の痴呆疾患との鑑別にも有用である。レビー小体を伴う痴呆（DLB）は臨床上しばしばアルツハイマー型痴呆との鑑別が困難となる。われわれはアルツハイマー型痴呆群と DLB 群の海馬ならびに扁桃体体積を計測比較し，海馬萎縮はアルツハイマー型痴呆群が DLB 群より強く，一方扁桃体の萎縮は 2 群間に差がないことを示した[14]。一方 Laakso らは前頭側頭型痴呆（FTD）群とアルツハイマー型痴呆群の間の海馬ならびに内嗅回の萎縮を定量的に検討し，アルツハイマー型痴呆では海馬は全長にわたって瀰漫性に萎縮している一方 FTD 群は海馬前方に萎縮が強く，萎縮のパターンに差があることを示している[15]。

2. 機能画像検査

痴呆疾患の診断に用いる機能画像検査としては，single photon emission computed tomography (SPECT) と positron emission tomography (PET) がある。機能画像検査は，形態異常より早く起こる機能異常を捉えることができ，初期診断には極めて有効な検査法である。

1）PET

PET とは陽電子を放出する核種（ポジトロン放出核種）で標識された薬剤を投与してその体内分布を PET カメラで画像化する診断法である。PET によるアルツハイマー型痴呆の研究については，1983 年，Frackowiac らが ^{15}O ガススタディにより痴呆の血流・酸素代謝率について報告して以来数多くの報告があるが，それらはいずれも頭頂側頭連合野ならびに前頭連合野の代謝低下を示す結果であった。この流れの中で Minosima らは，これまで注目されていなかった後部帯状回において，ごく初期のアルツハイマー型痴呆で糖代謝の低下がみられることを統計学的画像検査を行うことによって見いだした[16]。さらに Ishii らは，初期アルツハイマー型痴呆の後部帯状回では糖代謝のみならず血流低下も認められることを示し，この知見は SPECT を用いた診断にも応用されている[17]。

PET は SPECT と比較して定量性が優れており，脳血流量・代謝量を絶対値で測定して画像化できる。実際代謝量（酸素代謝，ブドウ糖代謝など）は SPECT で測定することはできず，これを生体で測定できるのは PET のみである。また PET は空間分解能，および感度において SPECT よりも優れている。これらの長所から痴呆の初期診断には SPECT よりも PET がより優れている。しかし PET はポジトロン放出核種の半減期が非常に短いため検査直前に合成して投与しなければならず，そのための大がかりな設備とスタッフが必要となる。そのため実際の臨床診断の目的で使用可能な施設は稀であり，通常は SPECT を用いている。

2）SPECT

SPECT はガンマ線放出核種で標識された薬剤を投与してその体内分布を断層像として画像化するものである。痴呆の検査に用いられる脳血流 SPECT 用の放射性薬剤は主に ^{123}I-IMP，^{99m}Tc-HMPAO，^{99m}Tc-ECD である。これらの薬剤は脳血流量に比例して脳内に分布するため，これを SPECT カメラで撮像して脳血流の分布画像を得ることができる。痴呆性疾患ではそれぞれ疾患に特異的な血流低下の分布パターンがあり，そのパターンの差によって鑑別診断を行う。

過去の SPECT 研究では，アルツハイマー型痴呆では頭頂・側頭葉の血流低下が認められることが報告されてきたが，病初期にこのような低下パターンを示すことは少ない。そこで PET で示された後部帯状回の血流低下を SPECT を用いて同定する試みがなされている。Kogure らは MMSE 25点以上の初期アルツハイマー型痴呆患者の脳血流量を ^{99m}Tc-ECD を用いて縦断的に検討し，初期アルツハイマー型痴呆患者群は正常被験者と比較して後部帯状回と楔前部の血流が優位に低下していることを報告した[18]。この結果は SPECT でも初期のアルツハイマー型痴呆の後部帯状回や楔前部の血流低下を捉えることが可能であることを示している。ただし，視覚的評価のみでこの部分の血流低下を捉えることは極めて難しく，Statistical Parametric Map（SPM）や NEUROSTAT などの統計学的処理画像を用いて，健常者群の血流分布との比較が必要となる[19]。この方法では読影者の習熟度や主観に影響されない客観的な診断ができるが，現時点でこの方法を用いて診断することができる施設は多くはない。

おわりに

今回紹介した MRI による volumetry，PET を用いた脳代謝の測定，PET や SPECT 画像の統計学的解析などはまだ診療に応用されていることは少ない。しかし抗痴呆薬の開発に伴う早期診断の必要性や，読影者の習熟度や主観に影響されない客観的な診断の可能性を考えればこれらの検査法が日常診療に浸透してくることは間違いないだろう。

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循環器疾患等総合研究事業

脳血管疾患の再発に対する高脂血症治療薬のHMGCoA阻害剤の予防効果に関する研究

(H14－効　果(生活)－023)
(H15－効　果(生活)－020)
(H16－循環器(生習)－003)

平成14年度～16年度　総合研究報告書

4/7

雑 誌 (II)

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雑　誌 (Ⅱ)

（平成14年度　つづき）

Original Article

A Single-Nucleotide Polymorphism in C-Type Natriuretic Peptide Gene May Be Associated with Hypertension

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We conducted an association study between genetic variants of C-type natriuretic peptide gene (*CNP*) and hypertension in a Japanese population. We found four genetic variants, two in the promoter region, one missense mutation, and one in the 3′-untranslated region (3′-UTR), and genotyped all four variants in 2,006 subjects recruited from the Suita study. One of the variants, G2628A in 3′-UTR, was found to be associated with blood pressure. Multiple logistic analyses indicated that the genotype of the G2628A polymorphism (GG=1, GA+AA=2) (p=0.0034), sex (p=0.0288), alcohol consumption (p=0.0002), age (p<0.0001), and body mass index (p<0.0001) were predictors of hypertension. The odds ratio of the GA+AA genotype over the GG genotype for hypertension was 1.40 (p=0.0034, 95% confidence interval (CI) 1.12–1.75). Multiple logistic analyses in a younger subpopulation aged below 65 years indicated that the odds ratio of the GA+AA genotype over the GG genotype for hypertension was 1.58 (p=0.0024, 95%CI 1.18–2.12). Thus, the *CNP* G2628A polymorphism made an even greater contribution to hypertension in the younger subpopulation.
(*Hypertens Res* 2002; 25: 727–730)

***Key Words*: genetic variants, blood pressure, epidemiology**

Introduction

Interactions between genetic and environmental factors are thought to play important roles in the pathogenesis of hypertension. The use of association studies in large epidemiological cohorts with a large number of single-nucleotide polymorphisms throughout a single gene or throughout the entire genome is a new strategy for identifying genes that contribute to high blood pressure (*1–3*). In the present study, we applied this strategy to the C-type natriuretic peptide (CNP) gene (*CNP*) to examine whether its genetic variants influence blood pressure.

The natriuretic peptides play important roles in cardiovascular homeostasis. A-type and B-type natriuretic peptides (ANP and BNP) are mainly produced in cardiac tissues and directly influence blood pressure and body fluid homeostasis (*4*). Although CNP is produced in endothelial cells, its level in the peripheral circulation is very low and does not appear to be associated with blood pressure status (*5*). On the other hand, the expression level of CNP in the central nervous system has been reported to be high (*6*), and intracerebroventricular CNP has been reported to lower blood pressure (*7*). These observations strongly suggest that CNP functions as a neuropeptide in regulating blood pressure and body fluid homeostasis. Thus, *CNP* is a candidate gene for human essential hypertension.

In the present study, we thoroughly searched for polymorphisms of *CNP* and performed an association study using a large epidemiological cohort. To our knowledge, this is the first report to investigate associations between *CNP* genetic variants and blood pressure.

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Table 1. Genetic Variants in *CNP*

Polymorphism	TaqMan probe	PCR primer
G733A	Fam-attgttcccacagaaggagttcaccagcgg	5′-ctctaggttcacgttcagccgg-3′
	Tet-attgttcccacagagggagttcaccagcg	5′-cctgtgaaagtgcacaggatactgg-3′
G1612C	Fam-cactgggaccctgctcgccct	5′-gcagctgggagagatgcatg-3′
	Tet-cactgggaccgtgctcgccct	5′-gagcagagtcacgggctcag-3′
G2347T	Fam-agattggcgcccccgccc	5′-gtcagaagaagggcgacaag-3′
	Tet-agattggcgaccccgcccc	5′-gttggctcctttgtatttgcg-3′
G2628A	Fam-ccgcccagccagccttcgga	5′-cctcaagctggaccgaatcg-3′
	Tet-cgcccagccggccttcgga	5′-cctagcacaacttgagcaaaggc-3′

The nucleotide numbers of polymorphisms are given according to the sequence in the GenBank database (accession No. E03598). G733A and G1612C are in the promoter region, G2347T is in exon 2 and shows an amino-acid change of Gly61Val, and G2628A is in the 3′-untranslated region of CNP mRNA.

Table 2. Linkage Disequilibrium among Polymorphisms

	G733A	G1612C	G2347T	G2628A
G733A		945**	118**	55**
G1612C			2	25**
G2347T				6*

The degree of linkage disequilibrium (LD) was estimated by contingency table analysis (Pearson). χ^2 values are shown. * $p=0.06$, ** $p<0.0001$.

Table 3. Characteristics of the Study Population

	Control	Hypertension
N (%male)	1,235 (46.4)	771 (50.5)
Age**	57.1 (0.3)	64.8 (0.4)
BMI**	22.3 (0.1)	23.4 (0.1)
Alcohol*	14.4 (0.8)	16.4 (0.8)
TChol*	208.2 (0.8)	213.3 (1.2)
TG**	119.2 (2.9)	138.2 (3.7)
HDL	58.8 (0.4)	58.1 (0.6)
MI (%)**	0.57	2.46
CVA (%)**	1.21	3.63
G733A (GG/GA/AA)	830/365/40	512/238/21
G1612C (GG/GC/CC)	1033/186/16	652/114/5
G2347T* (GG/GT)	1210/25	764/7
G2628A** (GG/AG/AA)	972/243/20	555/196/20

Values are expressed as the mean (SEM). BMI, body mass index (kg/m^2); Alcohol, alcohol consumption (ethanol mg/day); TChol, total cholesterol (mg/dl); TG, triglycerides (mg/dl); HDL, high density lipoprotein cholesterol (mg/dl); MI, myocardial infarction; CVA, cerebrovascular accident. * $p<0.05$, ** $p<0.01$.

Methods

Subjects

The selection criteria and design of the Suita study have been described previously (2). In the present study, subject information was made anonymous. 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 3,700 subjects, and the genotype of *CNP* was determined in 2,006 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

Genomic DNA from 24 subjects was used as a template for sequence analyses. The promoter (up to −1 kb) and coding regions (exons 1 and 2) were sequenced. The region of the promoter and exon 1 was amplified by the following primers: 5′-ggaagttgaccacctgtcacggct-3′ (570–594 according to GenBank accession No. E03598) and 5′-cttccctctctc ctgggtcctgc-3′ (1904–1882). The region of exon 1, intron 1, and exon 2 was amplified by the following primers: 5′-ctgcaaatggagttcccctgtg-3′ (1339–1360) and 5′-gaagccaggtgg gttcaaccag-3′ (2792–2770). Polymorphisms were determined by the TaqMan system. The primers and probes are summarized in Table 1.

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. Residuals of blood pressure values were calculated by adjusting for sex, age, and body mass index (BMI). Differences in numerical data among the groups were calculated by one-way analysis of variance (ANOVA) and the unpaired *t*-test. Differences in frequencies and the degree of linkage disequilibrium were tested by contingency table analysis.

Table 4. Characteristics of a Younger Subpopulation (<65 years)

	GG	GA	AA	*p*
N (%male)	981 (44.4)	272 (45.6)	27 (44.4)	ns
SBP	124.1 (0.6)	127.3 (1.2)	128.1 (3.7)	0.0344
DBP	79.4 (0.4)	81.4 (0.7)	80.1 (2.1)	0.0318
PR	67.1 (0.2)	67.0 (0.5)	66.9 (4.5)	ns
Age	52.9 (0.3)	53.4 (0.5)	54.9 (1.7)	ns
Alcohol	17.1 (0.8)	17.1 (1.6)	16.9 (4.5)	ns
BMI	22.8 (0.1)	23.2 (0.2)	23.0 (0.6)	ns
HTN (%)	27.7	38.2	44.4	0.0013
HTN medication (%)	10.9	12.9	14.8	ns

SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); PR, pulse rate per minute; HTN, presence of hypertension; HTN medication, medication for hypertension. Other abbreviations are the same as in Table 3.

Results

CNP Polymorphisms

We found 4 polymorphisms in *CNP*, which are summarized in Table 1. We found two polymorphisms in the promoter region, one polymorphism in the coding region (exon 2) that accompanied an amino acid change from Gly to Val at amino acid position 61, and one polymorphism in the 3′-non coding region. The pairwise linkage disequilibriums are summarized in Table 2. The genotype frequencies are given in Table 3.

Association Study

Table 3 shows characteristics of the study population. The GT genotype of the G2347T polymorphism is more frequent in hypertensives. The frequencies of the AG and AA genotypes in G2628A polymorphism are higher among hypertensives. Multiple logistic analyses which included sex, age, BMI, alcohol consumption, and genotype of *CNP* indicated that sex ($p=0.0288$), age ($p<0.0001$), BMI ($p<0.0001$), alcohol consumption ($p=0.0002$), and A2628G genotype (GG=1, GA+AA=2) ($p=0.0034$) were predictors of hypertensive status. The odds ratio of the GA+AA genotype of the G2628A polymorphism for hypertension was 1.40 (95% confidence interval (CI) 1.12–1.75) over the GG genotype.

It is generally accepted that genetic effects are more evident among younger subjects, whereas the phenotypes of older subjects are more strongly influenced by environmental factors. Therefore, we analyzed the effects of genotypes on blood pressure among younger subjects. Table 4 shows characteristics of the study population aged below 65 years according to the G2628A polymorphism. Multiple logistic analysis in this younger subpopulation indicated that age ($p< 0.0001$), BMI ($p< 0.0001$), alcohol consumption ($p=0.0029$), and A2628G genotype (GG=1, GA+AA=2) ($p=0.0024$) were predictors of hypertensive status. The odds ratio of the GA+AA genotype of the G2628A polymorphism for hypertension was 1.58 (95% CI 1.18–2.12) over the GG genotype. Thus, the *CNP* G2628A polymorphism made an even greater contribution to hypertension in the younger subpopulation.

Discussion

CNP is abundantly expressed throughout the brain, with particularly high concentrations found in the anterior pituitary (*6*). Since the receptor for CNP with guanylyl cyclase activity (natriuretic peptide receptor B; NPRB) is abundant in tissues of neural origin and is scarce in peripheral vasculature, CNP has less of a direct peripheral depressor effect than ANP and BNP (*8, 9*). However, intracerebroventricular infusion of CNP has been reported to lower blood pressure and aldosterone secretion (*7*). Therefore, it is possible that genetic variations of *CNP* may influence blood pressure through their effects on the central nervous system.

In terms of salt sensitivity, we have studied the influence of genetic variants of *SCNN1A*, *SCNN1G* and *CYP11B2* on blood pressure in the same study population (*10–12*). Our results showed that a genetic variant of *SCNN1A* had a significant influence on blood pressure. In future studies, it would thus be intriguing to study the relationship between the effectiveness of diuretic therapy and genetic variants of *SCNN1A* and *CNP*.

In the present work, we studied 4 polymorphisms of *CNP*, two in the promoter region, one in the coding region that is accompanied by an amino-acid change, and one in the 3′-untranslated region (3′-UTR). The missense mutation is outside the loop structure that is very important for biological activity (*4*), and may not have biological significance. Only the G2628A genotype in 3′-UTR was associated with blood pressure. We speculated that this 3′-UTR polymorphism may influence the stability of CNP mRNA (*13*). We constructed artificial luciferase genes which had CNP 3′-UTRs. Transient expression analyses indicated that this 3′-UTR poly-

morphism had no significant influence on luciferase activity, thereby dismissing the hypothesis described above (data not shown). The biological significance of this polymorphism is unclear at present. Variations in linkage disequilibrium with this 3′-UTR polymorphism may be more important and may have biological significance.

CNP has been reported to be located at chromosome 2q24-qter. Four other genes with unknown functions are reported to exist within 50 kb of *CNP* (contig NT_022157.7). The distance for tight linkage disequilibrium may vary according to the chromosomal region and race, and may be beyond 50 kb (*14*). In this sense, it may be necessary to sequence and genotype a wider range of this chromosomal region to identify the genetic variations that truly influence blood pressure.

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Letter to the editor

A GPVI polymorphism is a risk factor for myocardial infarction in Japanese

Glycoprotein (GP) VI plays an important role in platelet activation and aggregation. A polymorphism in the GPVI gene has been reported to confer an increased risk of myocardial infarction [1]. In the present study, we assessed whether variation in the GPVI gene was associated with myocardial infarction in Japanese. Table 1 summarizes variations in the GPVI gene. The T3147C, G3179A, G1040A, T661C, and G644477T polymorphisms (#1–5) were in tight linkage disequilibrium. The T2419T, A763G, C645213T, A535T polymorphisms were also in tight linkage disequilibrium (#6–9). We selected the C645213T and G644477T polymorphisms as representatives of each group. The study population consisted of 1080 control subjects and 376 subjects with myocardial infarction. The control subjects, who were free from ischemic heart diseases and cerebrovascular accidents, were randomly selected from the Suita study [2]. Patients with myocardial infarction were recruited from the outpatient clinic of the National Cardiovascular Center. Informed consent on genetic analyses was obtained from all of the subjects. The clinical characteristics of the study subjects are summarized in Table 2. The C645213T polymorphism, but not G644477T, was associated with myocardial infarction. The frequency of the C allele in the C645213T polymorphism was significantly higher in subjects with myocardial infarction than in control subjects. Multiple logistic analyses indicated that the C645213T (CC/CT/TT) polymorphism, diabetes mellitus, sex current smoking status, and body mass index (BMI) affected the occurrence of myocardial infarction. The odds ratio of the CC allele for myocardial infarction was 2.34 ($P = 0.0166$, 95% CI: 4.68–1.15). Thus, a variation in the GPVI gene is a risk factor for myocardial infarction in Japanese. Whether, this polymorphism is associated with functional changes in platelet function remains to be determined.

Table 2
Characteristics of subjects

	Control	MI	P
N	1080	376	
Male (%)	48.5	84.6	< 0.0001
Age	59.7 (0.3)	59.0 (0.6)	Ns
DM (%)	4.0	44.7	< 0.0001
Smoking (%)	26.0	55.1	< 0.0001
HDL chol (mg/dl)	59.3 (0.5)	44.1 (0.8)	< 0.0001
T-Chol (mg/dl)	211.2 (1.0)	207.0 (1.8)	0.0381
Triglycerides (mg/dl)	126.6 (2.7)	144.4 (4.7)	0.01
BMI (kg/m^2)	22.6 (0.1)	23.7 (0,2)	< 0.0001
GPVI G644477T (TT/TG/GG)	1/37/1042	0/15/361	ns
GPVI C645213T (CC/CT/TT)	31/347/702	21/129/226	0.0352

Values are expressed as mean (S.E.M.). DM, presence of diabetes mellitus; Smoking, current smoking habit; HDL Col, HDL cholesterol; T-Chol, total cholesterol; BMI, body mass index.

Table 1
Summary of SNPs in GP6 gene

GP6		GenBank (NT_011225)	Amino acid	Region	Allel frequency
19q13.4	1	A668445G		Promoter	T = 98% C = 2%
	2	C668413T		Promoter	G = 98% A = 2%
	3	C657112T	Ser192Ser	Exon4	C = 98% A = 2%
	4	A654727G	Ser219Pro	Exon5	T = 98% C = 2%
	5	G644477T	His322Asn	Exon8	C = 96% A = 4%
	6	A669173C		Promoter	T = 77% G = 23%
	7	C648167T	Lys237Glu	Exon6	A = 82% G = 18%
	8	C645213T	Thr249Ala	Exon7	A = 83% G = 17%
	9	A644491T	Gln317Leu	Exon8	A = 84% T = 16%

The polymorphism sites are described according to the GenBank numbering (ContigNT_025920.7).

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Epsilon 4 allele of apolipoprotein E gene associates with lower blood pressure in young Japanese subjects: The Suita Study

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Objectives **The apolipoprotein ε4 allele (*APOE/ε4*) increases plasma cholesterol level and the risk for the late onset type of Alzheimer's disease. However, the correlation between hypertension and *APOE/ε4* has not yet been clarified. To examine the *APOE/ε4* effect in the general population of Japan, we performed a large genetic epidemiological survey (the Suita Study).**

Design and methods **The Suita Study was a cohort study based on a random sample of 14 200 Japanese residents of Suita city. Subjects who gave informed consent for genetic analysis were recruited in the current study ($n = 3997$). *APOE* polymorphism was clearly determined by the TaqMan polymerase chain reaction method.**

Results **Subjects with *APOE/ε4* were significantly ($P < 0.03$) more frequent (19.7%) in normotensives than in hypertensives (16.9%), the estimated odds ratio for hypertension (with *APOE/ε4* versus without *APOE/ε4*) being 0.83 [95% confidence interval (CI), 0.70–0.98]. The significance of the association (OR = 0.64; 95% CI, 0.48–0.86) was increased in young subjects (≤60 years old) but disappeared in old subjects. *APOE/ε4* also significantly contributed to a 2.9% increase of total cholesterol, 11.8% increase of triglyceride and 3.2% of decrease of high-density lipoprotein-cholesterol.**

Conclusions **We concluded that *APOE/ε4* was associated with an increase of plasma lipid levels and with a decrease of systolic blood pressure. The final conclusion on whether *APOE/ε4* contributes to the risk for cardiovascular disease will be clarified by analysis of the cumulative incidence, which will be obtained in the future Suita Study.**
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Introduction

Apolipoprotein E (apoE), a main apoprotein of the chylomicron, binds to a specific receptor on liver cells and peripheral cells. The three major isoforms of human apolipoprotein E (apoE2, E3, and E4) are coded for by three alleles (*ε2*, *3*, and *4*) at the structural locus in the apolipoprotein E gene (*APOE*) [1]. At codon 112/158, apoE2, E3, and E4 contain cysteine/cysteine, cysteine/arginine, and arginine/arginine, respectively. These *APOE* polymorphisms have been associated with risk of elevated serum lipids [2,3], coronary artery disease [4], and Alzheimer's disease [5,6]. However, even if it is commonly accepted that the *APOE/ε4* allele increases low-density lipoprotein (LDL)-cholesterol level and predisposition to Alzheimer's disease, the precise mechanism of its involvement in the genetic predisposition to cardiovascular disease has not yet been clarified. Especially, the obtained results concerning the association between the *APOE* polymorphism and stroke risk are inconsistent, suggesting that the *APOE* polymorphism is not only a modifier of lipid metabolism. On the other hand, Kimura *et al.* [7] reported that the survival rate of diabetic patients with renal disease in *APOE/ε4* carriers was higher than that in non-carriers. In their reports, the *APOE* polymorphism and hypertension were identified as independent risk factors for the progression to renal failure. Their results suggested that the *APOE* polymorphism is associated with the progression of diabetic nephropathy, and the presence of the *APOE/ε4* allele is a protective factor and other alleles are risk factors. To clarify the effect of *APOE* polymorphisms

on cardiovascular risk, we examined precisely their effect in a large Japanese general population.

Methods

Study population

The Suita Study was based on a random sample of 14 200 Japanese urban residents of Suita City, which is located in Osaka prefecture, the second largest urban area in Japan. Participants between the ages of 30 and 79 years were selected at random from the municipality population registry, and stratified by sex and age groups of 10 years [8]. Basic sampling of the population started in 1989 with a cohort study base, and 51.7% ($n = 7347$) of the subjects had paid an initial visit to the National Cardiovascular Center by February 1997. In addition to performing routine blood examinations, we extracted DNA from an extra 5 ml of blood withdrawn from those who visited the National Cardiovascular Center between May 1996 and February 1998. All participants were Japanese, and only those who gave informed consent for genetic analysis of 13 genes including *APOE* and storage of a DNA sample were enrolled in the present study.

Blood pressure measurement and criteria for hypertension

After more than 10 min of rest, systolic and diastolic blood pressure were measured twice. According to the recent criteria of the sixth report of the Joint National Committee on Prevention, Detection, and Treatment of High Blood Pressure (JNC/VI) [9], hypertension was defined as a mean systolic blood pressure (SBP) of ≥140 mmHg, a mean diastolic blood pressure of ≥90 mmHg, or current administration of antihypertensive medication. The remaining population was simply defined as normotensive. Family history of hypertension was defined as having a father, mother or sibling with a history of hypertension.

Determination of genotypes according to APOE/ε2, ε3, ε4 allele combinations

To deal with the 4013 samples, we used the TaqMan polymerase chain reaction (PCR) method [10]. The following primers and probes were included in the reaction: FC2 forward primer, 5′-CGG ACA TGG AGG ATG TGC-3′; FT3 forward primer, 5′-GCG GAC ATG GAG GAT GTG T-3′; R1 reverse primer, 5′-CTC GCG GAT GGC GCT GA-3′; *ε2* allele specific probe (158TF), 5′-Fam-CAC TGC CAG GCA CTT CTG CA-Tamra-3′; *ε3, 4* allele specific probe (158CT), 5′-Tet-CTG CCA GGC GCT TCT GCA-Tamra-3′. PCR was carried out using a Gene Amp 9700 (Applied Biosystems, Inc., Foster City, California, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 60 s. During the PCR cycles, two TaqMan probes hybridize competitively to a specific sequence of the target DNA, and the reporter dyes separate from the quencher dye, resulting in an increase of fluorescence of the reporter. The fluorescence level of PCR products was measured using an ABI PRISM 7200 or 7900 Sequence Detector (Applied Biosystems), resulting in clear identification of six pairs of *APOE/ε2, 3,* and *4* alleles. As the quality control of TaqMan PCR method, we determined *APOE* genotype of volunteers ($n = 212$), with informed consent, using both TaqMan and classical PCR-RFLPs (restriction fragment length polymorphisms) and confirmed identical results between them.

Statistical analysis

All statistical analyses were conducted using Stat View 4.5J (SAS Institute Inc., Cary, North Carolina, USA) and JMP 3.1.5 (SAS Institute Inc.). Differences in genotype or allele between normotensives and hypertensives were examined by chi-squared analysis. The association between the *APOE* polymorphisms and clinical variables was examined by one-way analysis of variance. We assessed the quantitative effects of covariates by multiple logistic regression analysis using JMP.

Results

Study population

There was no significant difference in age, sex or blood pressure between those who participated in the genetic analysis and those who did not. From the 3997 subjects, 1518 hypertensives and 2479 normotensives were defined according to the criteria described above. In the comparison of characteristics between hypertensives and normotensives, age, percentage of males, body mass index (BMI), family history of hypertension (FH), alcohol consumption, past smoking habit, systolic blood pressure (SBP), diastolic blood pressure (DBP), total cholesterol (T-chol), triglyceride (TG), fasting plasma glucose (FPG) and creatinine were significantly higher in hypertensives (Table 1). In contrast, current smoking habit and HDL-cholesterol (HDL-chol) level were significantly lower in hypertensives (Table 1).

APOE polymorphism and hypertension

TaqMan PCR clearly detected the genotype of *APOE* and the obtained allele frequency of *APOE/ε2*, *ε3*, and *ε4* was 0.05, 0.85, and 0.10, respectively. The genotype distribution significantly satisfied Hardy–Weinberg's expectation (*ε2*: $\chi_1^2 = 1.11$, $P = 0.29$; *ε3*: $\chi_1^2 = 0.003$, $P = 0.95$; *ε4*: $\chi_1^2 = 0.17$, $P = 0.18$). *APOE/ε4* allele distribution was significantly different ($P < 0.02$) between hypertensive and normotensive subjects (Table 2). Marginal significant difference ($P = 0.067$) was observed in *APOE/ε3* allele distribution, while *APOE/ε2* allele frequency was similar in hypertensives and normotensives (Table 2). The prevalence of hypertension significantly ($P < 0.02$) decreased according to the number of *APOE/ε4* alleles (Fig. 1). In subjects with the *ε4* allele, the calculated odds ratio for hypertension was 0.83 (95% confidence interval (CI), 0.70–

Table 1 Clinical features of study subjects

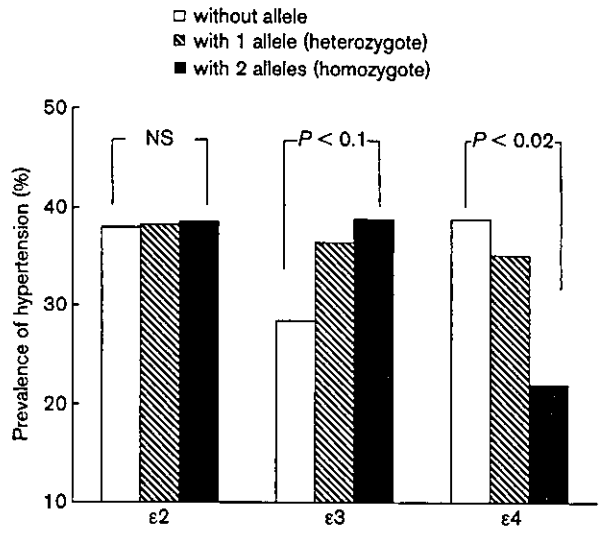
Variable	Hypertensives	Normotensives	*P* value
Number of subjects	1518	2479	
Age (years)	65 ± 10	57 ± 12	<0.0001
Sex (% male)	50	46	0.004
BMI (kg/m^2)	23 ± 3.2	22 ± 2.8	<0.0001
FH (%)	41	32	<0.0001
Drinking habit (%)	47	48	NS
Alcohol consumption (ml/day)	13 ± 24	11 ± 21	0.003
Smoking habit (%)			
Current	19	26	<0.0001
Past	22	15	<0.0001
Never	60	59	NS
SBP (mmHg)	149 ± 16	117 ± 12	<0.0001
DBP (mmHg)	89 ± 10	75 ± 7.9	<0.0001
T-chol (mmol/l)	5.5 ± 0.85	5.4 ± 0.85	<0.0001
TG (mmol/l)	1.6 ± 1.2	1.3 ± 0.95	<0.0001
HDL-chol (mmol/l)	1.6 ± 0.17	1.5 ± 0.18	0.0003
FPG (mmol/l)	5.5 ± 1.1	5.3 ± 1.0	<0.0001
Creatinine (μmol/l)	66 ± 26	62 ± 15	<0.0001

Variables are expressed as mean ± standard deviation.
FH, Family history of hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; T-chol, total cholesterol; TG, triglyceride; HDL-chol, HDL cholesterol; FPG, fasting blood glucose.
NS, no significant difference between hypertensives and normotensives.

Table 2 Genotype and ε2, 3, 4 allele distribution of *APOE* polymorphism in hypertensive and normotensive subjects

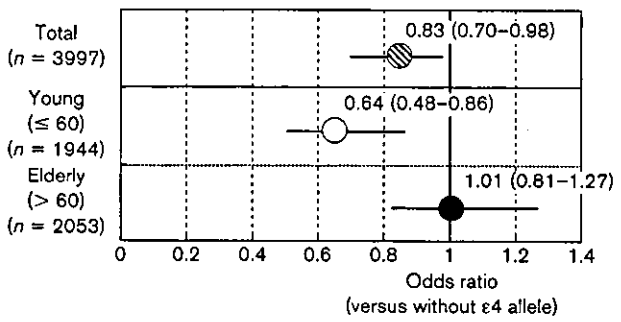
APOE Genotype	Number of ε2 alleles	Hypertensives *n*	Hypertensives %	Normotensives *n*	Normotensives %
ε3/ε3, ε3/ε4, ε4/ε4	0	1371	90.3	2242	90.4
ε2/ε3, ε2/ε4	1	142	9.4	229	9.2
ε2/ε2	2	5	0.3	8	0.3
		$\chi^2 = 0.017$, $P = 0.99$			
Genotype	Number of ε3 alleles				
ε2/ε2, ε2/ε4, ε4/ε4	0	25	1.7	63	2.5
ε2/ε3, ε3/ε4	1	367	24.2	640	25.8
ε3/ε3	2	1126	74.2	1776	71.6
		$\chi^2 = 5.4$, $P = 0.067$			
Genotype	Number of ε4 alleles				
ε2/ε2, ε2/ε3, ε3/ε3	0	1262	83.1	1990	80.3
ε2/ε4, ε3/ε4	1	247	16.3	457	18.4
ε4/ε4	2	9	0.6	32	1.3
		$\chi^2 = 8.3$, $P = 0.016$			

Fig. 1



Prevalence of hypertension and *APOE* genotype. The significance of the difference among the three groups was examined by ANOVA (analysis of variance). NS, there was no significant difference among three groups: subjects without the ε2 allele, with a single ε2 allele and with two ε2 alleles.

Fig. 2



Effect of *APOE/ε4* allele on hypertension in young and elderly subjects. The odds ratio of the risk for hypertension was calculated for the subject with the ε4 allele versus the subject without an ε4 allele.

0.98). Dividing the subjects into two groups by age, the significance was strongly enhanced in young subjects (≤60 years old) and the calculated odds ratio was only 0.64 (0.48–0.86) (Fig. 2). The calculated odds ratio was similar in young subjects between males (0.66) and females (0.59). The attributable risk of lack of *ε4* allele for hypertension was 0.118 in the whole population and 0.294 in young subjects. The positive association with hypertension remained after adjustment for age and sex ($P < 0.03$), and after full adjustment for confounding factors (age, sex, BMI, smoking habit, alcohol consumption, T-chol, TG, HDL-chol, creatinine, and FPG) ($P < 0.05$). The APOE ε4 allele was also significantly associated with systolic blood pressure ($P < 0.013$), pulse pressure ($P < 0.02$) and heart rate ($P < 0.045$), but not with diastolic blood pressure (Table 3).

APOE polymorphism and plasma lipid levels

The *APOE/ε4* allele was significantly associated with an increase of total cholesterol ($P < 0.0001$) and triglyceride ($P = 0.0002$) levels and with a decrease of HDL-cholesterol level ($P = 0.003$) (Table 3). *APOE/ε4* increased total cholesterol by 0.16 mmol/l and triglyceride by 0.16 mmol/l, and decreased HDL-cholesterol by 0.05 mmol/l. However, *APOE/ε4* was not associated with any other clinical parameters.

Table 3 Contribution of *APOE/ε4* allele to blood pressure and lipid levels

APOE genotype	ε4 allele (+)	ε4 allele (−)	*P* value
Number of subjects	745	3252	
SBP (mmHg)	127.6 ± 19.6	129.6 ± 20.7	<0.013
DBP (mmHg)	79.6 ± 10.4	80.3 ± 11.2	NS
Pulse pressure (mmHg)	47.9 ± 14.7	49.3 ± 14.9	<0.02
Heat rate (beats/min)	66.6 ± 8.01	67.3± 8.26	<0.045
T-chol (mmol/l)	5.55 ± 0.86	5.39 ± 0.85	<0.0001
TG (mmol/l)	1.54 ± 1.31	1.38 ± 1.00	0.0002
HDL-chol (mmol/l)	1.50 ± 0.41	1.55 ± 0.41	0.003

Variables are expressed as mean ± standard deviation. SBP, systolic blood pressure; DBP, diastolic blood pressure; T-chol, total cholesterol; TG, triglycerides; HDL-chol, high-density lipoprotein cholesterol.

Discussion

We have performed a large genetic epidemiological study of the Japanese general population and obtained the following results. (1) The estimated risk for hypertension in young subjects (≤60 years old) with the *APOE/ε4* allele was reduced by 36% compared with young non-carriers of *APOE/ε4*. (2) *APOE/ε4* carrier status versus non-carrier status was associated with a 2.9% increase of total cholesterol level, 11.8% increase of triglyceride level and 3.2% decrease of HDL cholesterol level.

Three common alleles of *APOE* have been stated as modulators of the lipid profile or as genetic predisposing factors for Alzheimer's disease. It has been considered that the *APOE/ε3* and *ε4* alleles increase plasma LDL-cholesterol level, suggesting that the *APOE/ε3* and *ε4* alleles are risk factors for atherosclerosis [11]. Therefore, gene polymorphism of *APOE* plays a key role in the development of cardiovascular disease [12], but the results obtained concerning *APOE* genotype and cardiovascular complications remain controversial. For cardiovascular disease, positive results for the association between coronary artery disease (CAD) and *APOE/ε4* allele have been frequently published [13]. A meta-analysis estimated that the odds ratio for CAD is 1.26 (95% CI, 1.13–1.41) in subjects with *APOE/ε4* [14]. In contrast, the association between *APOE* polymorphism and stroke has not been clarified. Kokubo *et al.* [15] recently reported, from genetic epidemiological results in a Japanese rural population, that the risk for cerebral infarction was increased in *APOE/ε2* carriers (odds ratio (OR) = 1.7) but not in *APOE/ε4* carriers (OR = 0.9). Despite the certain atherogenic effect of *APOE/ε4*, it is not clear why the results of investigations concerning the association between *APOE/ε4* and cerebral infarction have been inconsistent [16]. Our findings suggest a feasible explanation for the inconsistent results. *APOE/ε4* increased plasma LDL-cholesterol and triglyceride levels, and decreased HDL-cholesterol and blood pressure, with the result that the prevalence of hypertension was lower in young but not in elderly subjects in *APOE/ε4* carriers. In addition, Hanon *et al.* [17] examined the association between *APOE* polymorphism and arterial wall thickness, and found that *APOE/ε2* was related to hypertrophy of the carotid arterial wall despite having a favorable effect on the lipid profile. These results suggest that the unfavorable lipid profile did not simply reflect the progression of arteriosclerosis. Atherosclerosis in larger arteries is related mainly to lipid and blood pressure levels. Kessler [16] and Kokubo [15] reported that *APOE/ε4* was associated with atherothrombosis but not with lacunar infarction and cortical infarction. Therefore, we hypothesized that *APOE/ε4* enhances atherosclerosis in larger arteries via an unfavorable lipid profile, whereas it attenuates the risk for ischemia in smaller arteries via lower blood pressure. Another explanation for positive association in young subjects is that *APOE/ε4* might be associated with hypertensive death. However, it is difficult to arrive at a logical conclusion based on the current investigation.

On the other hand, the possible association with blood pressure only in systole, suggesting that the *APOE* polymorphism might be involved in modulation of peripheral arteriole resistance or cardiac output. Precise inspection of the results revealed that heart rate of *APOE/ε4* carriers was also lower than that of non-carriers ($P = 0.058$), suggested the possibility that sympathetic activity might be decreased in *APOE/ε4* carriers. A paper showed [18] that APOE elicits an increase in intracellular calcium levels and subsequent death of embryonic rat hippocampal neurons in culture, and similar effects on calcium were found when the APOE peptide was applied to chick sympathetic neurons. Of course, it is nothing more than a collateral evidence of our results but it can be a feasible explanation for the positive association with systolic blood pressure only in young subjects.

Previous investigations have always dealt with *APOE/ε4* as a 'risk' factor. However, the current study showed a protective effect against hypertension in young subjects, suggesting that the risk of genetic polymorphism should be discussed in the context of interaction with environmental factors, such as aging, excess salt intake, and obesity. Taking *APOE/ε4* carriers as an example, a genetically high cholesterol level and lower blood pressure offset each other in stroke susceptibility [19]. Presumably, an unfavorable lipid profile gradually reduces the effect of lower blood pressure, with the result that *APOE/ε4* shows no effect on hypertension in elderly subjects. Undoubtedly there are some study limitations in this cross-sectional analysis. The final conclusion on whether *APOE/ε4* modulates susceptibility to cardiovascular disease will be clarified in the analysis of cumulative incidence to be obtained in the future Suita Study.

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Association Between *SAH*, an Acyl-CoA Synthetase Gene, and Hypertriglyceridemia, Obesity, and Hypertension

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Background—The SA gene (*SAH*) has been isolated by differential screening from a genetically hypertensive rat strain as a candidate gene that may contribute to hypertension. Recently, the SA protein has been reported to be highly homologous to bovine xenobiotic–metabolizing medium-chain fatty acid:CoA ligase.

Methods and Results—To clarify the pathophysiological significance of *SAH*, we searched for polymorphisms of human *SAH* and performed association studies using a large cohort (4000 subjects) representing the general population in Japan. We found 2 polymorphisms in the promoter region and single-nucleotide polymorphisms in introns 5, 7, and 12 and exon 8. One of the variants, an A/G polymorphism in intron 12, just 7 bp upstream from exon 13, strongly affected plasma triglyceride, plasma cholesterol, body mass index (BMI), waist-to-hip ratio (W/H), and blood pressure status. The effect of this genotype on blood pressure seems to be conveyed through its effects on BMI and W/H. Transient expression of the SA protein in mammalian cells confirmed that it is expressed in mitochondria and has medium-chain fatty acid:CoA ligase activity. The A/G polymorphism was found to be associated with the expression level of SA mRNA in peripheral mononuclear cells in vivo.

Conclusions—The G allele of *SAH* was found to be associated with multiple risk factors, including hypertriglyceridemia, hypercholesterolemia, obesity, and hypertension. This observation should open a new area for future research in multiple-risk-factor syndromes. (***Circulation.* 2002;105:41-47.**)

Key Words: lipids ■ obesity ■ hypertension ■ genetics

Interactions between genetic and environmental factors are thought to play important roles in the pathogenesis of common diseases. The use of association studies in large epidemiological cohorts with a large number of single-nucleotide polymorphisms throughout a single gene or throughout the genome is a new strategy for identifying genes that contribute to common diseases.[1–3] In the present study, we applied this strategy to the SA gene (*SAH*) to examine whether it influences blood pressure.

SAH was isolated by differential screening from a genetically hypertensive rat strain, the spontaneously hypertensive rat.[4] The expression of *SAH* in the kidneys of the spontaneously hypertensive rat is markedly higher than that in kidneys of a normotensive control strain, the Wistar-Kyoto rat. *SAH* is expressed mainly in proximal tubules and hepatocytes.[5] Recently, SA protein has been reported to be significantly homologous to bovine xenobiotic–metabolizing medium-chain fatty acid (MCFA):CoA ligase.[6] Analyses of several F2 rat cohorts[7,8] and the establishment of several congenic rat strains[9,10] have confirmed that the SA gene locus contributes to blood pressure regulation in the rat. Thus, *SAH* is a candidate gene for human essential hypertension.

Several small-scale association studies, however, have given conflicting results regarding whether *SAH* contributes to hypertension in humans.[11,12] To clarify this issue, we thoroughly searched for polymorphisms of human *SAH* and performed association studies using a large cohort (4000 subjects) representing the general population in Japan.

Methods

Subjects

The selection criteria and design of the Suita Study have been described previously.[13] The genotype of *SAH* was determined in 4039 subjects (written informed consent was obtained).

The characteristics of the subjects analyzed in the present study are summarized in Table 1 according to the A/G genotype in intron 12. 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 using kits (L-TC Wako, Wako Pure Chemical, and Clinimate TG-2, Daiichi Chemicals).

DNA Studies

Genomic DNA from 32 subjects was used as a template for sequence analyses. The promoter region (up to −2.1 kb), exons 1 to 14, and

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TABLE 1. Characteristics of the Study Population (Total)

	AA	AG	GG	P_1	P_2
n	3904	151	4		
Male, %	47.3	45.7	50.0	NS	NS
Age, y	59.8 (0.2)	60.8 (1.0)	50.0 (6.0)	NS	NS
BMI, kg/m^2	22.63 (0.05)	23.39 (0.24)	24.02 (1.50)	0.0066	0.0017
AHT, %	16.8	23.8	0.0	0.0444	0.0434
SBP, mm Hg	129.0 (0.3)	133.2 (1.7)	124.0 (10.3)	0.0502	0.0225
DBP, mm Hg	80.1 (0.2)	82.5 (0.9)	79.8 (5.5)	0.0251	0.0076
HR, bpm	67.1 (0.1)	68.8 (0.7)	66.0 (4.1)	0.0456	0.0168
W/H	0.898 (0.001)	0.914 (0.005)	0.900 (0.034)	0.0187	0.0052
Plasma cholesterol, mmol/L	5.42 (0.01)	5.53 (0.19)	5.73 (0.43)	NS	0.0974
HDL cholesterol, mmol/L	1.54 (0.01)	1.54 (0.03)	1.55 (0.20)	NS	NS
Triglycerides, mmol/L	1.39 (0.02)	1.59 (0.09)	1.83 (0.52)	0.0571	0.0187
Blood glucose, mmol/L	5.36 (0.02)	5.62 (0.08)	5.27 (0.51)	0.0429	0.0155
%TG/T	0.5	0.7	0.0	NS	NS
%Chol/T	8.3	4.6	0.0	NS	NS
%HTN	37.6	47.9	25.0	0.0371	0.0166
%CVA	1.9	1.3	0.0	NS	NS
%OMI	1.8	2.7	0.0	NS	NS
Res. SBP, mm Hg	−0.13 (0.30)	3.2 (1.51)	2.0 (9.30)	0.0915	0.0290
Res. DBP, mm Hg	−0.09 (0.17)	2.29 (0.88)	0.83 (5.41)	0.0293	0.0082
Res. BMI, kg/m^2	−0.03 (0.05)	0.74 (0.24)	1.34 (1.49)	0.0053	0.0013
Res. W/H	−0.001 (0.001)	0.013 (0.005)	0.020 (0.032)	0.0205	0.0054
Res. TG, mmol/L	−0.01 (0.02)	0.19 (0.08)	0.44 (0.51)	0.0453	0.0146
Res. Chol, mmol/L	0.00 (0.01)	0.09 (0.07)	0.43 (0.41)	NS	NS

AHT indicates percentage of subjects with antihypertensive treatment; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BS, blood glucose; %TG/T, percentage of treated subjects with hypertriglyceridemia; %Chol/T, percentage of treated subjects with hypercholesterolemia; %HTN, percentage of subjects with hypertension; %CVA, percentage of subjects with cerebrovascular accident; %OMI, percentage of subjects with old myocardial infarction; Res. SBP, residuals of systolic blood pressure adjusted for age and sex; Res. DBP, residuals of diastolic blood pressure adjusted for age and sex; Res. BMI, residuals of BMI adjusted for age and sex; Res. W/H, residuals of W/H adjusted for age and sex; Res. TG, residuals of TG adjusted for age and sex; and Res. Chol, residuals of cholesterol adjusted for age and sex. P_1 was calculated by 1-way ANOVA; P_2 was calculated by unpaired *t* test (AA vs AG+GG).

their flanking regions were sequenced. The primer sequences will be provided on request. The polymorphisms were determined by use of the TaqMan system (PE Applied Biosystems) (Table 2).

Assessment of the Expression Level of *SAH* mRNA

The expression level of *SAH* mRNA was assessed by a competitive reverse transcription–polymerase chain reaction (RT-PCR) method.[14] The cRNA, which lacks the region between nucleotide 1064 and 1074 (GenBank accession D16350), was synthesized. RNA was extracted as previously described[14] from peripheral mononuclear cells purified by a Ficoll density gradient. Peripheral mononuclear cells were obtained from healthy doctors who understood the significance of this study (written informed consent was obtained). Total RNA (1 μg) combined with the deletion-mutated cRNA was reverse-transcribed, and the resultant cDNA mixture was amplified by primers covering the region between nucleotides 968 and 1111. The length of the PCR product from native mRNA was 144 bp, and that from the deletion-mutated cRNA was 133 bp. The expression level of mRNA is given as the ratio of the 144-bp PCR fragment to the 133-bp fragment.

Expression Study

The expression construct for human *SAH* was purchased from Invitrogen (GeneStorm expression-ready human clones). SA cDNA is expressed under the control of a cytomegalovirus (CMV) promoter. COS1 cells were transiently transfected by this expression vector by LipofectAmine Plus Reagent (Gibco-BRL). The transfected cells were suspended in buffer A (50 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 10% glycerol) containing a protease inhibitor cocktail (Sigma) and disrupted by sonication. The resulting supernatant was used for enzymatic assay for acyl-CoA synthetase. Acyl-CoA synthetase activity for octanoic acid and palmitoic acid was assayed according to the method of Vessey and Hu.[15]

To determine the cellular localization of the SA protein, the cDNA encoding human *SAH* with the C-terminal Myc tag was subcloned into pCI mammalian expression vector (Promega) and expressed in HeLa cells. To detect the SA protein and mitochondria simultaneously, the cells were incubated with anti–Myc-tag rabbit polyclonal antibody (MBL) and anti–human mitochondria mouse monoclonal antibody (Chemicon). The cells were then double-stained with Alexa Fluor 488–labeled anti-rabbit IgG and Alexa Fluor 568-labeled anti-mouse IgG (Molecular Probes).

To explore the significance of an A/G polymorphism in intron 12, we constructed a minigene that included the region between exons 11 and 14 (3′-untranslated region) under the control of the CMV promoter (pcDNA 3.1 as a vector). The correctly spliced mature transcript (M transcript) was detected as a PCR product of 204 bp by

TABLE 2. Primers and Probes Used for Genotyping

Genotype		Position
I/D (119801/119802)		
Primer/S	TAGAACTCAAAATGACCAAC	119402–119422
Primer/A	ATGGTGTATCTATTGCCTGGA	119554–119524
Probe/I	CCTCTTTAAGTAAA*TTTAAGTT*GGGTCTC	119428–119451
Probe/D	CCTCTTTAAGTAAA*GTT*GGGTCTCAC	119428–119453
G(119952)A		
Primer/S	CCTGTGCCAGATCATACAACTCC	119863–119885
Primer/A	CTAAATCTCATCAAGCCCATTGG	120030–120007
Probe/G	CAGGTTACTCCCCTGCTCAAATCTT	119964–119940
Probe/A	TCAGGTTACTCCC*T*TGCTCAAATCTTT	119965–119939
G(141420)C		
Primer/S	AACCAATTACCCCTGACGTGAC	141370–141391
Primer/A	CAGTGAGGTCAGGTACCGTTTC	141478–141457
Probe/G	AATGGAGAAACAAGACGGGCCTGGATA	141407–141433
Probe/C	AATGGAGAAACAACACGGGCCTGGATA	141407–141433
A(152718)G		
Primer/S	CACCAGGCATGTAGATTCTCAGA	152629–152653
Primer/A	GTGACTTGTAATCAGGATTTAGAACGA	152767–152741
Probe/A	TGTTTCAAATATTTATTTTAGGTAGTAAA	152704–152732
Probe/G	TGTTTCAAATATTT*G*TTTTAGGTAGTAA	152704–152731

Italicized nucleotides indicate polymorphic sites.

use of exon 12 and 14 primers. The unspliced transcript was detected as a PCR product of 229 bp with intron 12 and exon 13/14 primers. The ratio of M transcript to the unspliced RNA was expressed as the ratio of 209-bp to 229-bp PCR product. To assess the expression levels of M transcript of the A and G alleles, pRL-CMV vector (Promega), in which *Renilla* luciferase is under the CMV promoter, was included in the transfection mixture as an internal standard. The expression level of M transcript was assessed by the ratio of the PCR product from M transcript (204 bp) to that from luciferase (283 bp).

To explore the regulatory effects of an insertion/deletion polymorphism in the promoter region, we constructed *SAH* promoter/luciferase fusion genes. The polymorphisms were an insertion/deletion (alleles I and D) at −1037 and a G(119952)A polymorphism at −407. The transcription initiation site was determined by 5′-RACE, and the major site was numbered +1. The haplotypes determined were D/G, D/A, I/A, and I/G. The promoter region between −2052 and +253 was subcloned into pGL2-Basic (Promega), which does not contain any promoter sequence. Transfection was performed in MDCK cells with PRL-CMV vector (Promega) as an internal standard. *Photinus* and *Renilla* luciferase activities were measured with a kit (PG-DUAL-SP, Toyo Ink, Co).

A more detailed description of materials and methods, including primer sequences, will be provided on request.

Statistical Analyses

Values are expressed as mean±SEM or mean±SD. All statistical analyses were performed with the JMP and StatView statistical packages (SAS Institute Inc). Multiple linear regression and multiple logistic analyses were performed with other covariates (sex and age). Residuals of blood pressure values, waist-to-hip ratio (W/H), triglyceride, and cholesterol were calculated by adjustment for sex and age. Differences in numerical data among the groups were analyzed by 1-way/2-way ANOVA and the unpaired *t* test. Differences in frequencies and the degree of linkage disequilibrium were tested by contingency table analysis.

Results

SAH Polymorphisms

Polymorphisms found in *SAH* are summarized in Table 3. The genotypes in introns 5 and 7 were in complete linkage disequilibrium with the exon 8 polymorphism in the 96 subjects analyzed. Therefore, these 2 polymorphisms were not tested. The pairwise linkage disequilibrium of the I/D with the intron 12 polymorphism is shown in Table 4.

Association Study

We determined the genotypes of the promoter I/D, promoter G/A, exon 8 G/C, and intron 12 A/G polymorphisms in the entire study population. Table 1 shows the characteristics of the study population according to the intron 12 polymorphism. Because there were only 4 GG genotypes, the GG and AG genotypes were combined into one group.

The intron 12 polymorphism significantly affected body mass index (BMI), W/H, percentage of antihypertensive

TABLE 3. Polymorphisms of the *SA* Gene

Region	Base Number	Polymorphism	Frequency	AA Change
Promoter	119801/119802	TTTAA I/D	0.295/0.705	...
Promoter	119952	G/A	0.828/0.172	...
Intron 5	137221	T/C	Not determined	...
Intron 7	138162	A/T	Not determined	...
Exon 8	141420	G/C	0.829/0.171	Lys/Asn
Intron 12	152718	A/G	0.981/0.019	...

The base number was according to the numbering of the BAC clone covering the *SA* gene (AC004381). Frequency indicates allele frequency.

TABLE 4. Linkage Disequilibrium Between the I/D and Intron 12 Polymorphisms

	Intron 12 A/G Polymorphism			
	AA	AG	GG	Total
Promoter I/D				
II	374	1	0	375
ID	1603	39	0	1642
DD	1927	111	4	2042
Total	3904	151	4	4059

Number of subjects indicated according to genotype.

treatment, systolic and diastolic blood pressure, heart rate, fasting blood glucose, and triglyceride (Table 1). After adjustment for age and sex, the intron 12 polymorphism significantly affected BMI, W/H, triglyceride, and systolic and diastolic blood pressure (Table 1). The D allele in the promoter tended to affect the triglyceride level. Multiple regression analysis indicated that the triglyceride level was determined by age ($P<0.0001$), sex ($P<0.0001$), and the genotype of the I/D polymorphism (II+ID=1, DD=2, $P=0.0654$). None of the other polymorphisms of *SAH* significantly affected these phenotypic variables (data not shown). Multiple logistic analysis, in which age, sex, and the intron 12 polymorphism were included as independent variables, indicated that the odds ratio of the AG+GG genotype for the presence of hypertension was 1.49 (95% CI 1.05 to 2.10, $P=0.0235$).

The effects of the intron 12 polymorphism on various phenotypes were more evident in subjects <60 years old

TABLE 5. Characteristics of the Study Population (<60 Years Old)

	AA	AG+GG	*P*
n	1901	69+3	
Male, %	43.2	43.1	NS
Age, y	49.7 (0.2)	49.8 (0.9)	NS
BMI, kg/m^2	22.65 (0.07)	23.39 (0.35)	0.0384
AHT, %	7.5	12.5	NS
SBP, mm Hg	121.5 (0.4)	126.2 (2.2)	0.0362
DBP, mm Hg	78.8 (0.3)	82.0 (1.3)	0.0186
HR, bpm	66.2 (0.2)	68.2 (0.9)	0.0235
W/H	0.878 (0.001)	0.895 (0.005)	0.0327
Plasma cholesterol, mmol/L	5.34 (0.02)	5.65 (0.10)	0.0029
HDL cholesterol, mmol/L	1.57 (0.01)	1.59 (0.05)	NS
Triglycerides, mmol/L	1.35 (0.03)	1.81 (0.13)	0.0008
Blood glucose, mmol/L	5.30 (0.02)	5.66 (0.11)	0.0021
%HTN	22.9	36.1	0.0092
Res. SBP, mm Hg	−0.16 (0.39)	4.35 (2.00)	0.0275
Res. DBP, mm Hg	−0.11 (0.24)	3.02 (1.26)	0.0145
Res. BMI, kg/m^2	−0.03 (0.07)	0.71 (0.34)	0.0333
Res. W/H	−0.001 (0.001)	0.016 (0.006)	0.0197
Res. TG, mmol/L	−0.02 (0.03)	0.44 (0.13)	0.0005
Res. Chol, mmol/L	−0.01 (0.02)	0.29 (0.10)	0.0023

Abbreviations as in Table 1.

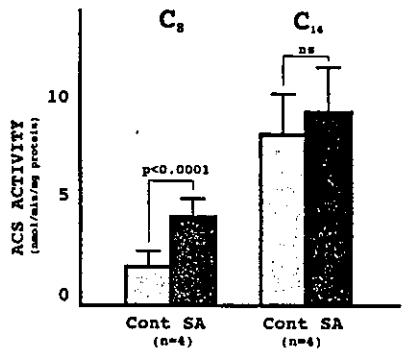

Figure 1. Acyl-CoA synthetase (ACS) activity of transfected Cos cells. COS1 cells were transfected with pcDNA/GS-SA (SA) or pcDNA/GS (cont). ACS activity for octanoic acid (C_8) and palmitoic acid (C_{16}) was assessed. Each value represents mean of 4 independent transfection experiments (mean±SD).

(Table 5). After adjustment for age and sex, the intron 12 polymorphism significantly affected BMI, W/H, triglyceride, cholesterol, and systolic and diastolic blood pressure in this younger subpopulation (Table 5). Multiple logistic analysis, in which age, sex, and the intron 12 polymorphism were included as independent variables, indicated that the odds ratio of the AG+GG genotype for the presence of hypertension was 2.01 (95% CI 1.19 to 3.35, $P=0.0081$).

Function of the SA Protein

The SA protein has been reported to be highly homologous to a bovine xenobiotic–metabolizing MCFA:CoA ligase.[6] COS1 cells transfected by pcDNA3.1/GS-human *SA* had significantly higher acyl-CoA synthetase activity for octanoic acid than those transfected with pcDNA3.1/GS, which confirmed

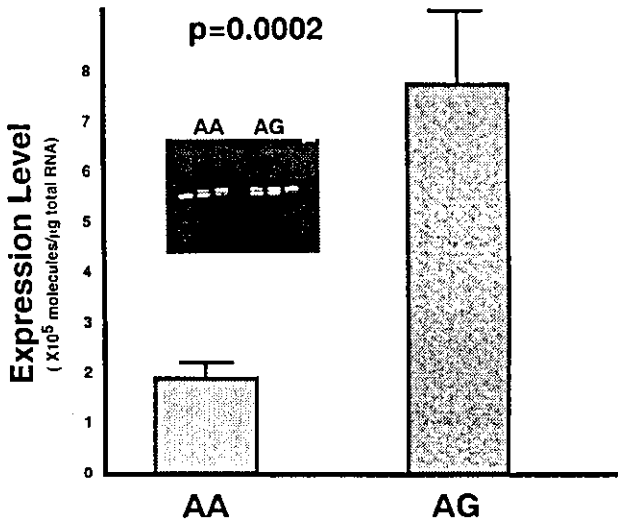

Figure 2. Expression level of SA mRNA according to A/G polymorphism. Expression level was measured by competitive RT-PCR in peripheral mononuclear cells. Inset, representative competitive RT-PCR with bands for SA mRNA (144 bp) and deletion-mutated cRNA (133 bp). Expression levels were assessed with 3 levels of deletion-mutated cRNA (1.0×10^5, 4.0×10^5, and 1.6×10^6 molecules/μg total RNA; left to right). Mean±SD data obtained from 8 subjects with AA genotype and 4 subjects with AG genotype.

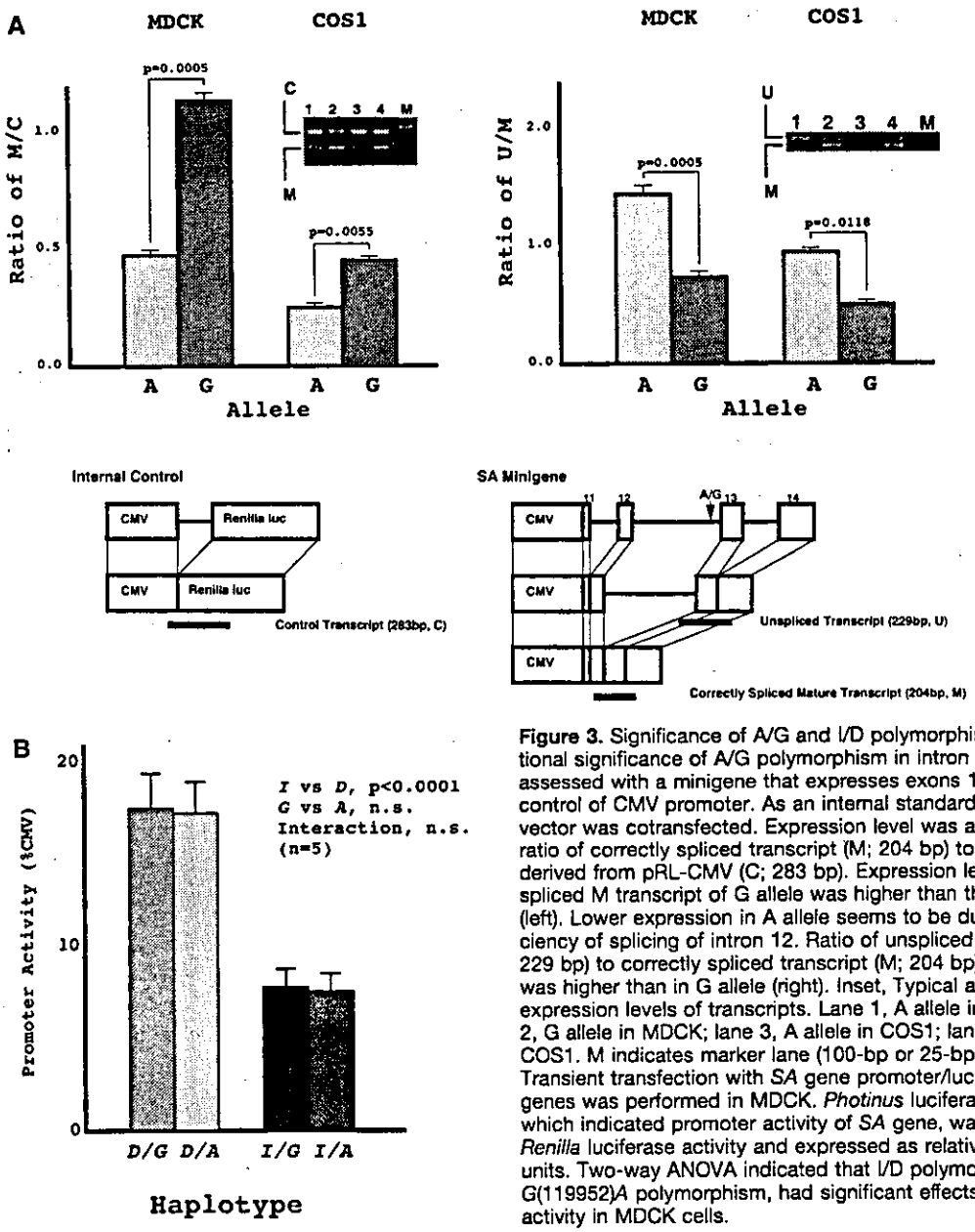

Figure 3. Significance of A/G and I/D polymorphisms. A, Functional significance of A/G polymorphism in intron 12 was assessed with a minigene that expresses exons 11 to 14 under control of CMV promoter. As an internal standard, pRL-CMV vector was cotransfected. Expression level was assessed by ratio of correctly spliced transcript (M; 204 bp) to transcript derived from pRL-CMV (C; 283 bp). Expression level of correctly spliced M transcript of G allele was higher than that of A allele (left). Lower expression in A allele seems to be due to inefficiency of splicing of intron 12. Ratio of unspliced transcript (U; 229 bp) to correctly spliced transcript (M; 204 bp) in A allele was higher than in G allele (right). Inset, Typical analyses of expression levels of transcripts. Lane 1, A allele in MDCK; lane 2, G allele in MDCK; lane 3, A allele in COS1; lane 4, G allele in COS1. M indicates marker lane (100-bp or 25-bp ladder). B, Transient transfection with *SA* gene promoter/luciferase fusion genes was performed in MDCK. *Photinus* luciferase activity, which indicated promoter activity of *SA* gene, was divided by *Renilla* luciferase activity and expressed as relative luciferase units. Two-way ANOVA indicated that I/D polymorphism, but not G(119952)*A* polymorphism, had significant effects on promoter activity in MDCK cells.

that human SA protein had MCFA:CoA ligase activity (Figure 1).

Anti-mitochondria antibody showed a spaghetti-like staining pattern in HeLa cells. Anti-Myc-tag antibody showed a similar pattern in the transfected cells. We concluded that human SA protein is associated with mitochondria (data not shown).

Functional Significance of the Intron 12 A/G and Promoter I/D Polymorphisms

We assessed the expression levels of SA mRNA according to the genotype of intron 12 A/G and the promoter I/D polymorphisms (Figure 2). The expression level of SA mRNA in mononuclear cells in subjects with the AG genotype (all DD genotype in the promoter) (n=4) was ≈4 times higher than that in subjects with the AA genotype (1 II, 4 ID, and 3 DD genotypes in the promoter) (n=8) (P=0.0002). The I/D polymorphism did not appear to have any significant effects on the SA mRNA level (data not shown).

The above observation suggested that the intron 12 A/G polymorphism might influence the expression level of SA mRNA. Because the A/G polymorphism is in the polypyrimidine tract of the intron and this tract has been suggested to influence splicing,[16] we examined the effects of this polymorphism on splicing efficiency, which might then affect the mRNA expression level.

A minigene that contained exons 11 to 14 under the control of the CMV promoter was constructed. The expression level of the correctly spliced M transcript compared with the control RNA level (*Renilla* luciferase RNA) was significantly higher in the G allele than in the A allele (Figure 3). The ratio of M transcript to the unspliced transcript was higher in the G