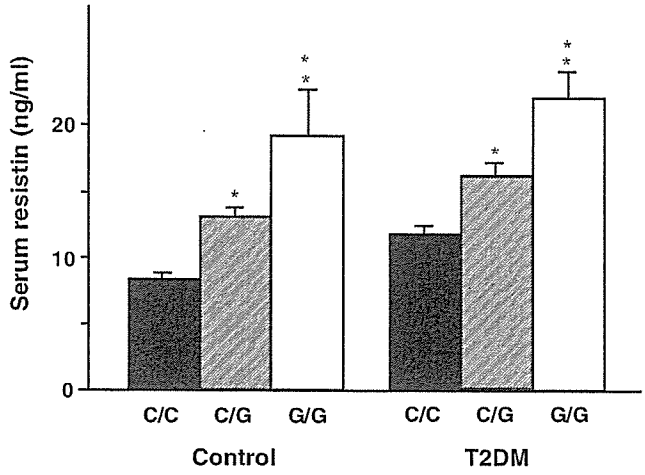

Fig. 1. Serum resistin levels of each genotype in T2DM and control subjects. Serum resistin levels were measured using a human resistin ELISA kit (Linco Research) as described in Materials and methods. Fasting serum samples from 198 T2DM and 157 control subjects were analyzed. The data represent means ± SE for each genotype in either control or T2DM subjects. *Significant difference compared to C/C, **significant difference compared to C/C or C/G. In controls, ANOVA: $F = 35.0$, $P < 0.0001$, Scheffe's test $P < 0.001$ between each pair. In T2DM, ANOVA: $F = 14.3$, $P < 0.0001$; Scheffe's test: $P = 0.0053$ (C/C vs C/G), $P < 0.0001$ (C/C vs G/G), and $P = 0.016$ (C/G vs G/G). When all genotypes were combined, serum resistin levels were significantly higher in T2DM than controls (means ± SE, control vs T2DM; 11.2 ± 0.5 vs 15.1 ± 0.7 ng/ml, Student's t test; $P < 0.0001$). Fasting serum resistin levels were also increased as the number of G alleles increased when T2DM and controls were combined (C/C 10.2 ± 0.4; C/G 15.0 ± 0.7; and G/G 21.1 ± 1.7 ng/ml, ANOVA: $F = 38.3$, $P < 0.0001$; Scheffe's test: $P < 0.0001$ between each pair).

analyzed. The resistin mRNA level in total RNA from human primary cultured adipocytes (Zen-Bio, NC) was quantitated as described above, but using three replicate wells, to compare resistin mRNA levels between human monocytes and adipocytes.

Statistical analysis. To examine the effect of the −420G/G genotype on serum resistin levels, a single regression analysis involving the genotype, gender, age, age of onset, duration of T2DM, BMI, maximum body mass index in life (max BMI), or HbA1c as an independent variable was performed. A multiple regression analysis was then performed using only the significant factors of these variables. In these regression analyses, the genotypes for −420C/C, −420C/G, and −420G/G were denoted by two dummy variables $(c1, c2) = (0,0)$, $(1,0)$, and $(0,1)$, respectively. To estimate the effects of serum resistin levels on T2DM, a multiple logistic regression analysis adjusted simultaneously for potentially confounding variables was performed. The variables considered in this model were age, gender, max BMI, and serum resistin. In the logistic regression analysis, the Wald test was used to assess statistical significance. Analysis of variance (ANOVA) followed by Sheffe's test is used in Figs. 1 and 2A. Student's t test is also used in Fig. 1 where indicated. Simple regression analysis is used in Fig. 2B.

Results

Serum resistin levels were higher in T2DM

We first compared serum resistin levels between 198 cases (SNP-420 genotype = n; C/C = 87, C/G = 87, and G/G = 24) and 157 controls (C/C = 80, C/G = 64, and G/G = 13) (Fig. 1). Serum resistin levels were significantly higher in T2DM than in controls (means ± SE,

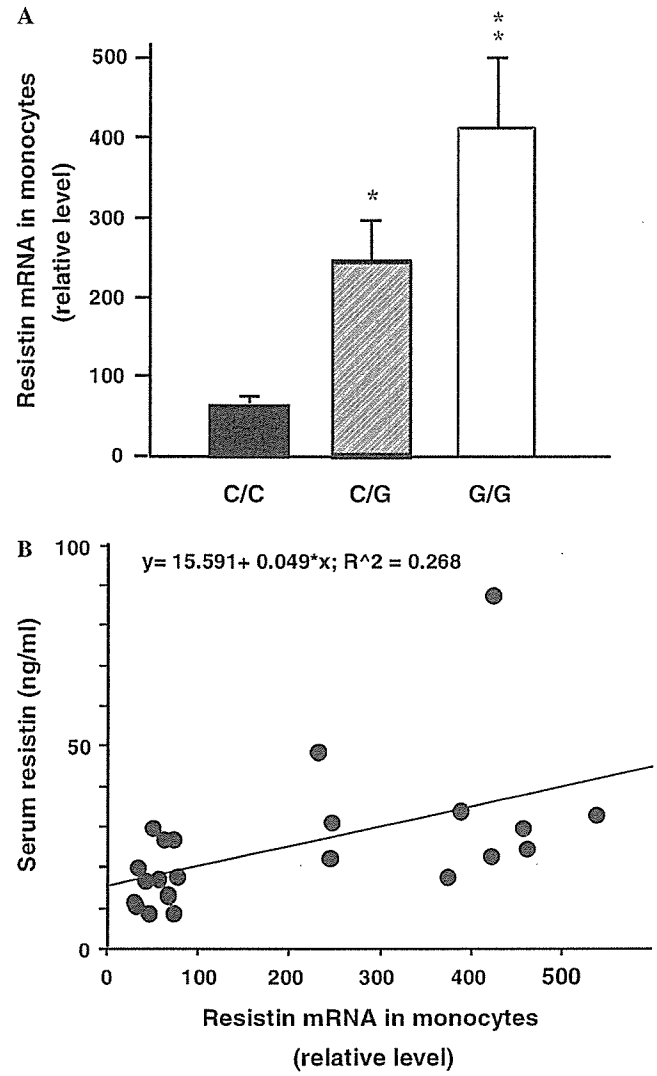

Fig. 2. Resistin mRNA levels in monocytes in healthy volunteers. Resistin mRNA levels in monocytes of 23 healthy volunteers were quantitated using the two-step TaqMan RT-PCR method as described in Materials and methods. The level of human resistin mRNA was normalized by that of human GAPDH mRNA in each sample for meaningful comparisons, and the relative amounts of resistin mRNA were determined by calculating from the threshold cycles. Resistin mRNA levels corrected by GAPDH mRNA levels in undifferentiated THP-1 cells are defined as 1. (A) Resistin mRNA in healthy volunteers with each genotype. The data represent means ± SE using duplicate wells for each subject. ANOVA and Scheffe's test were used for statistical analysis. C/C ($n = 9$), C/G ($n = 11$), and G/G ($n = 3$). ANOVA: $F = 8.87$, $P = 0.0018$; Scheffe's test: $^*P < 0.05$ and $^{**}P < 0.005$ compared to C/C genotype. (B) Correlation between resistin mRNA in monocytes and its simultaneous serum resistin levels. Fasting serum resistin levels at the time of monocyte isolation were measured as described in Materials and methods. Simple regression analysis was used for statistical analysis. Fasting serum resistin level = 15.59 + 0.049 × resistin mRNA in monocytes ($R^2 = 0.268$). $R = 0.518$, $P = 0.011$.

control vs T2DM; 11.2 ± 0.5 vs 15.1 ± 0.7 ng/ml, Student's t test, $P < 0.0001$). Fasting serum resistin levels increased with increasing number of G alleles in controls, T2DM, and both (both combined; C/C 10.2 ± 0.4; C/G 15.0 ± 0.7; and G/G 21.1 ± 1.7 ng/ml, ANOVA; $F = 38.3$, $P < 0.0001$, Scheffe's test; $P < 0.0001$ between each pair, see Fig. 1 legend for the other results).

SNP-420 genotype primarily determined serum resistin levels also increased with longer duration of T2DM and higher HbA1c

To examine which factors affect fasting serum resistin levels, we then analyzed 198 T2DM subjects (Table 2). A single regression analysis involving the genotype (C/G or G/G vs C/C), age, gender, age of onset, duration of T2DM, BMI, max BMI, or HbA1c as an independent variable revealed that only the genotype, duration of T2DM, and HbA1c were significantly associated with serum resistin levels.

A multiple regression analysis involving these three independent variables showed that serum resistin levels were ~4.4 ng/ml higher in C/G, and ~10.6 ng/ml higher in G/G than in C/C (Table 3). An increase in 1-year duration of T2DM and 1% of HbA1c was correlated with an increase in serum resistin at levels of 0.19 and 0.54 ng/ml, respectively.

A single regression analysis also revealed that serum resistin levels were determined by the genotype in 157 control subjects, whereas age, gender, BMI, max BMI, or HbA1c had no effects (data not shown). Neither BMI nor max BMI was associated with serum resistin levels, even when adjusted for genotype, age, gender, and HbA1c, either in the cases or the controls (data not shown). Therefore, serum resistin levels were strongly correlated with the SNP-420 genotype in both T2DM and controls. The duration of T2DM and HbA1c was positively correlated with these levels only in T2DM.

Table 2
Simple regression analysis involving fasting serum resistin level as a dependent variable in T2DM subjects

Variables	Parameter estimate	Standard error	P
CG	4.36	1.33	0.0012
GG	10.22	2.02	<0.0001
Gender (female)	0.93	1.33	0.488
Age	0.07	0.06	0.253
Age of onset	−0.09	0.06	0.145
Duration	0.24	0.08	0.002
BMI	−0.04	0.17	0.798
max BMI	0.14	0.16	0.373
HbA1c	0.86	0.38	0.023

Each of genotype of SNP-420, gender, age, age of onset of T2DM, duration of T2DM, BMI, max BMI, and HbA1c was involved in the analysis as an independent variable. Statistical analyses were performed as described in Materials and methods.

Table 3
Regression analysis for serum resistin in T2DM or T2DM as dependent variables

Variables	Estimate	Standard error	P
Serum resistin in T2DM			
Intercept	5.31	3.20	
C/G	4.42	1.36	0.0013
G/G	10.57	2.14	<0.0001
Duration of diabetes	0.19	0.07	0.0090
HbA1c	0.54	0.37	0.1486
T2DM (logistic regression)			
Intercept	−2.38	1.22	
Serum resistin	0.07	0.02	<0.0001
Age	−0.02	0.01	0.0725
Gender (female)	−0.29	0.24	0.2136
max BMI	0.13	0.03	0.0003

Each of serum resistin in T2DM, and T2DM was involved in the analysis as a dependent variable. The independent variables in each analysis are shown below each intercept. Statistical analyses were performed as described in Materials and methods.

Serum resistin level was an independent factor for T2DM

To determine whether serum resistin is associated with T2DM, a logistic regression analysis involving serum resistin level, age, gender, and max BMI was employed. Serum resistin level was found to be an independent determinant for T2DM (Table 3). Therefore, serum resistin levels, primarily determined by the SNP-420 genotype, could induce T2DM.

Resistin mRNA level in monocytes was higher in the G/G genotype and positively correlated with serum resistin levels

To determine whether the resistin SNP-420 genotype is associated with resistin gene expression in human monocytes, we analyzed its mRNA levels using RT-PCR (Fig. 2). To assess isolated effects of the SNP-420 genotype, 23 healthy volunteers were employed. Resistin mRNA was significantly higher in the C/G or G/G genotype than in the C/C genotype. Consistent with the data on serum resistin levels (Fig. 1), resistin mRNA in monocytes appears to be highest in the G/G genotype (means ± SE, C/C 62.6 ± 4.0; C/G 243.8 ± 54.0; and G/G 412.8 ± 87.5), although the difference did not quite reach the levels of significance when compared between G/G and C/G ($P = 0.07$) (Fig. 2A).

Finally, when these volunteers were analyzed together, resistin mRNA levels were positively correlated with serum resistin levels ($R = 0.518$, $P = 0.011$) (Fig. 2B). We also found that resistin mRNA level was more than ~100-fold higher in human monocytes than in human primary cultured adipocytes (resistin mRNA in human primary cultured adipocytes, means ± SE of three replicate wells; 0.61 ± 0.06). Therefore, the SNP-420 genotype determines resistin mRNA in monocytes and serum levels, which could induce T2DM.

Discussion

We report here that the resistin promoter SNP-420 genotype was associated with its monocyte mRNA and serum levels, and that T2DM subjects had higher serum resistin levels than controls. A logistic regression analysis revealed that serum resistin level was an independent factor for T2DM. Therefore, the SNP-420 determines monocyte mRNA and serum levels of resistin, which could induce T2DM.

We found that the SNP-420 genotype was a major determinant of serum resistin levels. Serum resistin levels were highest in the G/G genotype, followed by the C/G and C/C genotypes. This order was also confirmed in a report on Korean subjects [26]. Haplotypes including this SNP-420 showed a similar tendency in Japanese subjects [41]. We also found that resistin mRNA levels in monocytes were higher in healthy volunteers with the G/G genotype. Smith et al. [38] showed that obese human subjects with the G/G genotype also have higher resistin mRNA levels in their abdominal subcutaneous fat.

We found that resistin mRNA in monocytes was positively correlated with serum resistin levels. We also found that resistin mRNA was more than ~100-fold higher in monocytes than in primary cultured adipocytes in humans. Whereas it is dominantly expressed in adipose tissues of mice, resistin is most highly expressed in macrophages in humans [32–34]. Therefore, monocytes are promising candidates for the main source of serum resistin in humans, although other regulatory factors or secretory tissues could also affect serum resistin levels.

The association of resistin mRNA in adipose tissue with serum resistin or insulin resistance has been reported by other investigators. Heilbronn et al. [42] reported that serum resistin is positively correlated with resistin mRNA in the subcutaneous adipose tissue of obese subjects. The fat content in the liver and HOMA-IR has been also reported to be positively correlated with resistin mRNA in subcutaneous adipose tissues of obese subjects [38]. A total of four independent reports have shown that the activity of the mutant resistin promoter including −420G is higher than that of the wild type promoter including −420C [6,26,38,41]. Therefore, G of SNP-420 enhances resistin gene promoter activity, which could increase resistin mRNA levels in adipose tissues as well as monocytes, leading to whole body insulin resistance.

We have shown that serum resistin levels were associated with T2DM. The serum levels increased with the number of G alleles in both T2DM and control subjects. The duration of T2DM and HbA1c was also positively correlated with serum resistin in T2DM. Serum resistin levels have been reported to be increased or unchanged in human T2DM or obesity [14,26–31]. The discrepancy between previous reports may be resolved by considering the SNP-420 genotype as well as the duration of T2DM and HbA1c. It should be noted that serum resistin probably exists as a hexamer (major form) or trimer (a more biologically active form) in mice, which may also affect the assay results [43].

In summary, we elucidated factors correlated with serum resistin levels and effects of SNP-420 on resistin mRNA in monocytes. Fasting serum resistin was significantly higher in T2DM and its independent determinant. Resistin monocyte mRNA levels were positively correlated with their simultaneous serum levels. Therefore, the SNP-420 determines the monocyte mRNA and serum levels of resistin, which could induce T2DM. It is not presently clear how resistin induces insulin resistance in human subjects and whether adipocytes or macrophages are the main sources of serum resistin. Further experiments will be required to clarify these points.

Acknowledgments

This work was supported by grants from Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan. We thank M. Murase, T. Nishimiya, and Drs. M. Hashiramoto and H. Niiya for suggestions, and Drs. K. Ono, O. Ebisui, and Y. Kusunoki for collecting clinical data and samples.

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Radial Augmentation Index: A Useful and Easily Obtainable Parameter for Vascular Aging

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Background: It has been shown that the systolic augmentation index (AI) in the central arteries, including the aorta and carotid arteries, changes with age. The AI can also be obtained from the peripheral arteries. The possible usefulness of AI obtained from the radial artery as an index for vascular aging was investigated.

Methods: Radial arterial waveforms were obtained from 632 subjects with no cardiovascular disease, using radial tonometry. Radial AI was calculated as follows: (Second peak systolic blood pressure [SBP2] − diastolic blood pressure [DBP]) / (first peak SBP − DBP) ×100 (%).

Results: Radial AI was significantly higher in women than in men (81.1% ± 16.1% compared with 69.5% ± 16.3%, $P < .001$). Radial AI was positively related to age in healthy men and women ($r = 0.619$, $P < .001$, and $r = 0.644$, $P < .001$, respectively). When comparing subjects in their 20s to those in their 70s, radial AI increased 1.56 times (from 53.2% to 83.0%) in men and 1.49 times (from 64.6% to 96.4%) in women. Multiple regression analysis showed that age is a potent predictor of radial AI in addition to gender, DBP and pulse rate.

Conclusions: These findings indicate that simple and easily-obtainable radial AI is age-dependent and could be a useful index of vascular aging. Am J Hypertens 2005; 18:11S–14S © 2005 American Journal of Hypertension, Ltd.

Key Words: Augmentation Index, wave reflection, arterial stiffness, vascular aging.

Recently, several parameters have been introduced to assess vascular aging.[1–4] One of the parameters is systolic augmentation index (AI), which is obtained from the central arterial waveform as the ratio of augmentation pressure to the total pulse pressure.[1–3] Although many factors have been shown to affect AI, it has been shown that central AI is closely related to several risk factors for atherosclerosis[5–8] and for future cardiovascular events.[9,10] A close relationship between age and central AI has also been reported.[11] Because AI can also be obtained from radial arterial waveform,[1–3,12] radial AI itself could provide information on vascular aging. In the present study, we investigated the relationship of radial AI to age in healthy subjects free of cardiovascular disease.

Methods

Subjects

Subjects were selected among volunteers who agreed with the main purpose of this study and participated in either local resident or employee health examinations. A total of 632 subjects with no history of cardiovascular disease, diabetes, or hyperlipidemia were identified and included in the study. Anthropometric parameters, blood pressure (BP), and radial AI were measured in all participants. Written informed consent for the procedure was obtained from each subject, and the study was approved by the ethical committee of Ehime University School of Medicine.

Brachial BP and Pulse Wave Analysis

Blood pressure and radial AI were measured on the right upper arm using an oscillometric method after 5 min of rest in the sitting position (HEM-907; Omron Healthcare Co., Ltd., Kyoto, Japan). Immediately after measuring BP via the upper arm, the left radial arterial waveform was obtained using the tonometric method. Radial AI was calculated as follows: (Second peak systolic BP [SBP2] − diastolic BP [DBP]) / (first peak SBP − DBP) ×100 (%), which was automatically calculated using a fourth-order differential equation for radial arterial waveform (HEM-9010AI; Omron Healthcare Co., Ltd., Kyoto, Japan) (Fig.

Received September 30, 2004. Accepted October 4, 2004.

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0895-7061/05/$30.00
doi:10.1016/j.amjhyper.2004.10.010

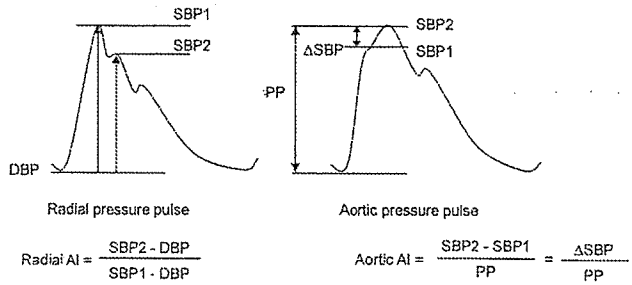

FIG. 1 An actual tracing of a radial arterial waveform. Radial augmentation index (AI) = (SBP2 − DBP)/(SBP1 − DBP) (%). DBP = diastolic pressure; PP = pulse pressure; SBP1 = first systolic blood pressure component; SBP2 = second systolic blood pressure component. Aortic AI is defined as (SBP2 − SBP1)/PP (%), ΔSBP = SBP2 −SBP1.

1). The HEM-9010AI device is programmed to determine automatically the pressure against the radial artery to obtain the optimal radial arterial waveform. The SBP2 was also calculated by calibrating with brachial BP as an index of the absolute values of reflection pressure wave.

To investigate the association between radial AI and aortic AI, we also measured aortic AI using the transfer function developed for the SphygmoCor apparatus (AtCor Medical, NSW, Australia) in 154 subjects. Aortic AI was calculated as follows: (SBP2 − SBP1) / pulse pressure (PP) × 100 (%) (Fig. 1).

Data Analysis

All values are expressed as mean ± SD, if not specified. The difference between men and women was evaluated by analysis of variance. Multiple regression analysis for AI was performed with the following parameters: age, gender, brachial SBP, DBP, pulse rate, body height, body weight, and body mass index (BMI). All statistical analyses were performed using the SPSS statistical software package (SPSS Inc., Chicago, IL). A probability value of $P < .05$ was considered to be statistically significant.

Results

Table 1 summarizes characteristics of the study participants divided by sex. The mean age was 47.0 ± 15.4 years (range 20 to 82 years). The radial AI in men was significantly lower than that in women (69.5 ± 16.3 compared with 81.1 ± 16.1, $P < .001$). Figure 2 illustrates the

Table 1. Clinical characteristics of the study participants

	Male (n = 348)	Female (n = 289)	P value
AI	69.5 ± 16.3	81.1 ± 16.1	<.001
SBP	125.9 ± 16.1	117.0 ± 18.0	<.001
DBP	75.9 ± 12.6	69.6 ± 11.9	<.001
Pulse rate	70.6 ± 11.0	71.5 ± 9.5	NS
Height	169.0 ± 6.0	155.4 ± 6.4	<.001
Weight	65.8 ± 9.5	52.6 ± 7.7	<.001
BMI	23.0 ± 2.9	21.8 ± 3.0	<.001

AI = augmentation index; BMI = body mass index; DBP = diastolic blood pressure; SBP = systolic blood pressure.
Values are mean ± SD.

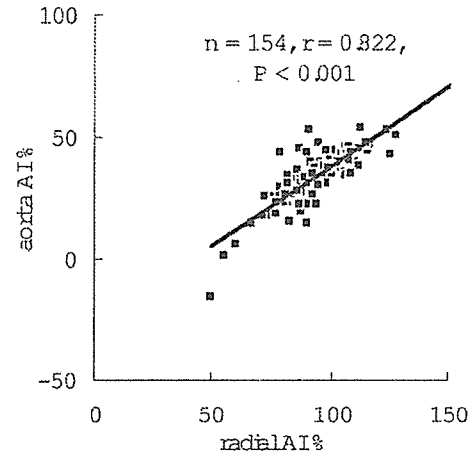

FIG. 2 Correlation between aortic augmetation index (AI) obtained by SphygmoCor and radial AI obtained by Omron HEM-9010AI. There was a highly significant correlation between radial AI and aortic AI.

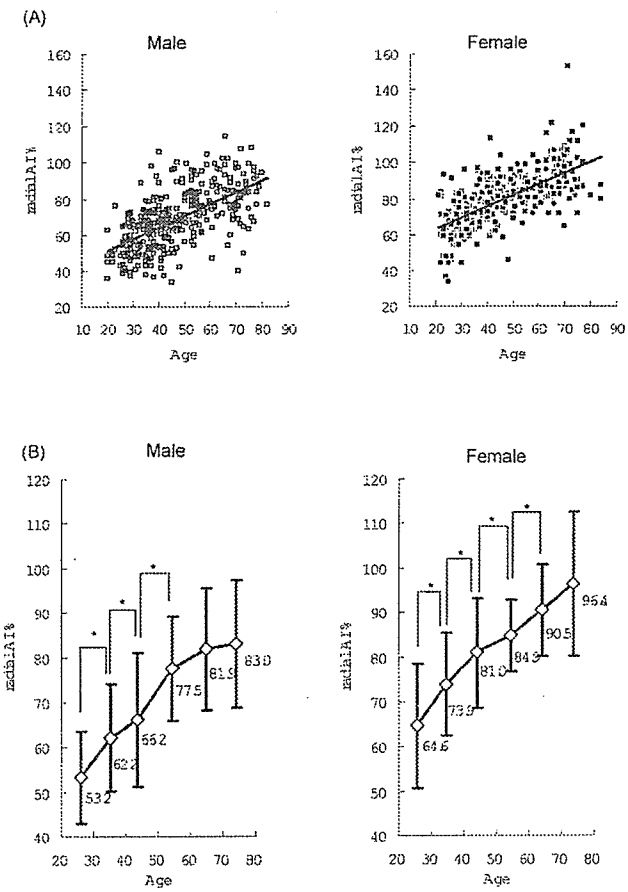

FIG. 3 Correlation between radial augmentation index (AI) and age (**A**) in men (r = 0.619, $P < .001$) and women (r = 0.644, $P < .001$). Age-related changes in radial AI in men and women are shown for each decade (**B**). Values are mean ± SD. *$P < .01$ between age groups.

Table 2. Multiple regression analysis for radial augmentation index

	β	t	P value
Age (y)	0.420	13.513	<.001
Sex (female)	0.218	5.452	<.001
SBP (mm Hg)	−0.082	−1.779	.076
DBP (mm Hg)	0.375	8.608	<.001
Pulse rate (beats/min)	−0.410	−16.276	<.001
Height (cm)	−0.194	−1.100	.272
Weight (kg)	−0.041	−0.148	.882
BMI (kg/m^2)	−0.080	−0.387	.699

Abbreviations as in Table 1.

correlation between radial AI obtained by HEM-9010AI and aortic AI determined by the SphygmoCor device. Radial AI showed a highly significant correlation with aortic AI ($r = 0.822$, $P < .001$).

Radial AI and Age

Figure 3 summarizes the relationship between radial AI and age. There was a significant positive correlation between age and radial AI for both men and women (men: $r = 0.619$, $P < .001$; women: $r = 0.644$, $P < .001$).

Age-related changes in radial AI, expressed as mean value in each decade of life, are also shown in Fig. 3. For men, there were significant age-dependent increases in radial AI from the 20s to the 50s. In women, an age-dependent increase in radial AI was observed from the 20s to the 60s.

Multiple regression analysis further revealed that radial AI was significantly associated with age in addition to pulse rate and DBP. However, body height, weight, BMI, and SBP were not associated with radial AI (Table 2).

Discussion

In the present study, we showed an age-related increase in radial AI in healthy men and women with no cardiovascular disease between 20 and 82 years of age. In a separate study, we evaluated the association between radial AI obtained from HEM-9010AI and aortic AI obtained by SphygmoCor. There was a highly significant and close association between AI obtained by two methods ($r = 0.822$, $P < .001$). This finding indicates the validity of radial AI semi-automatically obtained with HEM-9010AI, which detects the radial artery and determines the appropriate pressure of tonometry to obtain the most proper pressure waveform. The finding that radial AI significantly and closely associated with aortic AI obtained via transmission function is in accordance with the study by Millasseau et al.[12]

Several parameters have been shown to influence the AI, including body height,[13] heart rate,[14] postural change,[15] and gender.[16,17] Accordingly, it is generally necessary to take into account these confounding factors when assessing AI as an index for atherosclerosis. The difference in radial AI between men and women is consistent with previous findings.[13,16,17] Radial AI is higher in women than in men, and it has been suggested that the difference in body height is one of the underlying mechanisms.[13] However, the difference persists even after adjusting for body height. The smaller diameter of the radial artery and higher pulse wave velocity (PWV) in women has also been reported to account for the higher AI in women.[17]

The AI is determined by PWV, distance to the reflection point, and reflection coefficient.[1–3] The PWV increases with the age-related increase in arterial stiffness.[4] The reflection coefficient is influenced by area ratio (that is, the ratio of branches to parent vessel cross-sectional areas) and age. Atherosclerosis has been shown to be associated with a lower area ratio.[11] Body height, a simple estimate of the distance to the reflection point, diminishes somewhat with aging. These findings indicate that all factors defining AI are subject to age-dependent alterations.

In summary, the present study shows that radial AI could be an index of vascular aging. Because central AI reflects the difference between aortic BP and peripheral BP,[1–3] radial AI could also be used as an estimate of central BP. Recent guidelines also point out the potential usefulness in estimating central BP.[18] These findings further support the clinical usefulness of radial AI, a simple and easily obtainable parameter for vascular aging.

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An Association of 5,10-Methylenetetrahydrofolate Reductase (*MTHFR*) Gene Polymorphism and Ischemic Stroke

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Plasma homocysteine (Hcy) concentration has been shown to be influenced by a mutation in the gene coding methylenetetrahydrofolate reductase (*MTHFR*). Although plasma Hcy has been shown to be related to atherosclerotic disorders, the association between *MTHFR* gene polymorphism and ischemic stroke remains controversial. In the present study we investigated the association between *MTHFR* gene polymorphism and risk factor–dependent augmentation for ischemic stroke in subjects with several risk factors for atherosclerosis, with special emphasis on the risk factor–gene interaction. The diagnosis of cerebral infarction in each patient was confirmed by computed tomography (CT) findings of the brain. *MTHFR* C677T polymorphism was genotyped with a conventional method. In 97 stroke patients (48 cases of atherothrombotic infarction, 38 cases of lacunar infarction, 9 cases of cardiac embolism, 2 others) and 241 age-and sex-matched healthy control subjects, the frequencies of the T allele were 0.44 and 0.39, respectively. In patients with CT-proven atherothrombotic infarction, the T allele frequency was 0.54 (P = .033 vs controls). The adjusted odds ratio in subjects with TT genotype for atherothrombotic infarction was 3.87 (95% confidence interval = 1.27–11.8). A general linear model analysis showed that an interaction between the HDL-C and *MTHFR* genotype was significantly associated with atherothrombotic infarction (F = 5.695; P = .018). These findings indicate that the T allele of the *MTHFR* gene is significantly associated with atherothrombotic infarction. Furthermore, the analysis of risk factor–gene interaction could be a useful tool for deriving specific predictive information about ischemic stroke in an elderly Japanese population. **Key Words:** Risk factor—gene—interaction—homocysteine—polymorphism.

Homocysteine (Hcy) is a sulfur-containing amino acid generated as an intermediate product in methionine metabolism. Hyperhomocysteinemia has been substantiated as a risk factor for occlusive vascular disease in patients with cerebral, coronary, or peripheral arterial diseases.[1-3] Meta-analyses have revealed a consistent association between the plasma level of Hcy and atherosclerotic disorders.[2] Recent studies have suggested that the risk of ischemic stroke is increased in subjects with even slightly elevated Hcy concentrations, previously considered to be within the normal range.[4] Hcy can be transsulfurated to form cysteine or remethylated to form methionine. The latter reaction uses 5-methyltetrahydrofolate as a carbon donor; 5-methyltetrahydrofolate is synthesized from 5,10-methylenetetrahydrofolate through the action of the methylenetetrahydrofolate reductase gene (*MTHFR*),

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Received October 7, 2004; revised November 22, 2004; accepted November 24, 2004.

This study was supported in part by a grant-in-aid from the Foundation for the Development of the Community (2003).

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1052-3057/$—see front matter

doi:10.1016/j.jstrokecerebrovasdis.2004.12.003

which locates in endothelium or smooth muscle cell. Mutations in the gene coding for both of these enzymes leads to group of disorders in which marked elevation of circulating Hcy was observed.

There have been numerous genetic association studies of the *MTHFR* C677T variant, particularly in the homozygous state, which have shown both the presence[5-8] and the absence[3,9,10] of significant associations of such end points as coronary heart disease, myocardial infarction, and cerebrovascular disease. Although there are several subtypes in ischemic stroke, atherothrombotic infarction has been reported to be associated with Hcy.[11-13] Accordingly, the subtype of ischemic stroke enrolled in positive studies is thought to be mainly the atherothrombotic type. Recently, we and others observed that risk factor–gene interaction could have an influence on hypertension or carotid atherosclerosis.[14-20] It is also conceivable that the conflicts between *MTHFR* and atherosclerosis may suggest an interaction between of risk factor and gene. Furthermore, the studies of *MTHFR* polymorphism lack statistical power, and a *MTHFR* genotype may even modulate cardiovascular disease risk independently of Hcy.[2] We hypothesized that risk factor–dependent evaluation of the effect of *MTHFR* on a specific subtype of ischemic stroke could provide new information on the genetic predisposition to ischemic stroke. To address this hypothesis, we investigated the association between *MTHFR* polymorphism and subtype of ischemic stroke in elderly subjects with several risk factors for atherosclerosis, with special emphasis on the risk factor–gene interaction.

Materials and Methods

Subjects

The acute ischemic stroke patients defined with following criterion were consecutively enrolled from inpatients in the Internal Medicine Department of Nomura Municipal Hospital between August 1999 and December 2000. Patients with cerebral hemorrhage were not included. A total of 97 patients were enrolled in the study. A total of 241 control subjects with no clinical history of cerebrovascular disease or present neurologic abnormalities were randomly recruited at the time of annual health examination in Nomura-cho, in which Seiyo Municipal Nomura Hospital is a community hospital. Informed consent for the procedure was obtained from each patient. All procedures were approved by Ethics Committee of Ehime University School of Medicine.

Definition of Acute Cerebral Infarction

Clinical syndrome of ischemic stroke was defined as a rapidly developing clinical symptoms and/or signs of focal and at times global loss of brain function, with symptoms or leading to earlier death and with no apparent cause other than that of vascular origin. Acute ischemic stroke was defined as an acute onset of stroke with normal computed tomography (CT) brain findings followed by sequential change in CT lesions. A recent infarct in the clinically relevant area of the brain with CT scan evidence was also included. Patients were divided according to clinical diagnosis and CT findings into 4 groups: those with atherothrombotic infarction, those with lacunar infarction, those with cardiac embolism, and those unclassifiable.[21]

Evaluation of Risk Factors

Information on demographic characteristics and risk factors was collected from clinical files on all cases. Subjects with hypertension were defined as those who had previously been treated for hypertension. Subjects were classified as diabetic when they were being treated for diabetes mellitus. A smoker was defined as a subject with a pack-years index >0 with pack-years defined as packs of cigarettes per day multiplied by years smoked. In the blood biochemistry analyses, total cholesterol (T-C), triglycerides (TG), and High-density lipoprotein cholesterol (HDL-C) were measured after fasting within 24 hours after admission. Low-density lipoprotein cholesterol (LDL-C) level was calculated by the Friedewald formula. The use of antilipidemic drugs was assessed.

MTHFR *Genotype Analysis*

Genomic DNA was extracted from peripheral blood lymphocytes using standard procedures.[22] The DNA sample were subjected to amplification by polymerase chain reaction (PCR), and the restriction enzyme HinfI was used to identify those with the mutation, as described by Frosst et al.[23] The PCR reaction generated a fragment of 198 bp that contained codon 677. The point substitution of T for C at codon 677 created a HinfI recognition sequence with resulting 175- and 23-bp fragments. Alanine-coding alleles therefore produced a 198-bp fragment that was easily distinguished from the 175-bp fragment generated by valine alleles. Electrophoresis in a 4% agarose gel followed by ethidium bromide staining and UV illumination allowed detection of mutation alleles.

Statistical Analysis

Statistical analysis was performed using SPSS 10.0J (SPSS, Chicago, IL). The prevalence among the ischemic stroke and control subjects was compared using the χ^2 test. The differences among groups were analyzed using the Mann-Whitney *U*-test. The relation between ischemic stroke and risk factors, including genotype, were examined by logistic multiple regression analysis. A general linear model was used to evaluate the significant contri-

Table 1. *Baseline characteristics of control subjects and ischemic stroke patients*

Characteristics	Control (n = 241)	Ischemic stroke (n = 97)	*P*
Age	76 ± 7.1	78 ± 8.3	.081
≥ 75 years, n (%)	152 (63.1)	65 (67.0)	.532
Gender, male, n (%)	118 (49.0)	57 (58.8)	.118
Smokers, n (%)	60 (24.9)	42 (43.3)	.001
History of hypertension, n (%)	101 (41.9)	60 (61.9)	.001
History of diabetes mellitus, n (%)	41 (17.0)	26 (26.8)	.050
Antilipidemic drug use, n (%)	15 (6.2)	2 (2.1)	.168
Total cholesterol (mg/dL)	205.7 ± 31.9	181.1 ± 37.8	<.001
HDL cholesterol (mg/dL)	61.0 ± 14.8	47.7 ± 15.5	<.001
LDL cholesterol (mg/dL)	122.4 ± 28.4	114.2 ± 32.0	.011
Triglyceride (mg/dL)	111.3 ± 65.8	95.9 ± 45.7	.023

HDL, high-density lipoprotein; *LDL*, low-density lipoprotein.

bution of risk factor–gene interactions to ischemic stroke. A P value <.05 was considered significant.

Results

Background of Subjects

The baseline characteristics of the patients and control subjects are given in Table 1. The patients had a significantly higher prevalence of smokers and were more likely to have a history of hypertension (well-known major risk factors for stroke) than the control subjects. The patients had not only significantly lower mean HDL-C levels than controls, but also significantly lower mean T-C, LDL-C, and TG levels. There were no intergroup differences in age, gender, history of diabetes mellitus, or prevalence of antilipidemic drug use.

Distribution of MTHFR *Genotypes in Control Subjects and Ischemic Stroke Patients*

The distribution of the *MTHFR* genotypes in both control subjects and the patient groups is given in Table 2. The genotype distributions of both groups were in agreement with Hardy-Weinberg equilibrium, and the distribution of *MTHFR* genotypes was consistent with a published report in Japanese subjects.[6] The allele frequency of the T mutation was not significantly higher in the ischemic stroke patients compared with the control subjects. The association of the mutation with ischemic stroke was further studied in terms of the subtypes of stroke as well as CT findings of the lesions, and we found 48 cases of atherothrombotic infarction, 38 cases of lacunar infarction, 9 cases of cardiac embolism, and 2 others. The χ^2 test demonstrated a significant difference (P = .033) between the atherothrombotic infarction patients and control subjects in the distribution of the *MTHFR* genotype, suggesting a codominant effect of the T allele on the risk of atherothrombotic infarction.

Odds Ratio of Subtypes of Ischemic Stroke Associated With MTHFR *Genotypes*

In ischemic stroke patients with CT-proven atherothrombotic infarction, the T-allele frequency was 0.54 (Table 2). The unadjusted odds ratio (OR) and 95% confidence interval (CI) for atherothrombotic infarction are summarized in Table 3. Subjects with TT genotype as well as T carriers had a significant risk for atherothrom-

Table 2. *Distribution of MTHFR genotypes in control subjects and ischemic stroke patients*

		MTHFR genotype				
	n	CC, n (%)	CT, n (%)	TT, n (%)	C/T frequency	*P**
Control	241	91 (37.8)	110 (45.6)	40 (16.6)	0.61/0.39	—
Ischemic stroke	97	33 (34.0)	43 (44.3)	21 (21.6)	0.56/0.44	.530
Atherothrombotic infarction	48	10 (20.8)	24 (50.0)	14 (29.2)	0.46/0.54	.033
Lacunar infarction	38	17 (44.7)	15 (39.5)	6 (15.8)	0.65/0.35	.703
Cardiac embolism	9	6 (66.7)	2 (22.2)	1 (11.1)	0.78/0.22	.213
Other	2		2 (100)			

*χ^2 statistic test versus control group.

	Atherothrombotic Infarction	*P*-value
	Unadjusted OR (95% CI)	
CT vs CC	1.98 (0.90-4.37)	.094
CT + TT vs CC	2.31 (1.10-4.85)	.031
TT vs CC	3.19 (1.31-7.78)	.011
	Adjusted OR (95% CI)	
CT vs CC	2.30 (0.95-5.56)	.066
CT + TT vs CC	2.53 (1.10-5.82)	.030
TT vs CC	3.87 (1.27-11.8)	.017

CI, confidence interval.

Adjusted for age, gender, smoking habits, history of hypertension, history of diabetes mellitus, antilipidemic drug use, HDL cholesterol, and LDL cholesterol.

botic infarction even after adjustment with other known risk factors (Table 3).

Risk of Atherothrombotic Infarction in Subjects With MTHFR *T Carriers (CT + TT) According to Age, Gender, and Other Vascular Risk Factors*

We examined the possible synergistic effect between *MTHFR* T carriers and conventionally known risk factors in both control and atherothrombotic infarction subjects. Table 4 presents the risk of atherothrombotic infarction in subjects with *MTHFR* T carriers stratified according to age, gender, and presence/absence of other vascular risk factors. In this subgroup, significant risks of atherothrombotic infarction were observed in subjects with *MTHFR* T carriers of age $\geq$ 75 years, female sex, nonsmoker, no history of diabetes mellitus, HDL-C $<$ 40 mg/dL, and LDL-C $<$ 130 mg/dL. A possible interaction between *MTHFR* T carriers and specific risk factors in atherothrombotic infarction was suggested.

MTHFR *Genotypes and Conventional Risk Factors for Atherothrombotic Infarction*

To find possible gene–risk factor interactions for atherothrombotic infarction, multiple regression analysis for atherothrombotic infarction was performed with risk factors in subjects with a specific genotype of *MTHFR* gene polymorphism (Table 5). It was shown that smoker and HDL-C were significantly associated with atherothrombotic infarction in subjects with *MTHFR* CC genotype, and HDL-C in *MTHFR* T carriers. Analysis of covariance to demonstrate significant differences between 2 regression lines in smoker and atherothrombotic infarction ($F[1, 285] = 0.698$, $P = .404$).

*Risk Factors–*MTHFR *Gene Interaction*

To further investigate whether the interaction between *MTHFR* genotype and the conventional risk factors could have any influence on atherothrombotic infarction, a general linear model for presence of atherothrombotic infarction was analyzed with the following parameters: gender, age, smoking, history of hypertension and diabetes mellitus, HDL-C, LDL-C, and *MTHFR* gene polymorphism, including interactions between risk factor and *MTHFR* genotype (Table 6). This analysis revealed that interaction between HDL-C and *MTHFR* gene polymorphism ($F[1.275] = 5.695$, $P = .018$) was significantly associated with atherothrombotic infarction.

Discussion

The present study examined the association between *MTHFR* gene polymorphism and etiologic subtypes of ischemic stroke in patients with risk factors, and revealed that the T allele of the *MTHFR* gene was significantly associated with CT-proven atherothrombotic infarction in a Japanese patient population. A multivariate analysis demonstrated that this association was independent of other risk factors, including age, gender, smoking, history of hypertension, and diabetes mellitus, HDL-C, and LDL-C. This analysis also revealed the influence of an HDL-C–*MTHFR* gene interaction on atherothrombotic infarction.

The association between plasma Hcy concentration and atherosclerosis has been the subject of a number of clinical studies.[24,25] The British Regional Heart Study Cohort investigators reported that the mean Hcy level in 107 stroke patients was 13.7 μmol/L (95% CI = 12.7–14.8), a significantly higher level than the mean 11.9 μmol/L (95% CI = 11.3–12.6) in 118 control subjects, and that the OR for stoke increased with increasing Hcy level.[25] Eikelboom et al[12] also reported that higher Hcy level was a strong and independent risk factor for ischemic stroke (adjusted OR = 2.7, 95% CI = 1.4–5.1 for a 5-μmol/L increase in fasting plasma Hcy from 10 to 15 μmol/L).

Although there are several etiologic subtypes in ischemic stroke, an association between atherothrombotic infarction and plasma Hcy levels has been reported.[7,12] The underlying pathophysiology of lacunar infarction is less well understood but appears to involve cerebral

Table 4. *Odds ratios for atherothrombotic infarction in subjects with MTHFR T carriers subcategorized with known risk factor for ischemic stroke.*

Characteristics	Total (n = 289)	Control (n = 241)	Atherothrombotic infarction (n = 48) OR (95% CI)	P
Age				
<75 years	68/108	55/89	13/19 1.34 (0.47-3.86)	.794
≥75 years	120/181	95/152	25/29 3.75 (1.24-11.3)	.017
Gender				
Male	96/148	74/118	22/30 1.64 (0.67-3.99)	.392
Female	92/141	76/123	16/18 4.95 (1.09-22.5)	.032
Smoker				
Yes	55/83	39/60	16/23 1.23 (0.44-3.46)	.798
No	133/206	111/181	22/25 4.63 (1.34-16.0)	.008
History of hypertension				
Yes	86/131	63/101	23/30 1.98 (0.78-5.06)	.190
No	102/158	87/140	15/18 3.05 (0.84-11.0)	.115
History of diabetes mellitus				
Yes	33/56	22/41	11/15 2.38 (0.65-8.70)	.231
No	155/233	128/200	27/33 2.53 (1.00-6.42)	.048
HDL cholesterol				
<40 mg/dL	25/38	8/17	17/21 4.78 (1.13-20.3)	.042
≥40 mg/dL	163/251	142/224	21/27 2.02 (0.78-5.21)	.199
LDL cholesterol				
< 130 mg/dL	119/187	89/150	30/37 2.94 (1.21-7.12)	.014
≥ 130 mg/dL	69/102	61/91	8/11 1.31 (0.32-5.30)	1.000

OR, odds ratio; *CI*, confidence interval.

to subcortical vascular encephalopathy.[27] Li et al[7] found in a case-control study of 1823 stroke patients (807 with cerebral thrombosis, 513 with lacunar infarction, and 503 with intracerebral hemorrhage) and 1832 controls that total plasma Hcy levels were significantly higher in cases than in controls (median, 14.7 vs 12.8 μmol/L; $P < .001$) and were associated with an increased risk of 1.87-fold (95% CI = 1.58–2.22) for overall stroke, 1.72-fold (95% CI = 1.39–2.12) for cerebral thrombosis, 1.89-fold (95% CI = 1.50–2.40) for lacunar infarction, and 1.94-fold (95% CI = 1.48–2.55) for intracerebral hemorrhage. Based on these observations, it has been accepted that Hcy is an independent risk factor for stroke, regardless of the type of ischemic stroke.

Because it has been reported that thermolabile *MTHFR*, which is thought to be associated with *MTHFR* T allele and reduced enzyme activity, accounts for 25% to 30% of elevated Hcy levels in patients with premature vascular disease,[28] it has been strongly suggested that *MTHFR* is one of the candidate genes for ischemic stroke. Li et al[7] reported that the TT genotype of *MTHFR* was associated with an increased risk for overall stroke (OR = 1.27; 95% CI = 1.04–1.56) and thrombotic stroke (OR = 1.37; 95% CI = 1.06–1.78). In the present study, our observation of an association between *MTHFR* polymorphism and

Table 5. *Multivariate linear regression analysis for atherothrombotic infarction with conventional risk factors and the MTHFR genotype*

Characteristics	CC genotype (n = 101)	CT + TT genotype (n = 188)	Total (n = 289)
Age	−0.100 (.302)	−0.014 (.836)	−0.031 (.573)
Gender	−0.052 (.654)	−0.056 (.508)	−0.049 (.467)
Smoker	0.275 (.018)	0.052 (.536)	0.118 (.079)
History of hypertension	0.177 (.061)	0.116 (.081)	0.132 (.014)
History of diabetes mellitus	0.179 (.060)	0.037 (.585)	0.099 (.066)
Antilipidemic drug use	0.123 (.195)	−0.096 (.145)	−0.029 (.584)
HDL cholesterol	−0.223 (.023)	−0.413 (<.001)	−0.352 (<.001)
LDL cholesterol	−0.056 (.576)	−0.131 (.071)	−0.093 (.100)
MTHFR genotype	—	—	0.138 (.010)
r^2	0.215 (.003)	0.252 (<.001)	0.225 (<.001)

Values are the standard regression coefficients (*P* values). r^2, multiple coefficient of determination. An addictive model (CC = 0, CT + TT = 1) was used for MTHFR genotype.

atherothrombotic infarction are consistent with these results. But the nonsignificant relationship between the *MTHFR* polymorphism and ischemic stroke observed so far does not contradict the Hcy theory. One reason for this may be marked differences in nutritional status of the various subjects, because individuals with *MTHFR* TT genotype develop elevated Hcy only under conditions of impaired folate status.[29] In most studies, including our study, folate and Hcy concentrations were not measured,[30] and in several studies demonstrating no association between *MTHFR* and risk, the authors reported that their subjects were probably well nourished.[30] In contrast, in our study of ischemic stroke subjects who had a high prevalence of low T-C and low TG, the *MTHFR* TT genotype was a significant predictor of atherothrombotic infarction. Moreover, an association between *MTHFR* polymorphism and silent lacunar infarction was recently reported in a large Japanese general population.[8] The underlying pathophysiology of ischemic stroke due to microangipathy is less well understood, but appears to involve microatheroma formation as well as lipohyalinosis.[31] However, if the putative deleterious effects of hyperhomocysteinemia are mediated primary via a proatherogenic effect, then it is plausible that Hcy is not as strong a risk factor for lacunar stroke caused by microangiopathy as it is for atherothrombotic infarction. Although we did not find a positive association, the sampling of cases and controls might be too small to reach a conclusion.[8]

This finding may indicate the difference in genetic background between atherothrombotic infarction and lacunar infarction. It is also conceivable that different risk factor–gene interactions could take place in the etiology of ischemic stroke subtypes. Because ischemic stroke is a multifactorial disorder including genetic predisposition in addition to these risk factors, risk factor–gene interaction could decrease or enhance the absolute risk in individual subjects in various ways. The risk factor–gene interactions may also account for the contradictory results of previous studies of *MTHFR* polymorphism and ischemic stroke. In the present study, we found that the presence of atherothrombotic infarction on HDL-C differed significantly between *MTHFR* genotypes.

Unfortunately, we have no clear explanation of how the interaction between HDL-C and *MTHFR* affects atherothrombotic infarction. However, Hcy auto-oxidation has been shown to support the oxidation of LDL through generation of the superoxide anion radical.[32,33] It has also been reported that Hcy and oxidized LDL have distinct effects on endothelial cell thrombo-

Table 6. *General linear model for atherothrombotic infarction*

Characteristics	F	*P*
MTHFR T carrier	4.477	.035
Gender, male	0.311	.578
Age	0.382	.537
Smoker	4.092	.044
History of hypertension	5.463	.020
History of diabetes mellitus	2.591	.109
HDL cholesterol	29.49	<.001
LDL cholesterol	2.272	.133
MTHFR T carrier*		
Smoker	2.012	.157
HDL cholesterol	5.695	.018

An additive model (CC = 0, CT + TT = 1) was used for MTHFR genotype.

The net effect of each interaction was estimated using a general linear model including MTHFR T carriers, each conventional risk factor, and *the interaction between MTHFR T carriers and the factor.

genicity.[34] It has also been reported that VLDL and LDL demonstrate a high binding capacity for Hcy.[35] These findings may indicate synergistic interaction between *MTHFR* and lipid metabolism in the development of atherosclerosis.

We need to be aware of the limitations in interpreting the present results. We can not deny that the relatively small number of patients with atherothrombotic infarction limits the credibility of the results and that subjects might have a subclinical ischemic stroke (silent lacunar stroke), because the majority of the control group was elderly (age 70 years or older). Silent lacunar infraction could likely reduce the difference between cases and controls and thereby bias the results of the study toward the null, especially negative association between *MTHFR* and lacuar infarction. These points need to be addressed in a large population with more precise phenotyping including control subjects.

In summary, we have reported a significant association between *MTHFR* gene polymorphism and atherothrombotic infarction in subjects with risk factors for atherosclerosis. Furthermore, an interaction between HDL-C and *MTHFR* was observed. These findings further support the idea that risk factor–gene interaction could allow us to determine specific predictive information about the development of atherosclerosis.

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Original Article

Interaction between Serotonin 2A Receptor and Endothelin-1 Variants in Association with Hypertension in Japanese

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Serotonin has been implicated in the pathogenesis of hypertension because of its ability to induce vasoconstriction *via* stimulation of serotonin 2 (5-HT2) receptors. Recently, an association between the T102C functional polymorphism of the serotonin 2A (5-HT2A) receptor gene and hypertension in the UK has been reported. Another association study, however, failed to replicate this association in a Chinese population. We therefore investigated the possible association between the 5-HT2A T102C polymorphism and hypertension in two large Japanese populations (n=2,968 total). We also investigated the possible interaction between the 5-HT2A T102C polymorphism and the G/T (Lys198Asn) polymorphism of the endothelin-1 (ET-1) gene, based on robust biological evidence for the existence of an interaction between the serotonin and endothelin systems. The results showed that there was no significant difference in the frequencies of the alleles and genotypes between the hypertensive and normotensive subjects. However, a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension (p=0.0040) and with diastolic blood pressure (p=0.0013) was revealed. A marginally significant interaction in the association with systolic blood pressure was also shown (p=0.045). The associations of the 5-HT2A T102C polymorphism with hypertension and diastolic blood pressure in ET-1 T allele carriers were significant (p=0.0056 and 0.021, respectively). The association of the 5-HT2A T102C polymorphism with systolic blood pressure in ET-1 T allele carriers was marginally significant (p=0.054). Thus, the present study suggests that the 5-HT2A T102C and ET-1 G/T polymorphisms are interactively associated with hypertension. (*Hypertens Res* 2006; 29: 227–232)

Key Words: **serotonin receptor, endothelin, hypertension, genetics, polymorphism**

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a naturally occurring vasoactive monoamine and is widely distributed in the human organism (*1*). Serotonin executes diverse cardiophysiological actions, which are mediated by different subtypes of serotonin receptors. Currently, serotonin receptors are divided into seven groups (5-HT1–7). Among these groups, 5-HT2 receptors mediate the vasoconstrictive actions of serotonin, and these are further categorized into three subtypes (A, B, and C). Among these three subtypes, the 5-HT2A

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This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C), "Medical Genome Science," from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on the Human Genome, Tissue Engineering, and Food Biotechnology from the Ministry of Health, Labour and Welfare of Japan.

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Received August 15, 2005; Accepted in revised form January 13, 2006.

Table 1. Characteristics of Participants According to Hypertension Status

Variable	Population 1		Population 2	
	Normotensive (n=1,364)	Hypertensive (n=852)	Normotensive (n=502)	Hypertensive (n=250)
Sex (male %)	84.8	89.4*	77.1	78.2
Age (years)	49.5±9.0	53.8±6.6*	52.4±8.8	57.2±8.3*
Body mass index (kg/m^2)	22.6±2.8	24.2±3.2*	22.5±2.8	23.8±2.7*
Systolic blood pressure (mmHg)	122.7±10.7	148.7±12.7*	112.4±10.6	143.4±17.0*
Diastolic blood pressure (mmHg)	72.3±7.3	87.3±8.4*	72.1±8.9	89.4±9.3*
Total cholesterol (mg/dl)	195.4±32.1	203.2±31.6*	198.5±30.9	201.8±36.3
High density lipoprotein cholesterol (mg/dl)	60.8±13.3	60.8±13.2	54.1±14.7	52.1±14.8
Triglyceride (mg/dl)	127.0±75.9	155.3±85.4*	107.9±76.3	137.3±126.8*

Data are mean±SD. *$p<0.05$ *vs.* normotensives. Blood pressure readings before the start of antihypertensive medication were not available for 705 hypertensive subjects whose values were measured under treatment.

receptor is the primary receptor mediating vasoconstriction under conditions of normal blood pressure (*2*). Thus, the 5-HT2A receptor may play an important role in the regulation of blood pressure.

The 5-HT2A receptor gene is located on chromosome 13. Given the biological evidence for a relation between the 5-HT2A receptor and blood pressure, it is important to evaluate how variations in the 5-HT2A receptor gene are associated with blood pressure as genetic factors. In this context, a functional polymorphism (T102C) of the 5-HT2A receptor gene has been investigated in relation to hypertension. An initial study showed that increased frequency of the 102C allele was significantly associated with hypertension in female UK residents (*3*). Another association study, however, failed to show a significant association between the 5-HT2A T102C polymorphism and hypertension in a Chinese population (*4*).

Generally, inconsistent associations could result from various factors, including racial difference, insufficient statistical power, and interactions of polymorphisms with other genetic and environmental factors (*5*). In this context, it may be of significance that the serotonin system has been shown to biologically interact with the endothelin system (*6–14*). This interaction could modify the association between the 5-HT2A T102C polymorphism and hypertension. However, whether genetic interactions between polymorphisms corresponding to the biological interaction significantly influence blood pressure in the general population remains to be assessed. We therefore analyzed the association between the 5-HT2A T102C polymorphism and hypertension in two large Japanese populations, with consideration of the interaction between the 5-HT2A T102C polymorphism and the G/T (Lys198Asn) polymorphism in exon 5 of the endothelin-1 (ET-1) gene, because the ET-1 system (*15*), especially the ET-1 G/T polymorphism (*16*), has been shown to be involved in the development of hypertension.

Methods

Subjects

The clinical characteristics of the subjects included in the study are shown in Table 1. Population 1 (n=2,216) originated from the Ehime region of Japan, and population 2 (n=752) from the Hyogo region of Japan (*17*). All subjects were Japanese urban residents. Subjects in population 1 participated in medical check-ups 1–11 times (average 6.2 times per person), and the mean values of variables in their personal health records were used in the analyses. Subjects in population 2 also underwent a medical check-up, and the values of variables in their personal health records were used in the analyses. All subjects provided informed consent for participation in the molecular-genetic studies. The ethics committee of Ehime University approved the study.

Diagnostic Categories

Each subject was assigned to one of the blood pressure diagnostic categories defined by the following criteria. Hypertensive subjects had a previous diagnosis of hypertension and were being treated with antihypertensive medication, or their systolic/diastolic blood pressure (SBP/DBP) was >140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was <140/90 mmHg. Blood pressure was measured in the sitting position with the use of a standard sphygmomanometer during medical check-ups.

DNA Analysis

The TaqMan chemical method, which is an established and frequently used method (*18–21*), was used to detect the 5-HT2A T102C polymorphism. The forward primer was 5′-AAATGATGACACCAGGCTCTACAGT-3′, the reverse

Table 2. 5-HT2A Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Population 1			Population 2			Populations 1 and 2		
	Normo-tensive	Hyper-tensive	*p* value	Normo-tensive	Hyper-tensive	*p* value	Normo-tensive	Hyper-tensive	*p* value
5-HT2A genotypes (*n* (%))									
CC	344 (25.2)	230 (27.0)		123 (24.5)	68 (27.2)		467 (25.0)	298 (27.0)	
CT	645 (47.3)	409 (48.0)		254 (50.6)	108 (43.2)		899 (48.2)	517 (46.9)	
TT	375 (27.5)	213 (25.0)	0.38	125 (24.9)	74 (29.6)	0.15	500 (26.8)	287 (26.0)	0.48
5-HT2A alleles (*n* (%))									
C	1,333 (48.9)	869 (51.0)	.	500 (49.8)	244 (48.8)		1,833 (49.1)	1,113 (50.5)	
T	1,395 (51.1)	835 (49.0)	0.17	504 (50.2)	256 (51.2)	0.71	1,899 (50.9)	1,091 (49.5)	0.30

5-HT2A, serotonin 2A.

primer was 5′-TGTCCAGTTAAATGCATCAGAAGTG-3′, the T-allele specific probe was 5′-FAM-AACTCTGGAGAA GCT-MGB-3′, and the C-allele specific probe was 5′-VIC-AACTCCGGAGAAGC-MGB-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. The ET-1 G/T polymorphism was previously determined in our populations (*17*).

Statistical Methods

Comparisons of categorical variables were performed using the χ^2 test. Analysis of variance was used to assess differences in the means and variances of continuous variables. Because of a skewed distribution of data, logarithmically transformed plasma triglyceride values (TG) were used in the analysis. Logistic regression models were used to assess whether the 5-HT2A T102C polymorphism made a statistically significant contribution to the prediction of hypertension, with consideration of the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms. General linear models were used to assess whether the 5-HT2A T102C polymorphism made a statistically significant contribution to the prediction of blood pressure, with consideration of the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms. *p* values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software.

Results

Association of the 5-HT2A T102C Polymorphism with Hypertension

Table 1 presents the clinical characteristics of the participants in populations 1 and 2. In population 1, the relative frequencies of the CC, CT and TT genotypes were 25.9%, 47.6% and 26.5%, respectively. In population 2, the relative frequencies of the CC, CT and TT genotypes were 25.4%, 48.1% and 26.5%, respectively. In population 1, the allele frequencies were 49.7% and 50.3% for the C and T alleles, respectively. In population 2, the allele frequencies were 49.5% and 50.5% for the C and T alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium. There was no significant difference in the frequencies of the alleles ($p=0.17$) and genotypes ($p=0.38$) between the hypertensive and normotensive subjects in population 1 (Table 2). There was no significant difference in the frequencies of the alleles ($p=0.71$) and genotypes ($p=0.15$) between the hypertensive and normotensive subjects in population 2. Finally, there was no significant difference in the frequencies of the alleles ($p=0.30$) and genotypes ($p=0.48$) between the hypertensive and normotensive subjects in the combined group of populations 1 and 2.

Interaction between the 5-HT2A T102C and ET-1 G/T Polymorphisms in Association with Hypertension

We next analyzed the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. This analysis showed a significant interaction in population 1 ($p=0.012$, odds ratio [OR]=0.74, 95% confidence interval [95% CI]=0.58–0.94) and failed to show a significant interaction in population 2 ($p=0.14$, OR=0.73, 95% CI=0.48–1.11). Finally, analysis combining populations 1 and 2 yielded a lower *p* value of 0.0040 (OR=0.74, 95% CI=0.60–0.91) for the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. The interaction was also significant after adjustment for sex and age ($p=0.0022$), and for sex, age, body mass index (BMI), plasma total cholesterol, high density lipoprotein (HDL)–cholesterol, and TG ($p=0.050$). Table 3 shows the opposite directions of the association of the 5-HT2A T102C polymorphism with hypertension between the ET-1 genotypes. The association of the 5-HT2A T102C polymorphism with hypertension in ET-1 T allele carriers was significant ($p=0.0056$). The association of the ET-1 G/T polymorphism with hypertension also showed opposite directions between the 5-HT2A genotypes (Table 4). The association of the ET-1 G/T polymorphism with hypertension in 5-HT2A CC homozygotes was significant ($p=0.028$). The