Table 4　Clinical characteristics of the patients with recurrent stroke or TIA

	Age/Sex	ASA	Aortic arch lesions	Treatment	INR *	CI/TIA	Topography		m-RS (1st hospitalization → 2nd hospitalization)
DVT (+)									
Case 1	71/F	(+)	(−)	WF	1.56	CI	MCA	cortical/subcortical	1 → 4
Case 2	67/M	(+)	(−)	WF	1.39	CI	MCA-PB	φ 1.5 cm	0 → 1
Case 3	70/M	(−)	(+)	WF	1.67	TIA			3 → 3
DVT (−)									
Case 4	71/M	(−)	(+)	WF	1.96	CI	PCA?	not detected	1 → 1
Case 5	73/M	(−)	(+)	AP		CI	MCA-PB	φ 1.0 cm	1 → 1
Case 6	62/M	(−)	(+)	AP		CI	MCA-PB	φ 1.0 cm	0 → 1

TIA, transient ischemic attack; ASA, atrial septal aneurysm; INR, international nomalized ratios; m-RS, modified Rankin scale; DVT, deep vein thrombosis; WF, warfarin; AP, antiplatelet therapy; CI, cerebral infarct; MCA, middle cerebral artery; MCA-PB, perforating branches of middle cerebral artery; PCA, posterior cerebral artery.
* INR values at the time of the recurrent events.

Table 5　Frequency of deep vein thrombosis in stroke patients with patent foramen ovale reported by previous workers

References	Prevalence of DVT	Diagnostic strategies	Day of examinations
Ranoux, et al	3%	Venography/venous Doppler	< 4 weeks
Gautier, et al	13%	Venography	2 days—7 months
Stollberger, et al	57%	Venography	1—90 days
Bogousslavsky, et al	5.5%	Venography/venous Doppler	?
Hashimoto, et al	0%	RIvenography	2—23 days
Lethen, et al	9.5%	Venography	1—15 days

DVT, deep vein thrombosis

た．全例 ASA の合併はなく，大動脈弓粥腫病変（4.6mm～7.1 mm）をみとめた．病巣は，2例が穿通枝小梗塞，1例は病巣不明で，転帰も良好だった．

考　察

本研究では，DVT 検出率は 41% であり，その 92% が下腿，そのうち 71% は末梢の筋肉枝単独に存在した．これまでの報告では，奇異性脳塞栓症における DVT の検出率を 10% 程度とするものが多く，本研究結果の検出率にくらべて低い値を示している（Table 5）[7)～11)]．これらの報告は，RIvenography，もしくは静脈造影による検討であり，本研究によって多く発見された末梢の筋肉枝などの血栓の描出が困難であったことがその理由と考えられる．Labropoulos らは，臨床的に DVT がうたがわれる症例において，従来の評価法では偽陰性とされるような血栓が，末梢の筋肉枝単独に存在する例が少なくないことを示し，下肢静脈エコーにより筋肉枝まで評価をおこなう重要性を強調している[12)]．今回われわれがおこなった下肢静脈エコーは，ベッドサイドで簡便に施行することが可能であり，奇異性塞栓症がうたがわれる症例において DVT の評価をおこなうばあいのスクリーニング法として有用と思われた．

本研究では，DVT は高齢者，女性に多くみとめられた．これは従来の報告と一致していた[13)14)]．凝固系では，D-dimer は DVT 陽性例で高い傾向にあったが，TAT，AT III では差はみとめられなかった．DVT の有無のスクリーニングとして，D-dimer がもちいられる報告がみられるが，本研究のように筋枝静脈のみに血栓をみとめた 17 例中 14 例では D-dimer が正常であることから，末梢だけに血栓が存在するばあい，DVT の発見には血液凝固検査のみでは不十分であることが示唆された[15)]．

これまで，奇異性脳塞栓症の二次予防として，抗凝血薬療法，抗血小板療法，外科的治療が報告されている（Table 6）．内科的治療では，DVT を合併するばあいにはワルファリンによる抗凝血薬療法が第一選択とされている．DVT 所見をみとめないばあい，抗凝血薬療法と抗血小板薬のどちらを選択するか，明確な結論はえられていない[8)16)17)]．Orgera らによる二次予防に関する meta-analysis では，ワルファリンは抗血小板療法よりすぐれていたとしている[18)]．Homma らの報告では，ワルファリン群とアスピリン群とで，再発，死亡率に有意差はなかったとしている[19)]．また，ASA 合併例では再発のリスクが高く，抗凝血薬療法を必要とする報告がみられるが，抗血小板薬と差がなかったとする報告もある[19)20)]．外科的治療について，PFO のカテーテル的閉鎖や外科的閉鎖術が有効であったと報告されているが，十分なランダム化比較試験はおこなわれていない[21)～27)]．本研究における，DVT 陽性群での再発例

Table 6 Stroke recurrence rates following various preventive therapies in the patients with patent foramen ovale

Preventive therapies	Annual recurrent events rate	References
Medical treatment		
Aspirin or warfarin	0%	Hanna, et al
	1.9%	Bogousslavsky, et al
Aspirin	2.3% (PFO), 15.2% (PFO & ASA)	Mas, et al
Aspirin and warfarin	9.5% (WF), 17.9% (Asp)*	Homma, et al
Surgical treatment		
Percutaneous PFO closure	0%	Bridges, et al
	3.2%	Hung, et al
	3.4%	Windecker, et al
Surgical PFO closure	0%	Devuyst, et al
	7.5%	Dearani, et al
	19.5%	Homma, et al

PFO, patent foramen ovale; ASA, atrial septal aneurysm; WF, warfarin; Asp, aspirin, *Two-year rate

は，ワルファリンを服用していたものの，INR が全例 1.7 以下であり，ASA を合併したものが多く，再発後の転帰も不良であった．DVT 陽性例，とくに ASA 合併例では，より厳密なワルファリンコントロールの必要性が示唆された．一方，DVT 陰性群での再発例では，全例大動脈弓部粥腫病変をみとめ，穿通枝小梗塞または病巣不明であった．この群は，動脈硬化の関与が少なくないことが推測され，DVT の有無により，その後の再発の基礎となる病態がことなる可能性が考えられた．これらのことから，PFO を有する脳塞栓症患者の二次予防を考える際には，積極的に DVT の検索をおこなうことが重要である．DVT 陽性例では肺塞栓症予防の観点からもワルファリンが第一選択，DVT 陰性例では再発例で動脈硬化の関与が少なくなかったことを考慮に入れ，奇異性脳塞栓症以外の病態の検索を十分におこなうことが大切で，個々のケースで治療方針を決定する必要があると考えられた．

しかし，本研究は，症例数が 63 例と少なく，治療薬の割付がランダム化されていないため，結果の解釈には注意が必要である．また，DVT 検査の多くは脳塞栓症発症早期におこなわれているが，一部に入院後の治療や臥床期間の影響を受けている症例がある可能性は否定できない．また，抗血栓薬の使用に関して，効果的に再発を予防し，出血性合併症のリスクを減少させるには，適切な患者選択基準を確立する必要がある．症例の蓄積が必要であるが，発症早期に下肢静脈エコーによる DVT のスクリーニングを積極的におこなうことは，PFO を有する脳塞栓症患者の治療方針決定や予後の推測に有用であるものと思われた．

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Original Article

Lack of Association between Angiotensin II Type 1 Receptor Gene Polymorphism and Hypertension in Japanese

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Angiotensin II type 1 (AT_1) receptor mediates the vasoconstriction and growth-promoting effect of angiotensin II in humans. It has been reported that a polymorphism of the AT_1 receptor gene (an A/C transversion at position 1166; A1166C) may be associated with essential hypertension (HT). However, several conflicting results have also been reported. Therefore, we conducted an association study between A1166C variants of the AT_1 receptor gene and hypertension in the Japanese population. We genotyped this variant in 3,918 subjects (1,492 hypertensive subjects and 2,426 normotensive subjects) recruited from the Suita study. In subjects not receiving antihypertensive medication, the influence of the genotype on blood pressure values adjusted for clinical covariates was analyzed. The genotype distribution did not differ between hypertensive and normotensive subjects in either men (frequency of the C allele: 8.1% *vs.* 7.8%, $p=0.74$) or women (8.1% *vs.* 7.7%, $p=0.60$). There were no significant differences in systolic blood pressure, diastolic blood pressure, or pulse pressure among the three genotypes in either men or women who had not received hypertensive medication. Our data suggest that the A1166C polymorphism of AT_1 receptor is unlikely to influence blood pressure status in the Japanese population. (*Hypertens Res* 2003; 26: 131–134)

***Key Words*: epidemiology, genetics, blood pressure**

Introduction

Angiotensin II is an important effecter controlling blood pressure and volume in the cardiovascular system. Its importance is reflected by the efficacy of angiotensin-converting enzyme inhibitors in the treatment of hypertension and congestive heart failure. Angiotensin II interacts with two pharmacologically distinct subtypes of cell surface receptors, types 1 and 2. Angiotensin II type 1 (AT_1) receptors seem to play a key role in mediating the vasoconstrictor and growth-promoting effects of angiotensin II (*1*). It has been reported that a polymorphism of the AT_1 receptor gene (an A/C transversion at position 1166; A1166C) occurs more frequently in hypertensive subjects with a positive family history of hypertension than in control subjects (*2*). On the other hand, it has also been reported that this locus is not linked with this disease, and recent studies have demonstrated that the distribution of the genotypes did not differ between normotensive and hypertensive subjects (*3*, *4*). Moreover, a recent population-based survey of Caucasian hypertensives reported lower blood pressure values in CC homozygotes than in heterozygotes and AA homozygotes (*5*). In response to these controversial results, we performed an association study in a large epidemiological cohort to examine whether A1166C genetic variants influence blood pressure in the Japanese population.

Methods

Subjects

The selection criteria and design of the Suita study have

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Table 1. Characteristics of Men and Women Subjects by AT_1 Receptor Genotype

Characteristics	AT_1 receptor genotype		
	AA	AC	CC
Men (n=1,854)			
n	1,575	267	12
Age (years)	60.7±12.1	61.4±12.3	59.4±12.3
Body mass index (kg/m^2)	23.1±2.8	22.7±2.7	22.3±2.60
Waist-to-hip circumference ratio	0.91±0.06	0.90±0.06	0.94±0.04
Alcohol consumption (ml/day)	25.2±26.2	26.4±29.7	23.5±22.5
Smoking habit (%)	38.9	39.7	75
Ischemic heart disease (%)	4.75	6.37	0
Diabetes mellitus (%)	22.2	21.7	33.3
Proteinuria (%)	7.6	6.4	16.7
Use of antihypertensive drugs (%)	18.4	17.2	16.7
Women (n=2,062)			
n	1,755	290	17
Age (years)	58.8±11.8	59.1±11.6	57.2±13.7
Body mass index (kg/m^2)	22.3±3.2	22.5±2.9	21.1±2.7
Waist-to-hip circumference ratio	0.89±0.08	0.89±0.07	0.86±0.07
Alcohol consumption (ml/day)	5.1±11.2	4.6±10.0	6.1±13.3
Smoking habit (%)	8.1	8.6	11.8
Ischemic heart disease (%)	2.9	4.8	0
Diabetes mellitus (%)	14.2	13.1	5.9
Proteinuria (%)	3.9	4.5	5.9
Use of antihypertensive drugs (%)	16.1	16.2	17.6

Values are the mean±SEM. AT_1, angiotensin II type 1.

been described previously (*6*, *7*). The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Genetic Therapy of the National Cardiovascular Center. Informed consent on genetic analysis was obtained from about 4,000 subjects, and the genotype of A1166C was determined in 3,918 consecutive subjects. Subjects were categorized as hypertensives when they had a systolic blood pressure of ≧140 mmHg or a diastolic blood pressure of ≧90 mmHg. Subjects who were taking hypertensive medication were also categorized as hypertensives.

DNA Studies

DNA was isolated from peripheral leukocytes according to standard procedures. Polymorphisms were determined by the TaqMan system. The primers and probes for genotype determination were as follows: Sense 5′-CATTCCTCTGCAGCA CTTCACT-3′, Antisense 5′-CGGTTCAGTCCACATAATG CAT-3′, Probe for A(1166) 5′-Fam-AAATGAGCATTAGCT ACT-MGB-3′, Probe for C(1166) 5′-Fam-AAATGAGCCTT AGCTACT-MGB-3′. Each reaction included 20 ng of genomic DNA, 30 pmol of each primer, 12.5 pmol of each TaqMan probe and 1X TaqMan Universal Master Mix (PE Biosystems, Foster City, USA) in a volume of 50 μl. Polymerase chain reaction (PCR) cycling conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The results were analyzed using an ABI PRISM 7700 Sequence Detection System (PE Biosystems) using allelic discrimination software supplied by the manufacture.

Statistical Analyses

Values are expressed as the means±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc., Cary, USA). Multiple regression and multiple logistic analyses were performed with other covariates. Differences in numerical data among the groups were calculated by one-way analysis of variance (ANOVA) and unpaired Student's t-test. Values of $p<0.05$ were considered to indicate statistical significance. Differences in frequencies were tested by contingency table analysis.

Results

AT_1 Receptor Genotype and Allele Frequencies and Clinical Characteristics

The overall frequencies of the genotypes AA, AC, and CC were as follows: in men, 84.9%, 14.5%, and 0.6%, respectively; in women, 85.1%, 14.1%, and 0.8%; and overall, 85.0%, 14.1%, 0.8%. The allele frequencies for A and C were 92.1% and 7.9%, 92.0% and 8.0%, and 92.0% and 8.0%, respectively, in men, in women, and

Table 2. Genotype and Allele Distribution of AT1 Receptor A1166C Polymorphism in Hypertensive Subjects and Normotensive Subjects

	Men (*n* (%))		Women (*n* (%))	
	Hypertensive	Normotensive	Hypertensive	Normotensive
Genotype				
AA	627 (84.4)	948 (85.2)	632 (84.4)	1,123 (85.5)
AC	112 (15.1)	157 (14.1)	112 (15.0)	178 (13.6)
CC	4 (0.5)	8 (0.7)	5 (0.6)	12 (0.9)
	$\chi^2=0.55, p=0.76$		$\chi^2=1.09, p=0.58$	
Allele				
A	1,366 (91.9)	2,053 (92.2)	1,376 (91.9)	2,424 (92.3)
C	120 (8.1)	173 (7.8)	122 (8.1)	202 (7.7)
	$\chi^2=0.11, p=0.74$		$\chi^2=0.27, p=0.60$	

Table 3. Blood Pressure of Subjects Not Receiving Antihypertensive Medication by AT1 Receptor Genotype

	AT1 receptor genotype			ANOVA
	AA	AC	CC	*p*
Men ($n=1,518$)				
n	1,285	223	10	
SBP (mmHg)				
Unadjusted	125.8±0.5	126.4±1.2	126.2±5.8	0.88
Age-adjusted	125.8±0.5	126.5±1.1	128.0±5.4	0.93
Age and BMI-adjusted	125.7±0.5	127.1±1.1	128.6±5.3	0.73
DBP (mmHg)				
Unadjusted	80.3±0.3	79.5±0.7	77.1±3.3	0.40
Age-adjusted	80.4±0.3	78.8±0.7	74.3±3.3	0.38
Age and BMI-adjusted	80.4±0.3	79.2±0.7	74.7±3.2	0.69
PP (mmHg)				
Unadjusted	45.5±0.4	46.9±0.9	49.1±4.1	0.22
Age-adjusted	45.3±0.3	47.7±0.8	53.8±3.6	0.20
Age and BMI-adjusted	45.3±0.3	47.8±0.8	53.9±3.6	0.17
Women ($n=1,730$)				
n	1,473	243	14	
SBP (mmHg)				
Unadjusted	125.0±0.5	126.1±1.2	123.6±5.2	0.67
Age-adjusted	125.0±0.5	126.9±1.1	124.1±4.7	0.73
Age and BMI-adjusted	125.0±0.5	126.6±1.1	125.5±4.7	0.80
DBP (mmHg)				
Unadjusted	77.5±0.3	79.0±0.7	78.4±2.8	0.12
Age-adjusted	77.3±0.3	80.2±0.7	79.5±2.8	0.12
Age and BMI-adjusted	77.3±0.3	80.0±0.7	80.7±2.7	0.17
PP (mmHg)				
Unadjusted	47.5±0.4	47.2±0.9	45.3±3.6	0.77
Age-adjusted	47.6±0.3	46.8±0.8	44.7±3.2	0.82
Age and BMI-adjusted	47.6±0.3	46.7±0.8	44.9±3.2	0.80

Values are the mean±SEM. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; BMI, body mass index.

overall. The observed genotype frequencies were in agreement with those predicted by Hardy-Weinberg equilibrium.

The clinical characteristics of male and female subjects are summarized in Table 1. There were no significant differences among the three genotypes in any characteristics in either men or women.

Relationship between the AT1 Receptor Genotype and Blood Pressure

The genotype distribution and the allele distribution of the A1166C variants of AT1 receptor gene polymorphisms in hypertensive subjects were compared to those in normotensive

subjects for both men and women (Table 2). No significant differences in the genotype distribution or the allele distribution were observed between hypertensive subjects and normotensive subjects in either men or women.

The influence of the AT1 receptor genotype on residuals of blood pressure values after adjusting for age and body mass index (BMI) is shown in Table 3. No significant influence of the genotype on blood pressure was observed.

Discussion

Bonnardeaux *et al.* identified a positive association between A1166C variants of AT1 receptor gene polymorphisms and hypertension in 206 Caucasian patients with essential hypertension (*2*). Wang *et al.* did a case-control study of the A1166C variant in 108 Caucasian hypertensive subjects and found that the A1166C variant was associated with hypertension (*8*). On the other hand, Bonnardeaux *et al.* failed to detect such an association using affected sibling pair analysis, and recent studies have found no difference in the distribution of the genotypes between normotensive and hypertensive subjects (*3*, *4*). Two other studies conducted in Japan also failed to show a significant association between this polymorphism and hypertension (*9*, *10*). Moreover, a recent population based survey of Caucasian hypertensives reported lower blood pressure values in CC homozygotes than in heterozygotes and AA homozygotes (*5*).

In the original report with Caucasian subjects, the frequencies of the 1166C allele were 0.36 in hypertensives and 0.28 in normotensives (*2*). However, in the present study, the frequencies of the 1166C allele were 0.09 in hypertensives and 0.08 in controls. Since similar results have also been reported in other studies in Japan, it would appear that the C allele is less frequent in Japanese than in whites. This different allele frequency may have affected the different results between Japanese and whites.

Since a population of only several hundred subjects may be too small to draw statistically certain conclusions, and because independent confirmation is of critical value, we genotyped this variant in a large cohort representing the general Japanese population (the Suita study) consisting of 3,918 subjects.

Our results indicate that the genotype distribution did not differ between hypertensive and normotensive subjects, and that the genotype had no significant effects on blood pressure values in either men or women. However, the present observations do not necessarily exclude the possibility that the AT1 receptor gene is involved in hypertension in Japanese. It is still possible that some other polymorphisms in this gene may influence blood pressure. Takahashi *et al.* identified seven polymorphisms in the 5′-flanking region of the AT1 receptor gene and found a significantly higher frequency of the (−535)T allele in hypertensive subjects (*11*). However, the sample size was small and further confirmation may still be required.

In future studies, it will be necessary to identify a large number of polymorphisms throughout the AT1 receptor gene in Japanese and to perform association studies between these polymorphisms and blood pressure.

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An Acyl-CoA Synthetase Gene Family in Chromosome 16p12 May Contribute to Multiple Risk Factors

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Abstract—We recently reported that genetic polymorphisms of SAH, an acyl-CoA synthetase for fatty acids, might contribute to multiple risk factors, especially hypertriglyceridemia. There are at least 4 members in this SAH gene family, *SAH*, *MACS1*, *MACS2*, and *MACS3*, and these 4 members are clustered in human Ch16p12. It is possible either that the previously observed associations were due to linkage disequilibrium with truly important polymorphisms in other members of the SAH gene family or that other polymorphisms in this gene family may also influence multiple risk factors. Thus, we performed association studies between genetic polymorphisms in this *SAH* region and multiple risk factors, using a large cohort representing the general population in Japan. The L513S polymorphism in *MACS2* was shown to significantly influence the triglyceride level and the waist-to-hip ratio. The previously observed associations between an *SAH* polymorphism and the waist-to-hip ratio appear to be due to linkage disequilibrium with the L513S polymorphism. Haplotype analysis indicated that a haplotype defined by the I/D polymorphism of *SAH* and the L513S polymorphism in *MACS2* was highly significantly associated with the triglyceride level. This study confirmed the importance of this chromosomal region in the pathogenesis of hypertriglyceridemia and visceral obesity. (***Hypertension.* 2003;41:1041-1046.**)

Key Words: epidemiology ■ fatty acids ■ genetics ■ hyperlipidemia ■ obesity

Differential screening was used to isolate *SAH* (*S*pontaneously hypertensive rat–Clone *A*–*H*ypertension-associated) from a genetically hypertensive rat strain, spontaneously hypertensive rat (SHR).[1] The expression of *SAH* in the kidneys of SHR is markedly higher than that in the kidneys of a normotensive control stain, Wistar-Kyoto rat. The rat *SAH* is localized on chromosome 1 near the most prominent QTL for blood pressure and had been expected to contribute to hypertension in SHR.[2,3] However, subsequent congenic analysis excluded rat *SAH* from the genes that contribute to hypertension in SHR.[4,5]

Recently, SAH protein has been reported to be significantly homologous to bovine xenobiotic-metabolizing medium-chain fatty acids: CoA ligase.[6] We revealed that human SAH had acyl-CoA synthetase activity toward medium chain fatty acids and that a genetic polymorphism of *SAH* might contribute to multiple risk factors, including hypertriglyceridemia, obesity, and hypertension.[7] It is likely that a genetic polymorphism of *SAH* might influence triglyceride metabolism, energy expenditure, and fat metabolism by influencing fatty acid metabolism.

A homology search of *SAH* in the human genome indicates that there are at least 4 members in this *SAH* gene family, *SAH*, *MACS1*, *MACS2*, and *MACS3* (Figure). Moreover, these 4 appear to be clustered in chromosome 16p12 (see Results). It is possible that the associations seen between the *SAH* polymorphism and multiple risk factors in the preceding study[7] might be due to linkage disequilibrium with genetic polymorphisms in other members of this gene family and that genetic polymorphisms in other members of this gene family might also contribute to multiple risk factors. Thus, to extend our previous work, we searched for genetic variations in this chromosomal region and performed association studies between polymorphisms in this region and multiple risk factors using a large cohort representing the general population in Japan.

Methods

DNA Studies

Genomic DNA from 36 subjects was used for sequence screening for polymorphisms. The promoter region and all of the exons of the *MACS1*[8] (Medium Chain Acyl-CoA Synthetase 1; MACS1) gene were sequenced according to the human draft sequence. The genome structures of *MACS2* (GenBank accession; AX451437) and *MACS3* (GenBank accession; AK000588) had not been determined at the beginning of the present study. We determined exon-intron boundaries on the basis of homology to *SAH* and *MACS1* and amplified intronic sequences by primers residing on the neighboring exons to determine the flanking sequences of exons. Based on the flanking sequences, all of the coding exons of *MACS2* and *MACS3* were amplified and sequenced. Primer sequences can be provided on request. The polymorphisms were determined by use of the TaqMan system (PE Applied Biosystems). The sequences of the primers and probes used in the TaqMan method can be provided on request.

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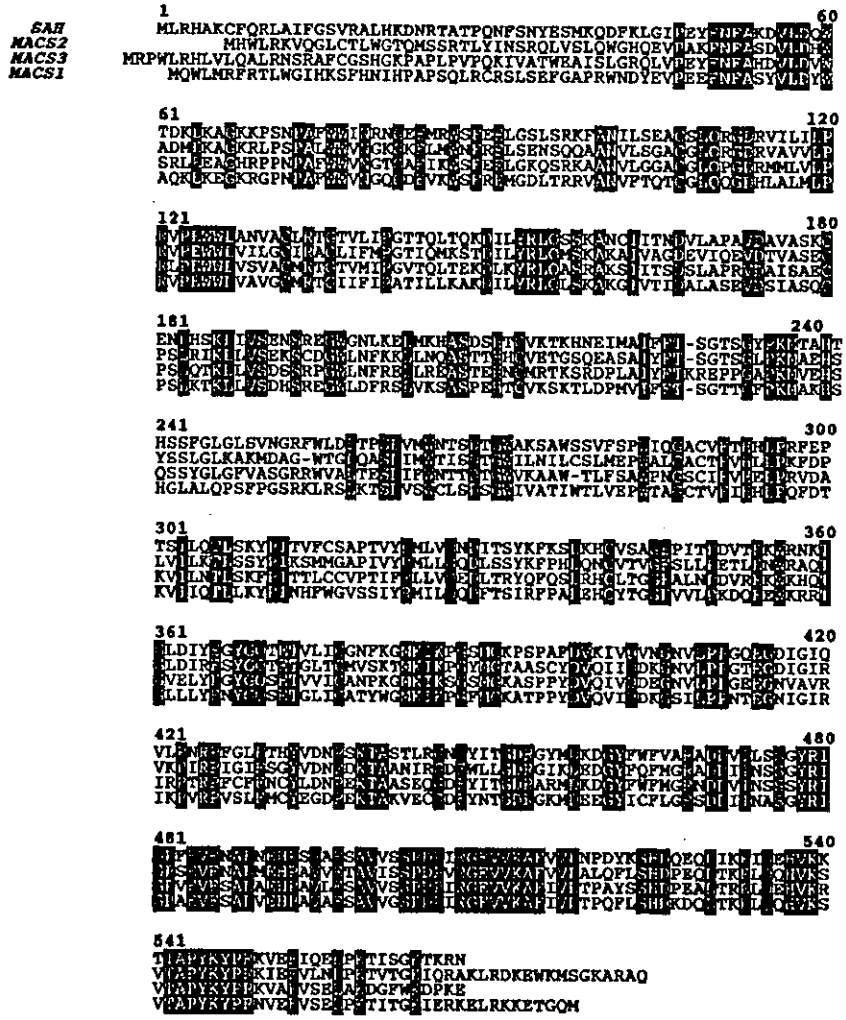

The *SAH* gene family. Amino acid sequences of *SAH* and *MACS1*, *MACS2*, and *MACS3* are shown. Identical amino acid residues among members are indicated. L513S polymorphism of *MACS2* is indicated by bold letter "**L**".

The expression levels of *MACS1*, *MACS2*, *MACS3*, and *SAH* mRNA were assessed by PCR, with the use of a human cDNA panel (Clontech) with 2 independent sets of primers.

Subjects

The selection criteria and design of the Suita Study have been described previously.[7] The genotypes were determined in 1976 consecutive subjects (written informed consent was obtained), who constituted the latter half of the study population in the preceding study. The study protocol was approved by the institutional ethics committee.

The characteristics of the subjects analyzed in the present study are summarized in Table 1, according to L513S polymorphism of *MACS2*. Hypertension was defined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or the current use of antihypertensive medication. Total cholesterol and triglyceride levels were determined by enzymatic methods and kits (L-TC WAKO, Wako Pure Chemical, and Clinimate TG-2, Daiichi Chemicals). Homeostasis model assessment of insulin resistance (HOMA) was calculated as follows[9]: HOMA=[fasting insulin (μU/mL)× fasting glucose (mmol/L)]/22.5. Total immunoreactive insulin was measured by a kit (TOSOH), with the use of a 2-site immunoenzymometric assay.

Statistical Analysis

Values are expressed as mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc). Multiple linear regression and multiple logistic analyses were performed with other covariates. Residuals of the waist-to-hip ratio and triglycerides were calculated by adjusting for age, gender, alcohol consumption (ethanol mL/d), and smoking (cigarettes/d). In some settings, the probability value was corrected (*Pc*) by the Bonferroni method. Principal component analysis was performed on the basis of correlations.

Linkage disequilibrium[10] and haplotype analyses were performed using the SNPAlyze statistical package (Dynacom Inc, http://www.dynacom.co.jp/; accessed March 5, 2003). Haplotype estimation was performed by the expectation-maximization algorithm.[11] To measure linkage disequilibrium between SNPs, Lewontin's D' was calculated.[12]

Results

Confirmation of the *SAH* Gene Family

A BLAST search revealed the existence of 3 transcripts homologous to *SAH*, namely *MACS1* to *MACS3*. The complete genome structure of *MACS1* has been described previously.[8] The genome structure of *MACS3* and part of the genome structure of *MACS2* have not been reported, and we determined flanking sequences of coding exons of *MACS2* and *MACS3* for sequence screening of polymorphisms.

The polymorphisms found in the present study are summarized in Table 2. Polymorphisms in introns were not studied in detail and are not included in Table 2.

TABLE 1. Characteristics of the Study Population

Phenotype	SS (n=125)	LS (n=731)	LL (n=1120)	P
Men, %	54.4	47.2	47.5	NS
Age, y	60.1 (1.1)	59.6 (0.4)	60.1 (0.4)	NS
Alcohol consumption, mL/d	15.6 (2.0)	15.6 (0.8)	14.6 (0.8)	NS
Smoking, cigarettes/d	5.4 (0.9)	4.4 (0.4)	4.3 (0.3)	NS
HTN, %	42.4	37.4	38.7	NS
HDL, mmol/L	1.40 (0.04)	1.51 (0.02)	1.53 (0.01)	0.0025
TChol, mmol/L	5.42 (0.08)	5.42 (0.03)	5.45 (0.03)	NS
TG, mmol/L	1.74 (0.09)	1.42 (0.04)	1.40 (0.03)	0.0059
R-TG, mmol/L	+0.36 (0.01)	0.00 (0.01)	−0.04 (0.04)	0.0089
W/H	0.914 (0.006)	0.905 (0.003)	0.897 (0.002)	0.0034
R-W/H	+0.011(0.006)	+0.005 (0.002)	−0.004 (0.002)	0.0011
BMI, kg/m^2	23.4 (0.3)	22.9 (0.1)	22.6 (0.1)	0.0090
FBS, mmol/L	5.56 (0.09)	5.47 (0.04)	5.42 (0.03)	NS
HOMA	2.43 (0.17) (n=60)	1.90 (0.07) (n=422)	1.79 (0.05) (n=624)	0.0015
Insulin, μU/mL	9.5 (0.6) (n=60)	9.7 (0.2) (n=422)	7.3 (0.2) (n=624)	0.0036

Characteristics of the study population are shown according to the L513S polymorphism of the *MACS2* genotype. HTN indicates hypertensive subjects; HDL, HDL cholesterol; TChol, total cholesterol; TG, triglycerides; R-TG, residuals of TG; W/H, waist-to-hip ratio; R-W/H, residuals of W/H; BMI, body mass index; FBS, fasting blood glucose; HOMA, homeostasis model assessment of insulin resistance. R-TG and R-W/H were calculated by adjusting for age, gender, alcohol consumption, and smoking.

The expression of *MACS1* was not detected, as described below, which may downplay the importance of this gene. The AC repeat polymorphism in the promoter may not be suitable for high-throughput genotyping and was neglected in the present study. The polymorphisms in exons 8, 11, 12, and 13 were in complete linkage disequilibrium in the 36 subjects sequenced, and we selected the exon 12 polymorphism for the association study.

We found 3 polymorphisms in the coding region of *MACS2*, which were selected for the association study. The L513S polymorphism may have some functional meaning, since hydrophobic leucine is replaced by hydrophilic serine.

We found 4 polymorphisms in the coding region of *MACS3*. The Q159H (exon 3) and P353R (exon 7 to 1) polymorphisms were in complete linkage disequilibrium with the T534M (exon 12) and H361R (exon 7 to 2) poly-

TABLE 2. Polymorphisms in the Ch16p12 SAH Region

Gene	Region		Sequence	AA Change	Minor Allele Frequency
MACS1					
	Promoter		TGTTTAGAAA (CA)n TTGGAGAGGT	...	0.417
	Ex8		CTCCACCCTA[C/T]GACGTCCAGG	TAC(Y)/TAT(Y)	0.417
	Ex10		GGGACAGAGG[A/T]AAGATGGATG	GGA(G)/GGT(G)	0.070
	Ex11		AGGTTGAAAG[T/C]GCTTTGGTGG	AGT(S)/AGC(S)	0.417
	Ex12		ACCCAAGGAA[A/G]GTGAGTGAGG	AAA(K)/AAG(K)	0.417
	Ex13	3'UTR	CTGCACACCT[A/G]AGGCAAATCC	...	0.417
MACS2					
	Ex9		CACAGGGATT[G/A]ACTTGCATGG	TTG(L)/TTA(L)	0.222
	Ex11		GGGACGGGCA[G/A]ATGATATCAT	GAT(D)/AAT(N)	0.097
	Ex13		GTCCTGGCCT[T/C]GCAGTTCCTG	TTG(L)/TCG(S)	0.208
MACS3					
	Ex3		ACCGGCTGCA[G/C]GCGTCCAGGG	CAG(Q)/CAC(H)	0.167
	Ex7(1)		GCCCTCAACC[C/G]TGACGTGAGG	CCT(P)/CGT(R)	0.457
	Ex7(2)		AAGTGGAAAC[A/G]CCAGACCGGT	CAC(H)/CGC(R)	0.457
	Ex12		AGAGGCACTA[C/T]CGCGGGAACT	ACG(T)/ATG(M)	0.167

Polymorphisms in the SAH region are shown. Minor allele frequencies are obtained from the 36 subjects sequenced. Polymorphisms indicated by bold letters are used for genotyping of the study population.

TABLE 3. Linkage Disequilibrium Between Polymorphisms

Genotype	SAH12	M1/E12	M2/E9	M2/E11	M2/E13	M3/E7	M3/E12
SAH I/D	**−0.9999**	**0.5302**	−0.2939	−0.0415	0.1851	−0.0337	0.4589
	15.5322	**241.7198**	15.4449	0.1314	56.3709	0.6984	93.1298
SAH12		**−0.6890**	0.1353	**−0.7164**	0.1830	0.4778	**−0.7965**
		16.1148	4.0146	**2.1522**	4.4001	9.8528	**1.5742**
M1/E12			0.1697	0.4467	0.4434	0.2454	**0.9778**
			12.8313	46.1666	140.5322	101.3379	**181.6169**
M2/E9				**0.9732**	**−0.8225**	**0.9480**	0.3467
				983.5424	**97.3028**	**463.9159**	104.8876
M2/E11					**−0.7896**	**0.9397**	0.2992
					45.6709	**233.7887**	147.0224
M2/E13						**0.6942**	−0.2010
						409.5116	2.6518
M3/E7							0.3393
							25.5663

Linkage disequilibriums between polymorphisms are shown. D' (upper) and χ^2 (lower) values are indicated. Bold letters indicate polymorphisms in strong linkage disequilibrium. The *SAH* I/D and intron 12 polymorphisms have been described previously.[7]

morphisms, respectively. Thus, we selected the H361R (exon 7 to 2) and T534M (exon 12) polymorphisms for the association study. We also determined 2 polymorphisms of *SAH*, I/D polymorphism in the promoter and A/G polymorphism in intron 12, which were concluded to be associated with multiple risk factors in the preceding study in 4039 subjects.[7]

Linkage disequilibrium among these polymorphisms is shown in Table 3. Although the locus for *MACS3* has not been clarified, strong linkage disequilibrium between the *MACS3* and *MACS2* polymorphisms indicates that *MACS3* may reside in this human chromosome 16p12 region near the *MACS2* locus.

Expression of the *MACS* Gene Family

RT-PCR analysis of expression levels of *MACS1, MACS2,* and *MACS3* and *SAH* revealed that *MACS2* and *MACS3* and *SAH* were expressed mainly in the kidney and liver. However, we could not detect PCR product from *MACS1* in any of the tissues examined including the spleen, thymus, prostate, testis, ovary, small intestine, colon, lymph node, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

Association Study

Association studies between the polymorphisms in Table 2 and various phenotypes in the 1976 subjects revealed that the L513S polymorphism in *MACS2* strongly influenced triglycerides (TG), HDL cholesterol, waist-to-hip ratio (W/H), and body mass index (BMI) (Table 1). More intriguingly, an index for insulin resistance (HOMA) was influenced by the L513S polymorphism. Since members of the *SAH* gene family appear to have acyl-CoA synthetase activity toward fatty acids, it is likely that principal phenotypes influenced by this gene family may be the triglyceride level and/or visceral obesity (waist-to-hip ratio).

The effects of other polymorphisms on the triglyceride level and W/H ratio are indicated in Table 4. Residuals of the triglyceride level (R-TG) and W/H ratio (R-W/H) were calculated by adjusting for age, sex, alcohol consumption, and smoking. Residuals of the triglyceride level were also calculated after excluding subjects with hypolipidemic drugs to correctly assess the influence of polymorphisms on the triglyceride level (R-TG'). The influence of a *SAH* polymorphism on triglycerides and W/H ratio, which was evident in 4039 subjects in the preceding study, was weak in the present group of 1976 subjects, who comprised a subset (latter part) of the preceding 4039 subjects.

To avoid the problems of multiple testing, a principal component analysis was also performed. After performing a correlation analysis among TG, HDL, W/H, and BMI, the principal components were identified. The first principal component explained 50.5% of the total variance, and the influence of genotype on this component was analyzed by 1-way ANOVA (Table 4). The first principal component was defined as [0.422 (TG)+0.499 (HDL)+0.531 (W/H)+0.539 (BMI)]. Although the pathophysiological meaning of this component is difficult to discern at a glance, it was significantly affected by the L513S polymorphism (Table 4).

To clarify the possible contribution of polymorphisms other than the L513S polymorphism to triglycerides and W/H ratio, diplotypes defined by L513S and another polymorphism were determined in the study population. The effects of various haplotypes on triglycerides and W/H ratio were also evaluated.

There are 4 haplotypes defined by the L513S and I/D (SAH) polymorphisms: L513-D (haplotype1, allele frequency 0.559, 95% CI, 0.533 to 0.585), L513-I (haplotype2, allele frequency 0.200, 95% CI, 0.180 to 0.216), S513-D (haplotype3, allele frequency 0.136, 95% CI, 0.119 to 0.155), and S513-I (haplotype4, allele frequency 0.105, 95% CI, 0.086 to 0.120). The effects of the diplotypes defined by these 4 haplotypes on the triglyceride level are shown in Table 5. One-way ANOVA indi-

TABLE 4. Polymorphisms of SAH Gene Family and Triglycerides and W/H Levels

Phenotype	SAH I/D	SAH12	M1/E12	M2/E9	M2/E11	M2/E13	M3/E7	M3/E12	10D	D33
R-TG (n=1976)										
F value	2.1400	0.6510	0.9534	3.0167	1.2921	4.7360	0.3173	2.5916	**3.0537**	**18.3860**
P	0.1179	0.5216	0.3856	0.0492	0.2749	0.0089	0.7281	0.0752	**0.0012**	**<0.0001**
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	0.2848	1.0000	1.0000	**0.0384**	**<0.0032**
df	2	2	2	2	2	2	2	2	**9**	**1**
R-TG′ (n=1898)										
F value	1.3562	0.6926	0.8046	4.2791	1.7569	**6.0809**	0.3752	3.0618	**3.5432**	**19.9302**
P	0.2579	0.5004	0.4474	0.0140	0.1713	**0.0023**	0.6872	0.0470	**0.0002**	**<0.0001**
Pc	1.0000	1.0000	1.0000	0.4480	1.0000	**0.0736**	1.0000	1.0000	**0.0064**	**<0.0032**
df	2	2	2	2	2	**2**	2	2	**9**	**1**
R-W/H (n=1976)										
F value	0.3093	0.8124	0.0952	1.0303	1.5800	**6.8456**	0.5537	0.8507	1.7752	2.4432
P	0.7340	0.4439	0.9092	0.3571	0.2062	**0.0011**	0.5749	0.4273	0.0681	0.1182
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	**0.0352**	1.0000	1.0000	1.0000	1.0000
df	2	2	2	2	2	**2**	2	2	9	1
1st PC (n=1976)										
F value	0.4884	0.7813	2.5228	0.8158	2.0186	**9.7314**	1.1155	0.2028	**2.8083**	**9.7354**
P	0.6137	0.4580	0.0805	0.4424	0.1331	**<0.0001**	0.3279	0.8165	**0.0028**	**0.0018**
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	**<0.0032**	1.0000	1.0000	**0.0448**	**0.0288**
df	2	2	2	2	2	**2**	2	2	**9**	**1**

The influence of polymorphisms on R-TG, R-TG′, R-W/H, and the first principal component (1st PC) were analyzed by 1-way ANOVA. R-TG and R-W/H were calculated by adjusting for gender, age, alcohol, and smoking (n=1976). R-TG′ was calculated after excluding subjects who were receiving hypolipidemic drugs (n=1898). The 1st PC was calculated as described in the text. The effects of the 10 diplotypes (10D) and the Diplotype 33 (D33) are also indicated. In D33, the 10 diplotypes (see TABLE 5) are recategorized into 2 groups, i.e., diplotype 33 and others. The haplotypes are defined in the text.

P values are corrected (*Pc*) by multiplying 32 [(8 genotypes+7 haplotypes+1 recategorization)×2 (possibly independent 2 phenotypes: triglyceride and waist-to-hip ratio)] (Bonferroni).

cated that the diplotype had significant effects on R-TG ($P=0.0012$) and R-TG′ ($P=0.0002$). As shown in Table 5, the diplotype 33 had significantly higher R-TG and R-TG′ levels. Thus, we recategorized the 10 diplotypes into 2 groups, that is, diplotype 33 and others. The influence of this diplotype 33 on the triglyceride level was highly significant even after correction by the Bonferroni method ($P<0.0001$ and $Pc<0.0032$, Table 4).

TABLE 5. Influence of Diplotype on Triglycerides Levels

Diplotype	n=1976 (n′=1898)	R-TG	*P*	R-TG′	*P*
11	632 (606)	0.01 (0.04)	<0.0001	−0.01 (0.04)	<0.0001
12	411 (393)	−0.12 (0.05)	<0.0001	−0.12 (0.05)	<0.0001
13	320 (306)	0.02 (0.06)	0.0001	0.01 (0.06)	<0.0001
14	332 (323)	0.02 (0.06)	0.0001	0.05 (0.06)	0.0001
22	75 (71)	0.13 (0.13)	0.0046	0.15 (0.13)	0.0074
23	4 (4)	0.41 (0.55)	NS	0.42 (0.54)	NS
24	81 (78)	−0.21 (0.12)	<0.0001	−0.23 (0.12)	<0.0001
33	46 (46)	0.69 (0.16)		0.70 (0.26)	
34	52 (49)	0.01 (0.15)	0.0023	0.03 (0.15)	0.0023
44	23 (22)	0.12 (0.23)	0.0444	0.19 (0.23)	0.0678

The influence of the diplotype on the triglyceride level was assessed by 1-way ANOVA. Haplotypes are defined in the text. The diplotype XY indicates the genotype with X and Y haplotypes. Thus, diplotype 23 indicates the genotype with one haplotype 2 and one haplotype 3. One-way ANOVA indicated that the diplotype had a significant influence on R-TG ($P=0.0012$) and R-TG′ ($P=0.0002$) (see TABLE 4).

P values indicate significant differences from the diplotype 33 group (by Fisher protected least significant difference test).

Discussion

We recently reported that genetic polymorphisms in *SAH* influenced multiple risk factors, including TG, HDL cholesterol, BMI, W/H ratio, and blood pressure status.[7] Since then, 3 other genes with high homology to *SAH* have been identified to cluster in the *SAH* region, chromosome16p12. Thus, it is possible either that the previously observed associations were due to linkage disequilibrium with truly important polymorphisms in other members of the SA gene family or that other polymorphisms in this gene family may also influence multiple risk factors.

In the present study, to evaluate the above-mentioned hypotheses, we performed extensive association studies between genetic polymorphisms in this region and multiple risk factors using a large cohort representing the general population in Japan. The L513S polymorphism in *MACS2* was shown to significantly influence TG, HDL, W/H, BMI, and HOMA index.

Because the L513S genotype appeared to influence various phenotypes including TG, HDL, W/H, and BMI, a principal component analysis was performed to avoid the problems of multiple testing. The L513S polymorphism had a highly significant influence on the first principal component. However, the pathophysiological meaning of this component is difficult to discern.

The members of the *SAH* gene family seem to have acyl-CoA synthetase activity toward medium chain fatty acids.[6–8] Thus, it is logically highly likely from the biological viewpoint that principal phenotypes influenced by this gene family may be the TG level and/or visceral obesity. Therefore, we studied the influence of polymorphisms on the TG level and W/H ratio (an excellent index of visceral obesity) (Table 4). Diplotype 33 had a highly significant influence on the TG level and the L513S polymorphism of *MACS2* had a weak but significant influence on the W/H ratio. Therefore, most of the previously observed associations between a *SAH* polymorphism and multiple risk factors appear to be due to linkage disequilibrium with the L513S polymorphism and haplotype 3.

In conclusion, the present study confirmed the importance of this chromosomal region, especially *MACS2* and *SAH*, in the pathogenesis of hypertriglyceridemia and visceral obesity. Intriguingly, this locus has been reported to be one of the suggestive loci for body mass index in the Framingham Heart Study.[13]

Perspectives

Human MACS1, human SAH, and bovine counterparts have been reported to act as acyl-CoA synthetases for various fatty acids, especially medium-chain fatty acids (MCFA).[6–8,14] MCFA are abundant in milk, coconut oil, and various synthetic oils. The activation of MCFA takes place mostly in the mitochondrial matrix by acyl-CoA synthetase for MCFA. Most of the MCFA incorporated into hepatocytes is subject to β-oxidation. Some of the acyl-CoA produced during MCFA oxidation is directed toward ketone body production, and the rest is directed to de novo synthesis of long-chain fatty acids, which are then incorporated into triglycerides or other complex lipids.[15,16] Recently, it has been proposed that medium-chain triglycerides may help to prevent obesity.[17] Therefore, it is highly likely that members of the *SAH* gene family (possible acyl-CoA synthetases for MCFA) may play some important roles in triglyceride metabolism, energy expenditure, fat metabolism, and, therefore, insulin resistance. However, the precise in vivo functions of the members of this gene family and the functional properties of the L513S polymorphism remain to be clarified and await further investigation.

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Association of Methylenetetrahydrofolate Reductase Gene Polymorphism With Carotid Atherosclerosis Depending on Smoking Status in a Japanese General Population

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Background and Purpose—The association of the *methylenetetrahydrofolate reductase* gene (*MTHFR*) with carotid atherosclerosis remains inconsistent. This may be due to small sample size and inappropriate analysis. We investigated the association of *C677T/MTHFR* with blood pressure and carotid atherosclerosis in a Japanese general population.

Methods—Subjects (30 to 89 years of age; 1693 women, 1554 men) who gave informed consent were randomly selected from a general population in Suita, Japan. *MTHFR* genotypes were determined by TaqMan polymerase chain reaction. Carotid atherosclerosis was evaluated by high-resolution ultrasonography with atherosclerotic indexes of intimal-medial thickness (IMT), maximum IMT in the common carotid artery (CCA), plaque score, and stenosis (>50%).

Results—Age-adjusted diastolic blood pressure was significantly higher in women with the *TT* genotype than in those with the *CC* genotype. In a recessive model (*CC+CT* versus *TT*), all adjusted odds ratios for hypertension and >50% stenosis in women were 1.42 and 3.42 (95% confidence intervals, 1.01 to 1.99 and 1.23 to 9.53), respectively. In women, maximum IMT in CCA for smokers with the *TT* genotype was significantly higher than for smokers with the *CC* genotype and nonsmokers with the *TT* genotype ($P<0.05$).

Conclusions—Our study suggests that the *MTHFR TT* genotype is a risk factor for hypertension and carotid stenosis in women. Significant interactions between *C677T/MTHFR* and smoking on maximum IMT in CCA were observed in women but not in men. Smoking cessation for subjects with the *TT* genotype is important in the prevention of cerebrovascular disease. **(*Stroke*. 2003;34:1628-1633.)**

Key Words: amine oxidoreductases ■ blood pressure ■ carotid arteries ■ Japan ■ risk factors

Hyperhomocysteinemia is associated with increased risk of atherosclerotic vascular disease.[1] The association of plasma total homocysteine concentration with atherosclerosis has been the subject of a number of clinical studies that have consistently linked moderate hyperhomocysteinemia with peripheral vascular disease, cerebrovascular disease, and coronary heart disease.[2–5]

Plasma total homocysteine levels are regulated mainly by 5,10-methylenetetrahydrofolate reductase, which is involved in the folate-dependent remethylation of homocysteine to methionine. Frosst et al[6] suggested that the *C677T* polymorphism in the *methylenetetrahydrofolate reductase* gene (*MTHFR*) is a candidate risk factor for vascular disease. The metabolic changes associated with *C677T/MTHFR* are postulated to modify the predisposition to diseases associated with folate deficiency.[7] Particular emphasis has been given to the role of *C677T/MTHFR* in cardiovascular[8] and cerebrovascular disease[9] and venous thrombosis.[10]

On the other hand, technical improvements in carotid ultrasonography have revealed new risk factors for stroke in its wide use. Some studies have demonstrated a close correlation between carotid ultrasound measurement, usually of carotid intimal-medial wall thickness (IMT), and the severity of extracranial carotid atherosclerosis.[11,12] Plasma total homocysteine levels have also been associated with more advanced carotid atherosclerosis in elderly subjects.[3,13] However, there have been controversies among their results. Most studies have failed to show an association between *C677T/MTHFR* and atherosclerotic disease.[14,15] These inconsistencies may be due to small sample size, combined-sex analysis, and lack of consideration of lifestyle. In this study, we examined the effect of *C677T/MTHFR* on carotid atherosclerosis and blood pressure (BP) in a large genetic epidemiological study, the Suita Study.

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Materials and Methods

Subject Population

The Suita Study was based on a random sample of 14 200 Japanese residents of Suita, a city located in the second-largest urban area in Japan, Osaka.[16] These 14 200 residents between 30 and 89 years of age were arbitrarily selected from the municipality population registry, stratified by sex and 10-year age groups. We sent these residents letters to ask if they were willing to participate in this study from 1989 with a cohort base; by February 1007, 51.7% of the subjects (n=7347) had paid an initial visit to the National Cardiovascular Center (NCVC). The participants have visited NCVC every 2 years since then for regular health checkups. In addition to routine blood examinations that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose, glycosylated hemoglobin A_{1C} (HbA_{1C}), systolic BP (SBP), and diastolic BP (DBP), DNA was extracted from an extra 5 mL blood withdrawn from those who underwent general examinations at NCVC between May 1996 and February 1998. Ninety percent of the subjects who visited NCVC during this period gave informed consent for genetic analysis of 13 genes including *MTHFR* and storage of a DNA sample and were enrolled in the present study. The study protocol of genetic analysis was approved by the ethics committee of Osaka University. Three physicians performed the carotid ultrasonic examinations. Finally, the subjects in the present study included 1693 women and 1553 men 30 to 89 years of age who attended regular health checkups and subsequently underwent ultrasonic examinations and genetic analysis.

Measurements

The subjects' BPs were measured after at least 10 minutes of rest in the sitting position. The mean value of 2 measurements of SBP or DBP obtained by a physician using a mercury sphygmomanometer (recorded >3 minutes apart) was used for the analysis. Hypertension was defined as a mean SBP of ≥160 mm Hg, a mean DBP of ≥95 mm Hg, or current use of antihypertensive medication.

The subjects were classified as current smokers or drinkers if they smoked or drank. Hypercholesterolemia was defined as serum total cholesterol levels ≥220 mg/dL or current use of antihyperlipidemic medication. Diabetes was defined as fasting plasma glucose levels ≥7.0 mmol/L (126 mg/dL) or nonfasting glucose levels ≥11.1 mmol/L (200 mg/dL), HbA1C ≥6.5%, or current use of antidiabetic medication. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Blood samples drawn from the subjects after 12 hours of fasting were collected in EDTA-containing tubes. Total cholesterol and HDL cholesterol levels were measured with an autoanalyzer (Toshiba TBA-80) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.[17] Among 3247 subjects, 1541 (820 women, 721 men) underwent measurement of fasting total plasma homocysteine levels by high-performance liquid chromatography.[18]

Carotid Ultrasound Measurements

Details of the carotid ultrasonic examination methods have been previously published.[16] We used a high-resolution B-mode ultrasonic machine with a 7.5-MHz transducer yielding an axial resolution of 0.1 mm. The regions from 30 mm proximal to the beginning of the dilation of the bifurcation bulb to 15 mm distal to the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the electronic caliper and were recorded on photocopies. IMT was measured on a longitudinal scan of the CCAs at a point 10 mm proximal to the beginning of the dilation of each carotid artery bulb. IMT was defined as the mean of the IMT of the proximal and distal walls at the point of measurement. Maximum IMT in the CCA and maximum IMT were defined as the maximum IMT in the scanned CCA area and the maximum IMT in the entire scanned area, respectively. We defined a plaque, a focal IMT thickening, as an area where IMT ≥1.1 mm and calculated plaque score by totaling the maximum thickness of all the plaques in the scanned area. Finally, we defined stenosis as a condition in which a plaque occupied more than half of the lumen circumference of an artery on a cross-sectional scan. We performed color-flow Doppler examination to confirm the presence of stenosis.

MTHFR Genotype Determination With TaqMan Polymerase Chain Reaction Method

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures with a QIAamp DNA Blood Kit (Qiangen Inc). To deal with a large number of samples, we introduced the TaqMan polymerase chain reaction (PCR) method (Applied Biosystems). In the current investigation, we prepared 2 probes: C allele–specific probe, 5' Tet-TCT GCG GGA GcC GAT TTC ATC ATC-Tamra-3', and T allele–specific probe, 5'-Fam-TCT GCG GGA GtC GAT TTC ATC ATC-Tamra-3'. Primer design for PCR of the flanking region of *C677T/MTHFR* was as follows: forward, 5'-GGC TGA CCT GAA GCA CTT GAA-3'; reverse, 5'-GCG GAA GAA TGT GTC ATC CT-3'. PCR was carried out with a thermal cycler (GeneAmp, PCR System 9700, Applied Biosystems). PCR was performed according to the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The fluorescence level of PCR products was measured with an ABI PRISM 7200 and 7900 Sequence Detector (Applied Biosystems), resulting in clear identification of the 3 genotypes of *C677T/MTHFR*.

Statistical Analysis

The number of subjects was restricted to 3247 who had complete data, including *C677T/MTHFR* and carotid ultrasonographic measurements. Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis.

Associations of *C677T/MTHFR* with BP were investigated by sex through logistic regression analysis considering potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. The genotype effect was examined according to a dominant (*TT+CT* versus *CC*) and a recessive (*TT* versus *CT+CC*) model. For multivariate risk predictors, the adjusted odds ratios (ORs) were given with the 95% confidence intervals (CIs). The relationships in men and women between *C677T/MTHFR* and hypertensive risk were expressed in terms of ORs adjusted for possible confounding effects. The association of *C677T/MTHFR* with carotid atherosclerotic index was also investigated by sex through logistic regression analysis considering potential confounding risk variables. Partial correlation coefficients between plasma total homocysteine and carotid atherosclerotic indexes by sex and *C677T/MTHFR* were determined. In addition, gene and environmental interactions were calculated with the following logistic regression model: logit $p=\beta_0+\beta_g x_g+\beta_e x_e+\beta_{ge} x_g x_e$, where x_g and x_e are genetic and environmental data, respectively; β_0 is an intercept term; β_g is the main effect due to genes; and β_e is the main effect of the environment. The coefficient β_{ge} of the product $x_g x_e$ estimates the gene and environmental interaction on the logit scale.[19] All analyses were performed with SAS statistical software (release 6.12, SAS Institute Inc).

Results

Basic Characteristics of Subjects in the Suita Study

As shown in Table 1, age, SBP, DBP, BMI, total cholesterol, HDL cholesterol, IMT, maximum IMT in CCA, plaque score, CCA stenosis (≥50%), percentage of current smokers, percentage of current drinkers, prevalence of hypertension, prevalence of diabetes mellitus, and total plasma homocysteine levels were significantly higher in men than in women.

TABLE 1. Basic Characteristics of Subjects in Suita, a Japanese Urban Population

	Women (n=1693)	Men (n=1554)
Age, y	58.2±12.2	60.4±12.8*
SBP, mm Hg	126.7±21.1	129.5±19.3*
DBP, mm Hg	78.0±11.0	80.7±11.0*
BMI, kg/m^2	22.3±3.2	23.0±2.8*
Total cholesterol, mmol/L	5.6±0.9	5.2±0.8*
HDL cholesterol, mmol/L	1.6±0.4	1.4±0.4*
IMT, mm	0.83±0.12	0.88±0.14*
Maximum IMT in CCA, mm	1.02±0.29	1.15±0.45*
Plaque score, mm	2.14±2.99	4.13±4.69*
Stenosis (≥50%), %	1.0	4.6†
Current smokers, %	8.0	39.6†
Current drinkers, %	28.2	70.6†
Present illness		
Hypertension	21.3	25.6†
Hyperlipidemia	48.0	30.2†
Diabetes mellitus	3.3	7.9†
Myocardial infarction	0.5	1.7†
Ischemic stroke	0.8	2.5†
Total plasma homocysteine, μmol/L	10.7±3.0 (n=820)	13.3±4.2* (n=721)

Values are mean±SD or percentage.

Hypertension indicates SBP ≥160 mm Hg and/or DBP ≥95 mm Hg or antihypertensive medication; hyperlipidemia, total cholesterol ≥5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥7.0 mmol/L (126 mg/dL), nonfasting plasma glucose ≥11.1 mmol/L (200 mg/dL), or antidiabetic medication.

*P<0.05 between female and male by Student's t test.

†P<0.05 between women and men by χ^2 test.

Only the frequency of hyperlipidemia was significantly higher in women than in men.

C677T/MTHFR, Hypertension, and Plasma Homocysteine Levels

The frequencies of *C677T/MTHFR* in women were 37.5% for *CC*, 47.2% for *CT*, and 15.3% for *TT* genotypes, whereas those in men were 36.2% for *CC*, 47.8% for *CT*, and 16.0% for *TT* genotypes. There was no significant difference in allele frequencies between age groups (χ^2=1.07, df=2, P=0.59). The genotype distribution of *C677T/MTHFR* was not significantly deviated from Hardy-Weinberg's expectation in men or women. In women, SBP and DBP increased according to the number of *T677* alleles of *MTHFR*, but the association was not statistically significant. Only DBP in *TT* women was significantly higher in those with the *C677* allele after age adjustment. In the recessive model (*CT+CC* versus *TT*), however, *C677T/MTHFR* was significantly associated with the prevalence of hypertension, and the all adjusted OR for hypertension was 1.42 (95% CI, 1.01 to 1.99) in women (Table 2).

Figure 1 shows plasma total homocysteine levels according to genotype of *C677T/MTHFR* in men and women. Mean plasma total homocysteine levels in subjects with the *TT* genotype was significantly higher than that in subjects with the *CC* or *CT* genotype.

TABLE 2. ORs of Presence of Hypertension in Men and Women by *C677T/MTHFR*

	Dominant Model		Recessive Model	
	CC	*CT+TT*	*CC+CT*	*TT*
Women (n=1693)				
Hypertensive, %	19.5	22.4	20.7	24.7
All adjusted OR*	1	1.15 (0.88–1.49)	1	1.42 (1.01–1.99)†
Men (n=1554)				
Hypertensive, %	25.9	25.3	25.5	25.7
All adjusted OR*	1	0.93 (0.73–1.20)	1	1.00 (0.72–1.40)

*Conditional logistic analysis, adjusted for age, BMI, SBP, smoking, drinking, antihypertensive drug use, hypercholesterolemia, and diabetes.

†P<0.05 vs *CC* or *CC+CT* subjects.

Carotid Atherosclerotic Index and *C677T/MTHFR*

Carotid atherosclerotic indexes (IMT, maximum IMT in CCA, maximum IMT, and plaque score) were evaluated in men and women separately, according to *C677T/MTHFR* genotype (Table 3). In women with the *CT* genotype, age-adjusted IMT, maximum IMT in CCA, and all adjusted maximum IMT in CCA were significantly thicker than in those with the *CC* genotype. However, there was no difference between subjects with the *TT* and *CC* genotypes in any atherosclerotic indexes.

In contrast, *C677T/MTHFR* gave a significantly increased risk for stenosis (>50%) of CCA in women. In a recessive model (*CC*+CT versus *TT*), the all adjusted OR for stenosis (>50%) was 3.42 (95% CI, 1.23 to 9.53) in women and 1.41 (95% CI, 0.76 to 2.63) in men.

Partial correlation coefficients between plasma total homocysteine levels and carotid atherosclerotic index by *C677T/MTHFR* genotype are shown in Table 4. Positive relationships were found between plasma total homocysteine levels and IMT in men with the *CC* genotype and maximum IMT in CCA for men. These associations were stronger in men than in women.

Interaction Between *C677T/MTHFR* and Lifestyle on Carotid Atherosclerotic Index According to Sex

Figure 2 shows the association of IMT and maximum IMT in CCA with *C677T/MTHFR* according to smoking and drink-

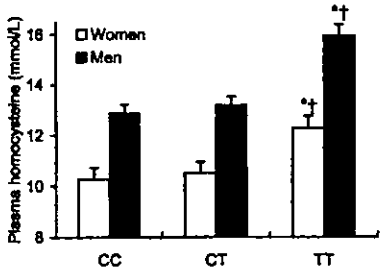

Figure 1. Plasma total homocysteine levels according to *C677T/MTHFR* by sex. Values are least-square mean±SE adjusted for age, BMI, smoking, drinking, antihypertensive drug use, hyperlipidemia, and diabetes. Bars indicate SE. *P<0.0001 vs CC subjects; †P<0.0001 vs CT subjects.

TABLE 3. Carotid Atherosclerotic Index in Men and Women by *C677T/MTHFR*

	MTHFR Genotype			
	CC	*CT*	*TT*	χ^2 *P*
Women (n=1693)				
IMT, mm				
Age adjusted	0.825±0.004	0.842±0.003‡	0.832±0.006	0.004
All adjusted*	0.861±0.009	0.874±0.009‡	0.866±0.010	0.030
Maximum IMT in CCA, mm				
Age adjusted	1.004±0.011	1.035±0.010†	1.023±0.018	0.122
All adjusted	1.075±0.026	1.100±0.025	1.094±0.029	0.231
Maximum IMT, mm				
Age adjusted	1.274±0.021	1.311±0.018	1.307±0.032	0.383
All adjusted	1.415±0.050	1.441±0.049	1.444±0.056	0.586
Plaque score, mm				
Age adjusted	1.990±0.108	2.259±0.096	2.141±0.169	0.178
All adjusted	2.915±0.262	3.114±0.254	3.026±0.293	0.369
Men (n=1554)				
IMT, mm				
Age adjusted	0.886±0.005	0.882±0.004	0.889±0.007	0.668
All adjusted	0.892±0.007	0.890±0.006	0.898±0.009	0.586
Maximum IMT in CCA, mm				
Age adjusted	1.162±0.020	1.140±0.017	1.144±0.030	0.713
All adjusted	1.173±0.027	1.163±0.026	1.165±0.036	0.916
Maximum IMT, mm				
Age adjusted	1.642±0.034	1.627±0.030	1.721±0.051	0.273
All adjusted	1.653±0.047	1.638±0.044	1.75±0.060	0.145
Plaque score, mm				
Age adjusted	4.201±0.178	4.010±0.155	4.308±0.268	0.550
All adjusted	4.413±0.246	4.215±0.232	4.625±0.316	0.363

*Values are least-square mean±SE adjusted for age, SBP, BMI, smoking, drinking, and medication (for hypertension, hyperlipidemia, or diabetes).
†$P<0.05$ vs *CC* subjects; ‡$P<0.005$ vs *CC* subjects.

ing status. In women with the *CC* or *CT* genotype, IMT in smokers was significantly higher than in nonsmokers. In women with the *TT* genotype, maximum IMT in CCA in smokers and drinkers was significantly higher than that in nonsmokers and nondrinkers, respectively (Figure 2–A2, $P<0.05$ for interaction; Figure 2–B2). In men with the *CC* or *TT* genotype, IMT and maximum IMT in CCA were significantly higher in smokers than in nonsmokers (Figure 2–A1).

TABLE 4. Partial Correlation Coefficient Between Plasma Total Homocysteine and Carotid Atherosclerotic Index by Sex and *C677T/MTHFR*

	CC	*CT*	*TT*
IMT			
Women	0.056 (0.334)	0.014 (0.784)	−0.027 (0.758)
Men	0.167 (0.001)	0.056 (0.300)	0.111 (0.253)
Maximum IMT in CCA			
Women	0.058 (0.398)	0.005 (0.935)	−0.098 (0.355)
Men	0.218 (0.003)	0.146 (0.016)	0.363 (0.002)

Figures in parentheses indicate *P* value adjusted for age, BMI, drinking, smoking, SBP, and medication for hypertension, hyperlidemia, and diabetes mellitus.

Discussion

The present study showed that the *TT* genotype of *C677T/MTHFR* was significantly associated with the prevalence of hypertension (OR, 1.15) and carotid stenosis (<50%) in women but not in men. In addition, the specific genotype of *C677T/MTHFR* affected maximum IMT in CCA in the interaction with smoking in women. These results show an association of *C677T/MTHFR* with BP and carotid atherosclerosis on the basis of gene and environmental interaction, which has not been previously reported.

Although previous studies showed that subjects with the *TT* genotype of *C677T/MTHFR* are associated with an increased risk of cardiovascular disease via an increase in plasma homocysteine levels,[2,6,20] the conclusion is still controversial.[7,14,15,21,22] The inconsistencies may be attributed to small sample size, combined-sex analysis, and no inclusion of lifestyle factors such as smoking and drinking. One should be aware that detecting gene and environmental interactions

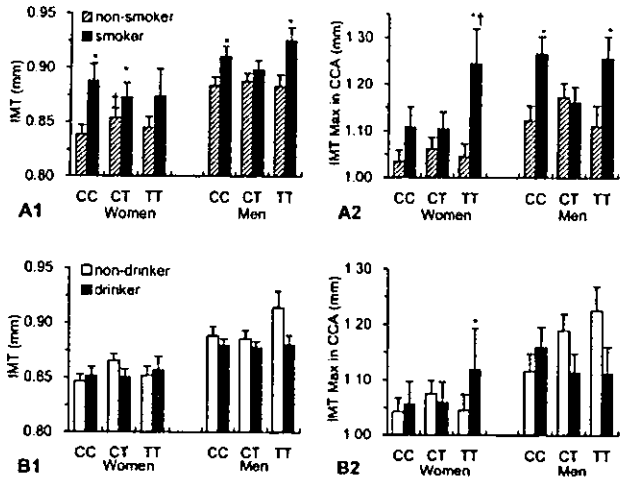

Figure 2. Association between *C677T/MTHFR* and carotid atherosclerotic indexes (IMT and maximum IMT in CCA) according to smoking (A) and drinking (B) status in men and women. Data are shown as the least-square mean±SE adjusted for age, BMI, SBP, smoking, drinking, and medication (for hypertension, hyperlipidemia, and diabetes). *$P<0.05$ vs nonsmokers (or nondrinkers) in subjects with the same genotype; †$P<0.05$ vs *CC* subjects with the same lifestyle (for smoking and drinking).

could require a substantially larger sample size than the sample size necessary for detecting genetic or environmental effects alone.[23] Thus, we examined the effect of *C677T/MTHFR* in a large general population with various phenotypes that included plasma homocysteine levels, atheromatous indexes, smoking and drinking status, and relevant basic characteristics.

It can be questioned why the *TT* genotype of *C677T/MTHFR* is not unequivocally associated with increased cardiovascular risk,[5] based on the argument that the gene is a strong predictor of hyperhomocysteinemia in general populations.[6,24,25] It could be attributed to the close relationship between plasma homocysteine levels and folate metabolism. Several reports revealed that plasma total homocysteine levels become elevated only in folate-deficient subjects with the *TT* genotype[7,25,26] and that the slope of regression lines relating total homocysteine to folate increases in the order of *CC*, *CT*, and *TT* genotypes.[15,24] In other words, if folate intake is sufficient, subjects with the *TT* genotype would not have increased risk of cardiovascular disease via hyperhomocysteinemia.

Under stratification by sex, we observed that the *TT* genotype was independently associated with DBP and carotid stenosis in women and showed a greater disadvantage in female smokers and drinkers. Even though homocysteine would injure the endothelium of small arteries at an early stage[27] and endothelial dysfunction plays a critical role in the early events of atherosclerosis,[28] we currently have no definitive answer to explain the results. However, it seems to be an important finding that most of the positive results in the present study were obtained only in women. As supporting data of our results, a female-specific significant association with the *TT* genotype was also reported in the predisposition to ischemic stroke[29] and asymptomatic carotid atherosclerosis.[30] Motti et al[31] reported that sex differentiation is independently associated with homocysteine. Plasma homocysteine levels are significantly higher in healthy men than in women, which is consistent with our results (Table 1). In addition, homocysteine levels are reported to be lower in premenopausal women than in men and postmenopausal women. Furthermore, a recent report suggested that total homocysteine levels were significantly correlated with fat-free mass and testosterone and inversely with estradiol. The sex difference with regard to total homocysteine levels was explained primarily by differences in fat-free mass but also by estradiol concentration. Those results might be a feasible explanation for the lack of association in men.[32] However, there was no association between *C677T/MTHFR* and carotid atherosclerosis in premenopausal and postmenopausal women (data not shown). This result suggests that estrogen might have a protective effect against homocysteinemia but not atherosclerosis via *C677T/MTHFR*. Indeed, previous reports did not find such a specific advantage in the relationship between *C677T/MTHFR* and coronary artery disease in young woman in a small Caucasian population.[33,34]

Disadvantages of our study design were that only half of the subjects had their total plasma homocysteine levels analyzed. This is not a serious limitation, however, because the association between *C677T/MTHFR* and plasma homocysteine levels has already been demonstrated in several large studies.[7,26] Another disadvantage was that we had no data on the physical activity and nutrition of the subjects, but these data were also supported by previous studies. The dietary intake of folate, vitamin B_6, and B_{12} is inversely (negatively) correlated with plasma homocysteine[35,36]; physical activity is also inversely associated with plasma homocysteine.[37] There is a need for additional prospective studies with data on relevant confounders that have sufficient power to examine the association between homocysteine concentration and stroke risk, whether linear or threshold, and to study interactions between homocysteine, other dietary markers, and established stroke risk factors such as smoking and hypertension. Similarly, the evidence linking hyperhomocysteinemia with hypertension is limited and inconsistent. Ultimately, the case for a causal role of elevated homocysteine levels in vascular disease, including hypertension and stroke, will depend on data from randomly controlled trials of homocysteine-lowering interventions.

In summary, the present study shows that the homozygous *T677* allele of *C677T/MTHFR* is a risk factor for hypertension and carotid stenosis in women. In addition, smoking increased IMT in CCA in women with the *TT* genotype. In the near future, physicians might use the genotypic data of *C677T/MTHFR* to modify their patients' lifestyles to prevent cardiovascular disease.

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Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women

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Objective To examine the relationship between the gene for haeme oxygenase (HO)-1 (*HMOX-1*) and human essential hypertension, because both the acute and systemic induction of *HMOX-1* have been suggested to attenuate vascular tone and blood pressure.

Methods We screened for sequence variations in *HMOX-1* and conducted an association study, using these polymorphisms, in a large cohort (1998 individuals) representing the general Japanese population.

Results We sequenced *HMOX-1* and found a *T(−413)A* polymorphism in the promoter region. The frequency of hypertensive individuals and the use of antihypertensive drugs were significantly greater in the *AA* genotype than in other genotypes among women: 45.5, 34.2, and 35.0% ($P = 0.0099$) and 23.4, 17.5, and 15.0% ($P = 0.038$), respectively, for the *AA*, *AT*, and *TT* genotypes, respectively. However, this association was not observed in men. Multiple logistic analyses indicated that the *T(−413)A* (*AA/TA+TT*) polymorphism, age, and body mass index affected the occurrence of hypertension in women. The odds ratio of the *AA* genotype for hypertension in women was 1.59 ($P = 0.0058$; 95% confidence interval 1.14 to 2.20). A luciferase reporter assay indicated that the *A* allele-promoter had eight-fold greater activity than the *T* allele promoter ($P < 0.01$).

Conclusions The *AA* genotype of *HMOX-1* is associated with an increased incidence of hypertension in women. Oestrogen attenuates vasoconstriction by increasing the expression of inducible nitric oxide synthase. As carbon monoxide, which is one of the products of HO-1, can attenuate nitric oxide-induced vasodilatation, a high expression of HO-1 may cause hypertension, especially in women. *J Hypertens* 21:1497–1503 © 2003 Lippincott Williams & Wilkins.

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Introduction

Haeme oxygenases, which are essential for haeme degradation, produce bile pigments, ferritin, and carbon monoxide [1]. Haeme oxygenases and carbon monoxide participate in the haemostatic control of cardiovascular functions, including the regulation of blood pressure [2].

Inhibition of haeme oxygenase (HO)-1 with metalloporphyrins is known to lead to increased blood pressure and augmented arterial neointimal development after balloon injury [3,4]. Enhancement of the HO-1 system with haeme oxygenase inducers decreased blood pressure and prevented neointimal development [3,5]. In contrast, transgenic mouse strains that chronically overexpress HO-1 site-specifically in vascular smooth muscle cells exhibited a significant increase in arterial blood pressure and reduced vasodilatory responses [6]. Thus it remains unknown whether chronic and systemic activation of HO-1 could affect vascular tone and blood pressure.

Carbon monoxide is well recognized as a physiologically important vasoactive substance. A previous in-vitro experiment showed that nitric oxide and carbon monoxide activated soluble guanylate cyclase by distinct mechanisms and that carbon monoxide is far less potent than nitric oxide [7]. Therefore, carbon monoxide may suppress the vasodilatory response to nitric oxide by competition, and thereby lead to an increase in arterial pressure when the concentration of carbon monoxide is not excessive. Even in greater concentrations, carbon monoxide begins to inhibit endothelium-dependent nitric oxide synthase (eNOS) activity and nitric oxide generation [8].

Recently, Johnson *et al.* [9] reported that increased concentrations of endogenous carbon monoxide contri-

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