 mmHg and 133.9 ± 24.3 mmHg; $P = 0.79$). Analysis of covariance adjusted with other risk factors also indicated that the two regression lines were significantly different (SBP*AGT F[1.196] = 7.20; $P = 0.0079$) (Fig. 3). It was shown that SBP was significantly associated with IMT in apoE4 carriers, but not in subjects without apoE4. However, analysis of covariance failed to demonstrate a significant difference between the two regression lines (SBP*apoE F[1.196] = 3.09; $P = 0.08$). On the other hand, such analysis revealed that the association between IMT and BMI was significantly different between apoE4 carriers and noncarriers (BMI*apoE F[1.196] = 6.78; $P = 0.0099$). Body mass index itself was not significantly different between apoE4 carriers and noncarriers (22.8 ± 3.2 and 22.8 ± 2.9 kg/m^2, respectively; $P = 0.99$).

Risk factor-gene interactions for intima-media thickness (IMT)

To further investigate whether risk factors and genotype interactions significantly influenced IMT, a general linear model for IMT was analyzed, with the following parameters; age, sex, SBP, BMI, total cholesterol, blood glucose, ACE genotype (D dominant), AGT genotype (M dominant), apoE genotype (E4 dominant), age*ACE genotype interaction, SBP*AGT genotype interaction, and BMI*apoE genotype interactions (Table 4). This analysis revealed that age*ACE genotype interaction, SBP*AGT genotype interaction, and BMI*ApoE genotype interaction were significantly associated with IMT, in addition to age, SBP, and BMI.

Table 4. General linear model for intima–media thickness

	F	*P*
Age	35.7	<0.0001*
Sex	2.81	0.095
Systolic blood pressure	10.72	0.001*
Body mass index	14.92	<0.0001*
Total cholesterol	1.83	0.18
Blood glucose	2.14	0.15
ACE genotype	3.48	0.064
AGT genotype	7.03	0.009*
ApoE genotype	4.42	0.037*
AT1R genotype	0.09	0.77
Age*ACE interaction	4.40	0.037*
Systolic blood pressure*AGT interaction	7.61	0.006*
Body mass index*ApoE interaction	6.11	0.014*

*Significant variables
ACE genotype, D allele dominant (ID + DD vs II); AGT genotype, M allele dominant (MT + MM vs TT); apoE genotype, E4 dominant (E2E4 + E3E4 vs E2E3 + E3E3); AT1R genotype, C allele dominant (AC + CC vs AA)

Discussion

Among *RAS* genes, insertion and deletion of the *ACE* gene is the most studied polymorphism in relation to several aspects of hypertension, as well as end-organ damage (Huang et al. 1998; Lindpaintner et al. 1995). In an earlier

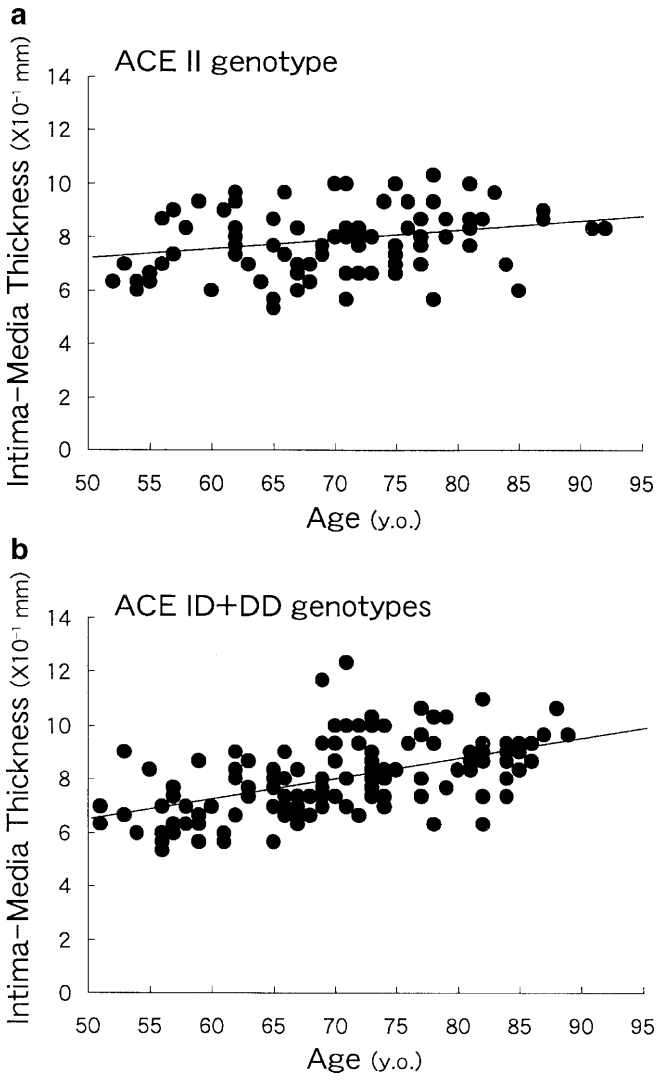

Fig. 2a,b. Genotype-dependent linear regression of carotid intima-media thickness on age. Analysis of covariance showed that the two regression lines were significantly different (F[1.196] = 4.97; P = 0.027), indicating that the effect of aging on IMT was more marked in ACE D carriers. *ACE*, Angiotensin-converting enzyme. **a** n = 83; r = 0.257; $P < 0.05$; **b** n = 122; r = 0.529; $P < 0.0001$

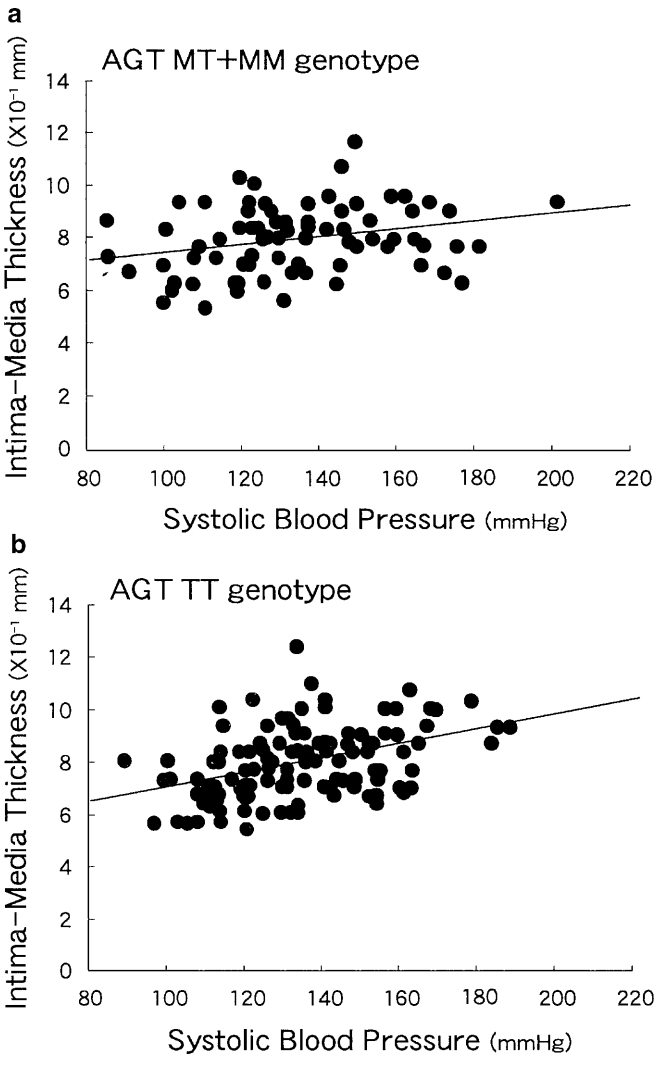

Fig. 3a,b. Angiotensinogen genotype-specific association between systolic blood pressure and carotid intima-media thickness. Analysis of covariance showed that the two regression lines were significantly different (F[1.196] = 7.20; P = 0.0079). **a** n = 79; r = 0.282; $P < 0.012$; **b** n = 126; r = 0.427; $P < 0.0001$

study, Castelanno et al. (1995) demonstrated an association between *ACE* I/D polymorphism and carotid IMT in a general population sampled in Vobarno, Italy. However, other, and more recent studies in the general population failed to demonstrate an association (Barley et al. 1995; Girerd et al. 1998; Zannad et al. 1998). A lack of association between *ACE* gene polymorphism and carotid IMT has also been demonstrated in a low-risk population (Dessi-Fulgheri et al. 1995). On the other hand, an association of polymorphisms of the *ACE* gene and IMT was reported in specific populations, such as noninsulin-dependent diabetes mellitus (NIDDM) patients (Hosoi et al. 1996), hemodialysis patients (Nergizoglu et al. 1999), and hypertensive patients (Jeng 2000). These findings suggest risk factor-genotype interactions of the *ACE* gene.

The association between carotid atherosclerosis and the AGT M235T polymorphism or the AT1R A/C1166 gene polymorphism has also been studied. No association between the AT1R A/C1166 gene polymorphism or the AGT M235T polymorphism and carotid wall thickness was reported in a population that included subjects who were free of cardiovascular disease (Barley et al. 1995; Girerd et al. 1998; Jeng et al. 1999; Zannad et al. 1998). In the present study, we also did not find a positive association between carotid atherosclerosis and the AGT M235T or the AT1R A/C polymorphism. Furthermore, any combination of the genes of *ACE* ID or DD, *AGT* TT, *AT1R* AC or CC, and *apo E4* failed to influence carotid IMT, suggesting that gene polymorphism itself did not have any effect on carotid atherosclerosis in the present population.

The relationship between phenotype and genotype is not constant over a broad range of environments, because many internal and external factors, including genetic factors, are involved in the pathophysiological expression of the phenotype (Turner et al. 1999). Gene-environmental interactions in the determination of blood pressure have been studied in relation to sex, and such environmental factors as salt sensitivity and drug treatment. These studies demonstrated that certain genotypes influenced blood pressure in male subjects (O'Donnell et al. 1998) to the same extent as interventions such as salt loading (Giner et al. 2000), salt restriction (Hunt et al. 1998), and antihypertensive treatment (Schunkert et al. 1997). Recently, Turner et al. (1999) investigated the context-dependent association of the ACE I/D polymorphism with blood pressure. They found that the ACE polymorphism itself was not related to the level of blood pressure; however, the polymorphism significantly affected the relationship between age and systolic blood pressure, indicating that the gene could influence blood pressure in a context-dependent way. Because atherosclerosis is a complex trait, with several risk factors involved in its development, the genetic predisposition to atherosclerosis could also be influenced by environmental factors. We found that the regression of IMT on age was significantly different between ACE genotypes. Judging from the regression slopes, the effect of aging on IMT thickening was more marked in subjects with the ACE D genotype than in subjects with the ACE II genotype. Similarly, the interactions between SBP and IMT were significantly different between subjects with the TT genotype and subjects with the MT or MM genotype. SBP and IMT were not significantly different between AGT TT and AGT M carriers; AGT was not a direct risk for IMT. However, the slope of the linear regression was steeper in subjects with the TT genotype, suggesting that the effect of an increase in SBP on IMT thickening is more prominent in subjects with the TT genotype.

ApoE has three isoforms, E2, E3, and E4. In an earlier study, an association between the E4 allele and carotid atherosclerosis was demonstrated in middle-aged asymptomatic subjects from a general population (Cattin et al. 1997). However, conflicting results have also been reported regarding the association between apoE4 and carotid atherosclerosis (Sass et al. 1998). In the present study, we also did not find a significant difference in IMT among apoE genotypes. Risk factor-gene interactions were also investigated in apoE. There was no significant difference in the regression lines of age and IMT among apoE genotypes. However, the apoE genotype showed an influence on the association between BMI and carotid atherosclerosis. The BMI-dependent augmentation of carotid IMT was prominent in apoE4 carriers compared with data in noncarriers. These findings indicate that an influence of risk factor-gene interaction on carotid atherosclerosis is also present in the *apoE* gene.

The finding in the present study that risk factor-gene interactions were observed in several genes that failed to show a positive association with IMT further supports the hypothesis that other factors could contribute to the effect of genetic variation to give rise to atherosclerosis. In other words, genetic variation itself could be associated with atherosclerosis in a particular population. The important information obtained from the present study is that risk factor-gene interaction could provide specific predictive information about when or how an individual is likely to develop atherosclerosis (Kardia 2000). We can not explain the exact mechanisms of the interaction between risk factors and genotype from the findings in the present study. However, the ACE DD genotype may share a common pathway with aging processes, such as endothelial dysfunction (Butler et al. 1999; DeSouza et al. 2000), by which atherosclerosis progresses. Similarly, the AGT genotype may influence the mechanisms underlying the process by which blood pressure promotes atherosclerosis. However, we need further study to explore these hypotheses.

In summary, our results suggest that known genetic predispositions to atherosclerosis, of RAS, as well as apoE4 alleles alone, were not strong enough to lead to the development of carotid arterial hypertrophy in community-dwelling healthy individuals. However, the interaction with age or blood pressure, two of the most potent risk factors for atherosclerosis, could promote the capability of the gene to lead to the development of atherosclerosis. A longitudinal study with a large number of subjects is needed to clarify the role of risk factor-gene interaction in the development of atherosclerosis.

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