Site	5'-Flanking					Ex. 1(5'-UTR)		Int. 1	Ex. 2	Int. 3	Ex. 4	Int. 4	Ex. 5		Int. 5	
Position[a]	-1847	-1789	-1491 _-1457	-1247	-371	-145	-129	IVS1 -78	49	IVS3 +36	144	IVS4 -25	304	325	IVS5 +76	IVS5 +123
Nucleotide change	T>C	G>A	delCAT CC	T>C	A>G	C>G	T>C	del G	T>C	C>T	G>T	G>T	G>C	G>A	T>G	A>G
Amino acid change									F17L		K48N		G102R	E109K		

Haplotypes		Tagging variations in the previous reports							Additional tagging variations in this study								No. of chromosome	Reported haplotype[j]	
Subgroup	Type																	Taniguchi et al (2003)	Takane et al (2004)
A	*1a type[b]																589		
	*1c type[c]								delG								85		
	*1d type[d]											T					46		
	*2a								delG					2			18	H1	H1
	*1n															G	11		
	*3a								delG				3				5		
	minors[e]								(other combinations of SNPs)								5		
B	*1b type[f]		A													G	126		
	*1f		A														30	H2 (low)	H4
	*5a?		A								5					G	1		
J	*1j		A			G											17	H5(nd)	H5
	*1k		A			G										G	16		
L	*1L		A				G										13		
	*1m		A				G									G	11	H2 (low) or H5 (nd)	H6 (low)
	minors[g]		A			(G)	G		(other combinations of SNPs)								3		
G	*1g	C			C	G		C		T				G			30		
	*1h	C			C	G		C		T		T					23		
	*4a?	C			C	G		C	4	T		T					1	H3 (low)	H2 (high)
	minors[h]	C			C	G		C	(other combinations of SNPs)								4		
E	*1e type[i]	C		del	C	G		C		T				G			32		H3 (high)

Figure 7 New classification of Block 1 haplotypes and comparison with reported promoter region haplotypes. Genetic variations (allele frequency >0.01) and nonsynonymous variations in Block 1 were sorted according to marker variation, and classified into 6 subgroups (A, B, J, L, G and E).
[a]The positions in other reports were adjusted to the nucleotide numbers used in this study.
[b]The *1a type includes *1a, *1o, *1s, *1t, *1v, *1w, *1x, *1y.
[c]The *1c type includes the *1c haplotype and an ambiguously defined *1 haplotype.
[d]The *1d type includes the *1d haplotype and two ambiguously defined *1 haplotypes.
[e]"Minors" include the *1u and *1r haplotypes and one ambiguously defined *1 haplotype.
[f]The *1b type includes the *1b and *1i haplotypes and three ambiguously defined *1 haplotypes.
[g]"Minors" include the *1q haplotype and one ambiguously defined *1 haplotype.
[h]"Minors" include the *1p haplotype and one ambiguously defined *1 haplotype.
[i]The *1e type includes the *1e haplotype and one ambiguously defined *1 haplotype.
[j]Altered promoter activity in the reporter gene assay is shown in parenthesis.
nd; not determined.

However, the frequency of the *2 group was much lower than that of the *1 group in Africans. The frequencies of *4 and *8 were higher in Japanese than in Caucasians, and the frequency of the *6 group was higher in Caucasians than in other ethnic groups. The most prominent characteristic of the Japanese population was the high frequency of *10 compared with the other ethnic groups. The variations that characterized *11 to *18 were only detected in our study, probably due to the relatively large number of subjects used. The haplotype distribution in Japanese was similar to that described for Asians, but with slight differences in the frequencies of *6, *8, *9, and *10 reported for a mixed Asian population (Kroetz *et al.* 2003).

Tagging SNPs for *ABCB1* Genotyping

For genotyping *ABCB1* in association studies it would be critical to select SNPs for the major haplotypes, including functional ones in Blocks 1 and 2. Table 6 shows the major tagging SNPs for genotyping which are applicable to Japanese and also to other ethnic populations. Genotyping with these SNPs can assign the diplotypes of Blocks 1 and 2 in more than 95% of Japanese. The nonsynonymous SNPs in Blocks 1 and 2, and the additional tagging variations in Block 1 obtained in our study (Fig. 7), could be included in the list for evaluation of their functional significance.

Table 4 Ethnic differences in *ABCB1* Block 1 haplotypes

	This study		Reported data (Takane *et al.* 2004)		
Marker site[a]	Subgroup (see Fig. 7)	Japanese (n = 1066)	Group	Japanese (n = 188)	Caucasian (n = 192)
	A	0.712	H1	0.665	0.964
−1789G>A	B	0.147	H4	0.191	nd
−1789G>A, −371A>G	J	0.031	H5	0.027	nd
−1789G>A, −145C>G	L	0.025	H6	0.032	nd
−1847T>C[b]	G	0.054	H2	0.043	nd
−1461delCATCC, −371A>G, −1847T>C[b]	E	0.030	H3	0.037	nd
−1154T>C		nd	H7	0.005	nd
−1753delGA		nd	H8	nd	0.010
−1347T>C, −129T>C		nd	H9	nd	0.016
−1085A>G		nd	H10	nd	0.010

[a]Each reported position was adjusted to the nucleotide numbers used in this study.
[b]This SNP is linked to −1347T>C and −129T>C.
n; 2 × number of subjects.
nd; not detected.

Table 5 Ethnic differences in the *ABCB1* Block 2 haplotypes

	This study	Reported data (Kroetz *et al.* 2003)[a]		
Group	Japanese (n = 1066)	Asian (n = 60)	Caucasian (n = 200)	African (n = 200)
*1	0.216	0.216	0.370	0.721
*2	0.386	0.365	0.410	0.075
*3	nd	nd	0.010	0.010
*4	0.016	0.016	0.005	0.090
*6	0.034	0.016	0.120	0.035
*7	nd	nd	0.015	0.005
*8	0.141	0.216	0.010	0.040
*9	0.020	0.082	0.025	0.010
*10	0.174	0.066	0.025	0.005
*11	0.005	nd	nd	nd
*12	0.002	nd	nd	nd
*13	0.002	nd	nd	nd
*14	0.001	nd	nd	nd
*15	0.001	nd	nd	nd
*16	0.001	nd	nd	nd
*17	0.001	nd	nd	nd
*18	0.001	nd	0.01	nd

[a]Reported haplotypes were re-assigned according to our haplotype nomenclature.
n = 2 × number of subjects.
nd; not detected.

Discussion

Extensive studies of *ABCB1* haplotypes and their functional significance have been conducted, mostly focused on the common SNPs of 1236C>T, 2677G>T/A, and 3435C>T. However, recent association studies on promoter region haplotypes have indicated the importance of haplotypes within this region (Taniguchi *et al.* 2003; Takane *et al.* 2004). The results of functional or P-gp expression analyses based on these polymorphisms/haplotypes have not always been consistent, possibly due to the small number of subjects used, different ethnic backgrounds, or insufficient haplotyping over a limited region. In the present study, we have conducted a re-assignment of Block 1 haplotypes by extending the region sequenced to the distal promoter, and

Table 6 Major tagging SNPs of *ABCB1* for genotype-phenotyhpe association studeis

i) Block 1 haplotypes (subgroups)					
Position	−1847	−1789	−1461_−1457	−371	−145
Nucleotide change	T > C	G > A	delCATCC	A > G	C > G
	a	a	a	a	a
A					
B		A			
J		A		G	
L		A			G
G	C			G	
E	C		del	G	

ii) Block 2 haplotypes					
Position	1236		2677	3435	
Nucleotide change	C > T	G > A	G > T	C > T	
Amino acid change		A893T	A893S		
		a			
*1					
*2	T		T	T	
*4	T			T	
*6				T	
*8	T				
*9	T		T		
*10		A			
*11	T	A			
*18		A		T	

[a]Specific for Asian populatioins.

added novel haplotypes in other blocks after assessing a large number of subjects.

LD analysis revealed that one of the marker SNPs in the promoter region, −1789G>A, was moderately linked to IVS5 + 123A>G, previously classified into Block 2. Therefore, we shifted the border between Block 1 and Block 2 and re-analyzed the Block 1 haplotypes. Two promoter haplotype classes associated with functional changes have been reported previously (Taniguchi *et al.* 2003; Takane *et al.* 2004). One class included the −1789G>A SNP, and the other included the three linked SNPs of −1847T>C, −1347T>C and −129T>C. In our analysis these SNPs were included in our Block 1 region.

The haplotype containing −1789G>A was reported to be associated with reduced P-gp expression levels in the colon and liver, and reduced promoter activity was shown in a reporter gene assay (Taniguchi *et al.* 2003) (see Fig. 7). However, another study found that a haplotype containing −1789G>A without −145C>G (subgroups B and J in our present study) showed no change in the reporter assay, while another haplotype that contained −1789G>A together with −145C>G (subgroup L) showed reduced promoter activity (Takane *et al.* 2004). Data on the functional effects of haplotypes harbouring the three linked SNPs (G and E subgroups) are also contradictory. While one study showed an association with reduced colon and liver P-gp expression levels in patients and reduced promoter activity in a reporter gene assay (Taniguchi *et al.* 2003), another study reported an association with increased P-gp expression levels in the placenta and liver, and with increased promoter activity in a reporter gene assay (Takane *et al.* 2004). By expanding Block 1 into intron 5 we identified additional types within previously reported wild-type sequences (corresponding to subgroup A in this study) and other variant haplotypes (subgroups B, E, G, J, and L) (Fig. 7). In total our data revealed 11 tagging variations in Block 1: −1789G>A, −1461_−1457CATCdel, −371A>G, −145C>G, −129T>C, IVS1 − 78delG, IVS4 − 25G>T, 304G>C (G102R), 325G>A (E109K), IVS + 76T>G and IVS5 + 123A>G. Thus, if some of these markers are of functional importance it is possible that

our subdivisions (types within A and other subgroups) might explain the discrepancies in P-gp expression levels in the previously reported studies. In fact, our preliminary observation has suggested possible influences of some of the tagging variations in Block 1 on pharmacokinetic parameters of paclitaxel (data not shown). However, this hypothesis requires further clarification in large scale clinical studies.

Several novel haplotypes were added to the other 3 blocks (1, 38, and 4 new haplotypes in Blocks −1, 2, and 3, respectively). We identified a new haplotype *1d in Block −1, but this variant haplotype was very rare and the functional significance of uncommon Block −1 haplotypes remains unknown. We added 7 new groups to Block 2 haplotypes (*12 to *18), but their frequencies were also very low (0.002 and less). We also confirmed the previous finding that, in order of frequency, the major groups were *2d, *10a, *1e, and *8a. In our previous study we estimated the relative P-gp activity of the different haplotypes according to the renal clearance of irinotecan and its metabolites in Japanese cancer patients. While we found a significant association between *2, which contained the three common SNPs, and reduced renal clearance levels, associations with the *6, *8, and *10 groups that contained only one of the common markers remained unclear. For the *4, *9, and *11 groups, which harbour two marker SNPs in Block 2, functional evaluation was impossible due to the small number of subjects. Previously we showed that *1f may have been associated with reduced P-gp activity. The current study revealed that *1f in Block 2 was completely linked with the newly defined *1d in Block 1, which contained IVS4 −25G>T. A further association study is needed to clarify the effects of the linked *1d (Block 1) and *1f (Block 2) haplotypes. Regarding Block 3 we added several minor *1 haplotypes and confirmed the previous findings that *1a and *1b were the major haplotypes. We previously observed a trend for an association between *1b and higher P-gp activity. Taking into consideration the haplotype-combinations across the blocks this trend also needs to be confirmed in a larger number of subjects.

It is well recognized that there are large ethnic differences in the frequencies of functionally important haplotypes, including 1236C>T, 2677G>T, and 3435>T (corresponding to the *2 group in Block 2), and promoter region SNPs (corresponding to the variant Block 1 subgroups). Comparison of our data with the results from other ethnic groups indicated the existence of unique haplotype profiles in the Japanese population. As suggested by the previous report on the promoter region (Takane *et al.* 2004), Japanese samples exhibited large variations in Block 1 haplotypes. This suggested that not only *2 in Block 2 but also certain Block 1 haplotypes may be functionally important in the Japanese ethnic group. For Block 2 we confirmed our previous findings that the major groups were *1 and *2, and that *2d was the most frequent haplotype. While both groups were detected as the major types in other Asian and Caucasian populations, *1 was considerably more frequent than *2 in Africans (Kroetz *et al.* 2003). Another recent study found that the two major haplotypes were common to 5 ethnic groups (Tang *et al.* 2004). That study also revealed that the Chinese and Malay haplotype profiles were very similar, and that while some similarities were also observed between Caucasian and Indian populations, Africans differed from all other non-African populations. Furthermore, their study suggested that positive selection for 2677T-3435T had occurred in Chinese and Malays, and for 3435C in Africans. As pointed out previously, frequent occurrence of *10 (2677G>A) was unique to Japanese compared with Caucasians and African populations. Our study revealed higher frequencies of *10 (2677G>A) and *6 (3435C>T) and lower frequencies of *8 (1236C>T) and *9 (1236C>T and 2677G>T) than reported for Asian populations in Kroetz *et al.* (2003) (Table 5). This difference might be due to the mixed Asian population used in the report, as differences in the frequencies of 2677G>A between the Chinese, Malay, and Indian populations have been noted (Tang *et al.* 2004). The finding that the high frequency of 2677G>A is shared among Japanese, Koreans (Yi *et al.* 2004) and Chinese (Tang *et al.* 2004) suggests a close evolutionary relationship between these three populations.

A whole-genome haplotype database for three populations is now available at the Perlegen website (www.perlegen.com), which provides a good tool for investigation of the structures of human genetic variation within and between different populations (Hinds *et al.* 2005). For the *ABCB1* gene, however, we could not directly compare their data with ours because their

SNPs are mostly intronic and did not overlap with our SNP markers (<20%).

For genotype-phenotype association studies on the *ABCB1* gene, genotyping of the major functional key SNPs in Blocks 1 and 2 (Table 6) would be useful. Further studies on the clinical significance of the haplotypes described in the present study and elucidation of the haplotype-combinations across blocks, will be required to achieve the goal of personalized drug therapy.

Conclusions

We re-established *ABCB1* haplotypes in the Japanese population based on novel polymorphisms found in a large number of subjects, expanding the promoter region. Our current data added more detailed information on functionally-important haplotypes in Blocks 1 and 2 in the Japanese population, and identified differences in haplotype profiles between ethnic groups. The information provided in this study will be of use in further studies investigating the relationship between genetic markers and functional changes.

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Original Article

Association of Sixty-One Non-Synonymous Polymorphisms in Forty-One Hypertension Candidate Genes with Blood Pressure Variation and Hypertension

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We previously selected a group of hypertension candidate genes by a key word search using the OMIM database of NCBI and validated 525 coding single nucleotide polymorphisms (SNPs) in 179 hypertension candidate genes by DNA sequencing in a Japanese population. In the present study, we examined the association between 61 non-synonymous SNPs and blood pressure variations and hypertension. We used DNA samples taken from 1,880 subjects in the Suita study, a population-based study using randomly selected subjects. Analyses of covariance adjusting for age, body mass index, hyperlipidemia, diabetes, smoking, drinking, and antihypertensive medication revealed that 17 polymorphisms in 16 genes (*APOB, CAST, CLCNKB, CTNS, GHR, GYS1, HF1, IKBKAP, KCNJ11, LIPC, LPL, P2RY2, PON2, SLC4A1, TRH, VWF*) were significantly associated with blood pressure variations. Multivariate logistic regression analysis with adjustment for the same factors revealed that 11 polymorphisms in 11 genes (*CAST, CTLA4, F5, GC, GHR, LIPC, PLA2G7, SLC4A1, SLCI8A1, TRH, VWF*) showed significant associations with hypertension. Five polymorphisms in five genes, *CAST* (calpastatin), *LIPC* (hepatic lipase), *SLC4A1* (band 3 anion transporter), *TRH* (thyrotropin-releasing hormone), and *VWF* (von Willebrand factor), were significantly associated with both blood pressure variation and hypertension. Thus, our study suggests that these five genes were susceptibility genes for essential hypertension in this Japanese population. (*Hypertens Res* 2006; 29: 611–619)

Key Words: **genetic variants, hypertension, calpastatin, lipase, von Willebrand factor**

Introduction

Hypertension is one of the major risk factors for cardiovascular disease morbidity and mortality (*1–4*). In order to reduce events related to cardiovascular disease, control of hypertension is very important (*5, 6*). The clinical phenotypes of hypertension are known to be affected by both lifestyle and genetic factors (*1*). Although studies of Mendelian inheritance in hypertension are limited, the causative genes have recently been identified in cases with glucocorticoid-remediable aldosteronism, Liddle syndrome, and pseudohypoaldo-

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steronism (*7–10*). Essential hypertension, however, is a multifactorial disease caused by the interaction of environmental factors with specific genotypes of multiple genes.

To delineate the genetic factors underlying hypertension, numerous association analyses have been performed. In these studies, hypertensives and matched controls with normal blood pressure are genotyped for a marker such as a single nucleotide polymorphism (SNP) thought to be etiologically important, and then allele or genotype frequencies in cases and controls are compared. In this study design, cases and controls must be representative and must be matched as closely as possible, except for blood pressure. To achieve these criteria, a subject group from the general population is widely used (*11–13*).

The National Cardiovascular Center conducts the Suita Study for the purpose of identifying the most common risk factors or characteristics that contribute to cardiovascular disease, including hypertension, in the Japanese population. This study is based on a random sampling of 15,200 Japanese residents of Suita, a City near Osaka and part of the second-largest urban area of Japan. The residents, between 30 and 79 years of age, were arbitrarily selected from the city population registry and were stratified by sex and decennial boundaries. By February 1997, 53% of the selected subjects had paid an initial visit to the National Cardiovascular Center. Since then, participants have visited the National Cardiovascular Center every 2 years for regular health checkups.

SNPs have received much attention as a means of identifying the genotypes of multiple genes for common diseases, such as myocardial infarction, asthma, and hypertension. In particular, SNPs concomitant with a missense mutation (non-synonymous SNPs) can potentially alter the protein function and gene expression level. In the translated protein, the amino acid changes caused by the missense mutation have the potential to affect protein function. Therefore, non-synonymous SNPs are the primary targets when searching for DNA variations that are causative for hypertension (*14–16*).

We previously selected a group of hypertension candidate genes by a key word search using the OMIM database of NCBI and retrieved SNPs from the public database (*17*). We verified 525 coding SNPs in 179 hypertension candidate genes by DNA sequencing of samples from 32 Japanese individuals and successfully identified a total of 143 SNPs in 93 candidate genes, including 104 missense mutations in 65 genes. Some of the missense mutations including the C677T polymorphism in *MTHFR* (*18*) and the T268M substitution in angiotensinogen, *AGT* (*19*), have previously been examined for their association with hypertension in our population, but the others remain to be assessed. This study was undertaken to examine the association of these missense mutations with blood pressure variation or hypertension in a general population.

Methods

Subjects of the Population Study

The subjects of the Suita study consisted of 15,200 men and women (30–79 years of age), who were randomly selected from the municipal population registry and stratified by gender and age in 10-year intervals. They were all invited, by letter, to receive medical and behavioral examinations every 2 years at the Division of Preventive Cardiology, National Cardiovascular Center, Japan. DNA from the leukocytes was collected between April 2002 and February 2003 from participants who gave written informed consent for genetic analyses. A total of 1,880 samples were collected during this period. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. Routine blood examinations that included measurements of total serum cholesterol, high-density lipoprotein (HDL)–cholesterol, triglycerides, and glucose levels were performed. A physician or nurse interviewed each patient with regard to smoking and alcohol drinking habits and personal history of common diseases.

Blood pressures were measured after at least 10 min of rest in a sitting position. Systolic and diastolic blood pressure (SBP/DBP) values were taken as the mean of 2 measurements recorded by well-trained doctors using a mercury sphygmomanometer. Hypertension was defined as a mean SBP of ≥140 mmHg, a mean DBP of ≥90 mmHg, or current use of antihypertensive medication (*20*, *21*). Diabetes was defined as fasting plasma glucose levels ≥7.0 mmol/l (126 mg/dl), non-fasting plasma glucose levels ≥11.1 mmol/l (200 mg/dl), HbA1c ≥6.5%, or current use of antidiabetic medication. Hyperlipidemia was defined as total cholesterol levels ≥5.68 mmol/l (220 mg/dl) or current use of antihyperlipidemia medication. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared.

Genotyping of Polymorphisms

Non-synonymous SNPs with a minor allele frequency of greater than 3% described in our previous study (*17*) were genotyped by the TaqMan-polymerase chain reaction (PCR) system (*22*, *23*). However, some of these SNPs could not be genotyped in case of the nearest-neighbor sequence. Six SNPs (rs16027, rs362331, rs362272, rs1805020, rs1805021, and rs1982073) that were previously assigned as non-synonymous SNPs were here mapped in intron by the current version of dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), build 122. Thus, these SNPs were excluded from the present analyses, leaving a total of 61 non-synonymous SNPs that were genotyped in this study.

Statistical Analysis

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.

Analyses of covariance for SBP and DBP in each sex of genotypes were performed with consideration of potentially confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with the 95% confidence intervals. The association between genotype and risk of hypertension was expressed in terms of odds ratios adjusted for possible confounding effects including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SAS statistical software (release 8.2; SAS Institute Inc., Cary, USA) was used for statistical analyses.

Results

Basic Characteristics of Subjects in the Suita Study

The characteristics of the 1,880 participants (866 men and 1,014 women) are summarized in Table 1. Age, SBP, DBP, BMI, percentage of current smokers and drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, HDL-cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men.

Table 1. Basic Characteristics of Subjects in Suita, a Japanese Urban Population, 2002

	Women (n=1,014)	Men (n=866)
Age (years)	63.3±11.0	66.3±11.1*
SBP (mmHg)	128.0±19.7	131.8±19.4*
DBP (mmHg)	76.6±9.8	79.7±10.7*
Body mass index (kg/m^2)	22.3±3.2	23.3±3.0*
Total cholesterol (mg/dl)	215.7±30.6*	197.9±30.6
HDL-cholesterol (mg/dl)	64.3±15.5*	55.0±14.3
Current smokers (%)	6.3	29.9†
Current drinkers (%)	29.5	67.1†
Present illness (%)		
Hypertension	38.1	47.3†
Hyperlipidemia	54.5†	27.8
Diabetes mellitus	4.3	11.1†

Values are mean±SD or percentage. Hypertension indicates SBP ≥140 mmHg and/or DBP ≥90 mmHg or antihypertensive medication; hyperlipidemia, total cholesterol ≥220 mg/dl or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥126 mg/dl or non-fasting plasma glucose ≥200 mg/dl or HbA1c ≥6.5% or antidiabetic medication. *$p<0.05$ between women and men by Student's t-test. †$p<0.05$ between women and men by χ^2 test. SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein.

Susceptible Missense Mutations Related to Blood Pressure Variation and Hypertension

We genotyped 61 non-synonymous SNPs by the TaqMan-PCR system in 1,880 individuals; 796 of whom were hypertensives and 1,084 of whom were normotensives. Non-synonymous SNPs genotyped in this study in conjunction with the allele frequencies are listed in Table 2.

Analysis of covariance adjusting for age, BMI, hyperlipidemia, diabetes mellitus, smoking, drinking, and antihypertensive medication revealed that 17 polymorphisms in 16 genes (*APOB*, *CAST*, *CLCNKB*, *CTNS*, *GHR*, *GYS1*, *HF1*, *IKBKAP*, *KCNJ11*, *LIPC*, *LPL*, *P2RY2*, *PON2*, *SLC4A1*, *TRH*, *VWF*) were significantly associated with blood pressure variation in either a dominant or a recessive genetic model (Table 3). Among them, four SNPs (*GYS1*: glycogen synthase; *LIPC*: heptic lipase; *TRH*: thyrotropin-releasing hormone; *VWF*: von Willebrand factor) were associated with blood pressure in men or women on the basis of a probability value <0.01 in either a dominant or recessive genetic model.

Multivariate logistic regression analysis with adjustment for the same factors revealed that 11 polymorphisms in 11 genes (*CAST*, *CTLA4*, *F5*, *GC*, *GHR*, *LIPC*, *PLA2G7*, *SLC4A1*, *SLCI8A1*, *TRH*, *VWF*) showed significant association with hypertension (Table 4). Among them, two SNP, rs754615 in calpastatin (*CAST*) and rs9016 in a group-specific component (*GC*) were associated with hypertension in women on the basis of a probability value <0.01. When the controls were defined as SBP ≤120 mmHg, DBP ≤80 mmHg, or non-medication, and the hypertensives were defined as SBP ≥160 mmHg, DBP ≥100 mmHg, or current use of antihypertensive medication, 5 out of 11 SNPs showed positive association with hypertension after adjustment for the confounding factors described above as follows. Rs754615 of *CAST* was associated with hypertension in women (GG+GC *vs.* CC, odds ratio: 0.17, 95% confidence interval: 0.03–0.88, $p=0.035$). Rs9016 of *GC* was associated with hypertension in women (CC *vs.* CT+TT, odds ratio: 0.19, 95% confidence interval: 0.06–0.56, $p=0.003$). Rs1390938 of *SLC18A1* was associated with hypertension in women (TT+TC *vs.* CC, odds ratio: 0.60, 95% confidence interval: 0.38–0.92, $p=0.020$). Rs5036 of *SLC4A1* was associated with hypertension in men (AA *vs.* AG+GG, odds ratio: 0.57, 95% confidence interval: 0.34–0.96, $p=0.035$). Rs1063856 of *VWF* was associated with hypertension in men (AA *vs.* AG+GG, odds ratio: 0.51, 95% confidence interval: 0.28–0.92, $p=0.026$).

Association analysis using two different statistical calculations showed that five genes, *CAST* (calpastatin), *LIPC*

Table 2. List of Non-Synonymous SNPs Genotyped in this Study

Gene symbol	Reference SNP (dbSNP)	Allele 1/2	Amino acid change	Allele 1 Homo	Hetero	Allele 2 Homo	Allele frequency	
							Allele 1	Allele 2
ABCC8	rs757110	G/T	Ala1369Ser	296	841	729	0.384	0.616
ADRB2	rs1042713	G/A	Gly16Ala	473	902	461	0.503	0.497
APOA4	rs5104	A/G	Asn147Ser	776	882	220	0.648	0.352
APOB	rs1367117	C/T	Thr98Ile	1,581	267	20	0.918	0.082
	rs679899	C/T	Ala618Val	32	405	1,439	0.125	0.875
APOC4	rs1132899	T/C	Leu36Pro	182	808	885	0.313	0.687
	rs5167	G/T	Arg96Leu	432	960	484	0.486	0.514
CALCA	rs5241	C/A	Ser76Arg	1,777	99	0	0.974	0.026
CAST	rs754615	G/C	Cys408Ser	1,405	439	35	0.865	0.135
CCR2	rs1799864	G/A	Val64Ile	936	779	163	0.706	0.294
CDKN1A	rs1801270	C/A	Ser31Arg	523	947	406	0.531	0.469
CFTR	rs213950	G/A	Val470Met	722	878	280	0.618	0.382
CLCNKB	rs2015352	G/T	Arg27Leu	133	738	996	0.269	0.731
CPT2	rs1799821	G/A	Val368Ile	9	198	1,672	0.057	0.943
	rs1799822	A/G	Met647Val	1,670	199	9	0.942	0.058
CSF1	rs1058885	T/C	Leu408Pro	279	894	688	0.390	0.610
CTLA4	rs231775	G/A	Ala17Thr	722	877	281	0.617	0.383
CTNS	rs161400	T/C	Ile260Thr	1,662	211	7	0.940	0.060
CYP21A2	rs6474	G/A	Arg103Lys	857	799	222	0.669	0.331
F5	rs6020	G/A	Arg513Lys	230	854	795	0.350	0.650
F7	rs6046	G/A	Arg413Gln	1,647	224	8	0.936	0.064
GC	rs7041	T/G	Asp432Glu	1,064	679	137	0.747	0.253
	rs4588	A/C	Lys436Thr	148	746	979	0.278	0.722
	rs9016	C/T	Arg445Cys	1,786	90	2	0.975	0.025
GHR	rs6182	G/T	Cys440Phe	1,588	273	18	0.918	0.082
	rs6180	C/A	Leu544Ile	593	904	381	0.556	0.444
	rs6184	C/A	Pro579Thr	1,577	294	0	0.921	0.079
GIPR	rs1800437	G/C	Glu354Gln	1,147	634	96	0.780	0.220
GYS1	rs5447	A/G	Met416Val	1,512	342	23	0.897	0.103
HF1	rs800292	G/A	Val62Ile	657	915	304	0.594	0.406
	rs1061170	C/T	His402Tyr	6	222	1,643	0.063	0.937
	rs1065489	G/T	Glu936Asp	525	951	401	0.533	0.467
IKBKAP	rs1538660	C/T	Pro1158Leu	792	874	210	0.655	0.345
KCNJ11	rs5219	A/G	Lys23Glu	253	834	788	0.357	0.643
LIPA	rs1051339	G/A	Gly23Arg	1,650	219	11	0.936	0.064
LIPC	rs6078	G/A	Val95Met	1,083	691	105	0.760	0.240
	rs6083	A/G	Asn215Ser	14	284	1,574	0.083	0.917
LPL	rs328	C/G	Ser474Stop	1,412	435	33	0.867	0.133
NOTCH3	rs1044009	C/T	Ala2223Val	299	883	696	0.394	0.606
P2RY2	rs1626154	T/C	Cys334Arg	12	259	1,600	0.076	0.924
PCSK1	rs6234+	C/G	Gln665Glu	1,121	665	92	0.774	0.226
	rs6235+	G/C	Ser690Thr	1,122	666	92	0.774	0.226
PLA2G7	rs1805017	G/A	Arg92His	1,175	612	91	0.789	0.211
	rs1805018	T/C	Ile198Thr	1,179	620	79	0.793	0.207
	rs1051931	T/C	Val379Ala	24	358	1,498	0.108	0.892
PON1	rs854560	T/A	Leu55Met	1,525	294	10	0.914	0.086
	rs662	A/G	Gln192Arg	214	852	767	0.349	0.651
PON2	rs11545941	C/G	Ala148Gly	1,175	627	74	0.793	0.207
SELE	rs5368	C/T	His468Tyr	1,125	676	78	0.779	0.221
	rs5355	C/T	Leu575Phe	1,695	178	4	0.950	0.050

Table 2. (Continued)

Gene symbol	Reference SNP (dbSNP)	Allele 1/2	Amino acid change	Allele 1 Homo	Hetero	Allele 2 Homo	Allele frequency	
							Allele 1	Allele 2
SLC18A1	rs1390938	T/C	Ile136Thr	128	703	1,044	0.256	0.744
SLC2A2	rs1800572	G/A	Val101Ile	1,769	109	1	0.970	0.030
SLC4A1	rs5035	A/C	Asp38Ala	1,715	163	2	0.956	0.044
	rs5036	A/G	Lys56Glu	1,317	524	37	0.841	0.159
	rs2285644	C/T	Pro854Leu	1,697	176	7	0.949	0.051
TRH	rs5658	G/C	Val8Leu	210	812	856	0.328	0.672
VWF	rs1800377	G/A	Val471Ile	1,329	504	44	0.842	0.158
	rs1800378	A/G	His484Arg	238	855	785	0.354	0.646
	rs1063856	A/G	Thr789Ala	1,626	236	17	0.928	0.072
	rs216321	A/G	Gln852Arg	63	576	1,240	0.187	0.813
WRN	rs1346044	T/C	Cys1367Arg	1,608	263	8	0.926	0.074

+: SNPs in linkage disequilibrium. Present rs numbers of SNPs are obtained from dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), build 122. SNP, single nucleotide polymorphism.

(hepatic lipase), *SLC4A1* (band 3 anion transporter), *TRH*, and *VWF*, were significantly associated with both blood pressure variation and hypertension. The blood pressure variations by genotypes of these genes were 4.4 mmHg, 3.5 mmHg, 1.6 mmHg, 4.5 mmHg, and 5.5 mmHg, respectively.

Discussion

In this study, we performed an association of a large number of non-synonymous SNPs previously identified in Japan with blood pressures variation and hypertension in a general population. The results showed that 16 and 11 genes showed an association with blood pressure variation and hypertension, respectively, and five genes (*CAST*, *LIPC*, *SLC4A1*, *TRH*, *VWF*) showed an association with both blood pressure variation and hypertension.

Some of the SNPs showed relatively large blood pressure variation (>5 mmHg; Table 3). For example, the mean blood pressure variations contributed by the genotypes of *APOB* (apolipoprotein B), *CTNS* (cystinosin), *GHR* (growth hormone receptor), and *VWF* were 12.2 mmHg, 9.9 mmHg, 8.1 mmHg, and 5.5 mmHg, respectively. These SNPs have a minor allele frequency of below 0.1, suggesting that the blood pressure variation of these genes may be overestimated. *CAST*, *KCNJ11* (potassium channel, inwardly rectifying, subfamily J, member 11), *LPL* (lipoprotein lipase), *TRH*, and *VWF*, in which the minor allele frequencies were over 0.1, showed a moderate blood pressure change of between 4–5 mmHg by the genotypes.

CAST (5q14–q22) encodes an intracellular protease inhibitor, calpastatin, that regulates a calcium-dependent cysteine proteinase, calpain, ubiquitously present in a variety of tissues and cells (*24*). Calpain activity is tightly regulated with intracellular calcium concentration, and the calpain-calpastatin system governs the non-lysozomal intracellular degradation of proteins. Calpastatin consists of an N-terminal domain L and four repetitive calpain-inhibition domains (domains 1–4). The missense mutation we reported here is the Cys-to-Ser substitution at position 408 that is present in domain 2. In Milan hypertensive rats, calpastatin activity was decreased compared to that in Milan normotensive rats (*25*). Patients with essential hypertension showed lower calpastatin activity in red cells than normotensive subjects (*26*). These reports suggest a possible link between *CAST* and hypertension.

LIPC, located on chromosome 15q21, encodes hepatic lipase. It is a key enzyme in lipoprotein metabolism together with lecithin cholesterol acyl transferase. Hepatic lipase is synthesized by the liver and resides in the hepatic endothelial cell lining (*27*). Genetic polymorphisms in the promoter region of *LIPC* have been associated with high plasma HDL-cholesterol concentrations (*28*). In the current study, the Val149Met polymorphism in *LIPC* was associated with HDL cholesterol ($p=0.04$; data not shown). Here, we showed an association of Val49Met substitution with blood pressure variation and hypertension. The mechanisms by which this substitution affects the blood pressure variations are not clear.

SLC4A1 encodes a plasma membrane anion exchanger, termed band 3, abundantly present at the erythrocyte membrane. It performs electroneutral exchange of Cl^- for HCO_3^- across the membrane. It is also present in renal tubular cells, defects of which cause distal renal tubular acidosis characterized by defective urinary acidification by the distal nephron (*29*). We showed that the Lys-to-Glu substitution at position 56 in *SLC4A1* is associated with hypertension. This substitution has previously been reported as band 3 Memphis (*30*). This variant did not show functional difference towards the specific band 3 inhibitor, stilbenedisulfonates, although the detailed analysis has not been done (*31*). If the mutation affects the anion transport in a low amount, it might influence the cation transport. Long-term exposure to the variant may

Table 3. Association of Blood Pressure Variation with Genotypes

Gene	SNP amino acid change	Allele1/2 (allele freq.)	Sex	BP	Genotype group	BP mean±SEM (mmHg)	p*	Variation of mean BP (mmHg)
APOB	rs1367117	C/T	Men	SBP	CC+TC	132.0±0.6		
	T98I	(0.918/0.082)			TT	119.7±5.8	0.035	12.2
CAST	rs754615	G/C	Women	DBP	GG+GC	76.5±0.3		
	C408S	(0.865/0.135)			CC	80.9±2.2	0.042	4.4
CLCNKB	rs2015352	G/T	Women	DBP	GG	74.3±1.1		
	R27L	(0.269/0.731)			GT+TT	76.8±0.3	0.034	2.5
CTNS	rs161400	T/C	Men	DBP	TT+TC	79.8±0.3		
	I260T	(0.940/0.060)			CC	69.8±4.4	0.026	9.9
GHR	rs6182	G/T	Men	DBP	CC+CT	79.8±0.3		
	C440F	(0.918/0.082)			TT	71.7±4.0	0.046	8.1
GYS1	rs5447	A/G	Men	DBP	AA	80.2±0.4		
	M416V	(0.897/0.103)			AG+GG	77.8±0.8	0.006	2.4
HF1	rs800292	G/A	Men	DBP	GG+GA	79.4±0.4		
	V62I	(0.594/0.406)			AA	81.2±0.8	0.047	1.8
IKBKAP	rs1538660	C/T	Women	SBP	CC+CT	128.5±0.6		
	P1158L	(0.655/0.345)			TT	125.2±1.6	0.046	3.3
KCNJ11	rs5219	A/G	Men	SBP	AA	128.1±1.6		
	K23E	(0.357/0.643)			AG+GG	132.3±0.6	0.015	4.2
LIPC	rs6078	G/A	Men	SBP	GG	133.4±0.8		
	V95M	(0.760/0.240)			GA+AA	129.9±0.9	0.004	3.5
LPL	rs328	C/G	Women	DBP	CC+CG	76.5±0.3		
	S474X	(0.867/0.133)			GG	81.2±2.1	0.029	4.7
P2RY2	rs1626154	T/C	Women	DBP	TT+TC	75.0±0.8		
	C334R	(0.076/0.924)			CC	76.9±0.3	0.025	1.8
PON2	rs11545941	C/G	Women	DBP	CC	77.0±0.4		
	A148G	(0.793/0.207)			CG	76.0±0.5	0.032	2.5
					GG	74.6±1.5		
SLC4A1	rs5036	A/G	Men	DBP	AA	79.3±0.4		
	K56E	(0.841/0.159)			AG+GG	80.8±0.7	0.040	1.6
TRH	rs5658	G/C	Women	SBP	GG+GC	127.6±0.6		
	V8L	(0.328/0.672)			CC	132.1±1.5	0.006	4.5
VWF	rs1800377	G/A	Men	SBP	GG	132.8±0.7		
	V471I	(0.842/0.158)			GA+AA	129.5±1.1	0.009	3.4
VWF	rs1063856	A/G	Women	DBP	AA+AG	76.5±0.3		
	T789A	(0.928/0.072)			GG	82.0±2.7	0.045	5.5

*Analyses of covariate analysis, adjusted for age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), antihypertensive medication, and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; BP, blood pressure; SBP, systolic blood presssure; DBP, diastolic blood pressure.

result in a slight but significant dysfunction of anion exchange, thereby leading to hypertension.

TRH encodes the thyrotropin-releasing hormone (TRH), which is a tripeptide functioning as a regulator of the biosynthesis of thyroid-stimulating hormone. TRH also plays an important role in central cardiovascular regulation. Overexpression of the TRH precursor has been shown to induce hypertension in normal rats, which was reversed by TRH antisense treatment (*32*). This treatment also reduced the central TRH hyperactivity in spontaneously hypertensive rats and normalized blood pressure. TRH decreased leptin and mediated the leptin-induced pressor effect (*33*). The polymorphisms in the promoter region of the TRH receptor that belongs to the G protein–coupled seven-transmembrane domain receptor superfamily have been associated with essential hypertension (*34*, *35*). The Leu-to-Val substitution at position 8 in the thyrotropin-releasing hormone precursor is present in the signal sequence that is cleaved off during the formation of TRH. Thus, there would be a possible link between the Leu8Val substitution in TRH and hypertension

Table 4. Allele Frequency and Odds Ratio of Presence of Hypertension by Genotypes of Polymorphisms

Gene	SNP amino acid change	Allele1/2 (allele freq.)	Sex	Genotype group	Odds ratios (95% CI)	p^*
CAST	rs754615	G/C	Women	GG+GC	1	
	C408S	(0.865/0.135)		CC	0.25 (0.09–0.68)	0.007
CTLA4	rs231775	G/A	Men	GG+GA	1	
	A17T	(0.617/0.383)		AA	1.50 (1.00–2.24)	0.050
F5	rs6020	A/G	Women	AA+AG	1	
	K513R	(0.650/0.350)		GG	0.58 (0.39–0.88)	0.010
GC	rs9016	C/T	Women	CC	1	
	R445C	(0.975/0.025)		CT+TT	0.31 (0.15–0.66)	0.002
GHR	rs6180	C/A	Women	CC+CA	1	
	L544I	(0.556/0.444)		AA	0.70 (0.50–1.00)	0.048
LIPC	rs6078	G/A	Men	GG	1	
	V95M	(0.760/0.240)		GA+AA	1.42 (1.07–1.90)	0.016
PLA2G7	rs1805018	T/C	Women	TT+TC	1	
	I198T	(0.793/0.207)		CC	2.30 (1.14–4.64)	0.020
SLC18A1	rs1390938	T/C	Women	TT+TC	1	
	I136T	(0.256/0.744)		CC	0.73 (0.55–0.98)	0.033
SLC4A1	rs5036	A/G	Men	AA	1	
	K56E	(0.841/0.159)		AG+GG	0.70 (0.51–0.97)	0.031
TRH	rs5658	G/C	Women	GG+GC	1	
	V8L	(0.328/0.672)		CC	0.63 (0.41–0.98)	0.041
VWF	rs1063856	A/G	Women	AA	1	
	T789A	(0.928/0.072)		AG+GG	0.65 (0.43–0.97)	0.034

*Conditional logistic analysis, adjusted for age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; CI, confidence intervals.

due to the insufficient production of TRH.

VWF encodes von Willebrand factor, which is synthesized and stored in the endothelium and is an essential plasma protein for platelet plug formation at the site of vessel injuries. It is widely regarded as a marker of endothelial cell damage/dysfunction. Elevated levels of plasma VWF are related to adverse cardiovascular outcomes (*36*). Hypertensive patients with target organ damage are at high risk of adverse cardiovascular events, particularly myocardial infarction and stroke (*37*), and there is a relationship between target organ damage and endothelial damage/dysfunction in hypertension. Although the functional significance of the Val471Ile mutant remains to be determined, the mutant likely has adverse effects on the vasculature.

We would point out that SNPs positively associated with blood pressure/hypertension may be merely markers, and true DNA variation may be present in the other sites in linkage disequilibrium. It has been well established that the human chromosome is divided into discrete blocks of sequences called haplotype blocks, which are separated by hot spots of recombination (*38*). In haplotype blocks, a small number of common haplotypes are present. The size of the haplotype blocks occasionally extends to more than 100 kb (*39*). Therefore, the variation that actually confers the susceptibility to disease may be present in adjacent genes in the same haplotype blocks.

Given the relatively small number of tests performed in the present study, the association of individual SNPs with hypertension or blood pressure variation can be considered marginally significant at best. All the p-values were greater than 0.004 (Tables 3 and 4), but the significance vanished after correction by the Bonferroni method. However, these SNPs in the hypertension candidate genes are non-synonymous, which could potentially affect the protein function. In addition, these SNPs had a positive association with both blood pressure variation and hypertension. Taking these results together, we can regard these five genes as candidate genes for hypertension. Many reports of association study failed to be confirmed. Thus, the association between the SNPs identified in the present study and blood pressure/hypertension will need to be confirmed by another set of studies.

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ORIGINAL ARTICLE

Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers

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Summary. *Background:* Protein S (PS) is an anticoagulant protein that functions as a cofactor for activated protein C (APC), and congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT). Recently, we and others identified the K196E missense mutation in the second epidermal growth factor-like domain of PS as a genetic risk factor for DVT in the Japanese population. The incidence of this mutation is high in the Japanese population. *Objectives:* In the present study, we investigated the relationship between plasma PS activity and the presence of the K196E mutation. *Patients and methods:* We measured PS activity as a cofactor activity for APC in 1862 Japanese individuals and determined the PS K196E genotype in this population. *Results:* Individuals heterozygous for the mutant E-allele had lower plasma PS activity than wildtype subjects (mean ± SD, 71.9 ± 17.6%, $n = 34$ vs. 87.9 ± 19.8%, $n = 1828$, $P < 0.0001$). However, the PS activity of several heterozygous individuals ($n = 8$) was greater than the population average. In contrast, multiple wildtype subjects ($n = 26$) had PS activity less than 2 SD below the population mean, indicating that other genetic or environmental factors affect PS activity. *Conclusions:* Plasma PS activity itself is not suitable for identifying PS 196E carriers and other methods are required for carrier detection.

Keywords: deep vein thrombosis, missense mutation, protein S.

Introduction

Protein S (PS) is an important regulator of coagulation that serves as a cofactor for activated protein C (APC), the anticoagulant protease that proteolytically degrades activated factor (F) V and FVIII [1]. Individuals with homozygous or compound heterozygous deficiency for PS develop disseminated thrombosis after birth, and heterozygosity for PS deficiency increases the risk of deep vein thrombosis (DVT) [2,3].

Recently, we and others identified that a PS missense mutation prevalent in the Japanese population, which causes Lys196 to be replaced by Glu (K196E mutation, formerly known as PS Tokushima, and referred to as K155E mutation), is a genetic risk factor for the development of DVT [4,5]. This mutation lies within the second epidermal growth factor-like domain of PS, and, *in vitro*, K196E mutant PS has decreased APC cofactor activity and poorly accelerates prothrombinase inactivation [6–8]. This missense mutation was originally identified in Japanese patients with PS deficiency suffering from DVT [9,10]. However, the plasma PS activity in individuals with this mutation remained controversial. In one report, PS activity was decreased in carriers of the K196E mutation with normal PS levels [9]. In contrast, another study found PS activity within the normal range in affected individuals [10].

We identified 66 heterozygotes and no homozygotes for the mutant PS 196E-allele from a population of 3651 individuals [5]. Therefore, the frequency of the mutant E-allele in the Japanese population was about 0.009. Extrapolating from these values, we estimated that approximately one out of every 55 Japanese individuals is heterozygous for the E-allele [11]. Thus, a substantial number of Japanese carry the E-allele for PS and are at increased risk for the development of DVT. Given the relatively high frequency of this mutation and its strong correlation with DVT, it may be advisable to screen individuals for the presence of this mutation so that carriers can avoid additional environmental risk factors associated with DVT. An appropriate screening test is lacking, however, and we hypothesized that plasma PS activity levels may directly correlate with PS genotype. If this were the case, genetic testing

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would not need to be undertaken to determine the PS genotype of a large population.

In this study, we examined the relationship between PS activity and the presence of the K196E mutation. The mean PS activity of individuals heterozygous for the K196E mutation was significantly less than that of wildtype individuals. However, there was substantial overlap in PS activity between these populations, and, thus, PS activity is not an appropriate method to differentiate K196E carriers from the general population.

Methods

We previously measured the PS activity in a population of Japanese individuals as part of the Suita Study, and we determined their genotype with respect to the PS K196E mutation [5,12]. The ability of PS to act as a cofactor for PC activation was measured on the basis of the activated partial thromboplastin time assay using Staclot PS (Diagnostica Stago, Asnières, France) [12]. The plasma levels of PS activity were expressed as percentages of the levels obtained from commercially available standard human plasma (Behringwerke, Marburg, Germany). The intra-assay coefficient of variation for PS activity was 6.9% (n = 10). The PS K196E genotype was determined by the TaqMan genotype discrimination method [5], using the primers 5′-ACCACTGTTCCTGTAAAAATGGTTT/5′-TGTGTTTTAATTCTACCATCCTGCT and the probes 5′-VIC-CAAATGAGAAAGATTGTAAAG-MGB (the mutant E-allele)/5′-FAM-CAAATAAGAAAGATTGTAAAG-MGB (the wild-type allele). The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. PS activity was measured in 2690 population individuals [12] and the genotype was determined in 3651 individuals [5]. The 1862 individuals with both known PS activity and genotype were used for analysis in this study. Plasminogen activity was previously measured using the chromogenic assay method with streptokinase as the activator and the specific substrate S-2251 (Chromogenix AB, Stockholm, Sweden) [13]. Plasminogen activity was determined in 4517 individuals [13], and the plasminogen A620T mutation genotype was determined in 3295 out of 4517 individuals by the TaqMan method using the primers 5′-TGTGGAGGCACCTTGATATCC/5′-TGTCAATTGTCCCCTAAACATACTTC and the probes 5′-VIC-TGTTGACTACTGCCCACT-MGB (the mutant T-allele)/5′-FAM- TGTTGACTGCTGCCCACT-MGB (the wild-type allele). Analysis of variance was used to compare mean values between groups by Student's t-test using JMP v 5.1 software (SAS Institute Inc., Cary, NC, USA).

Results

We measured the PS activity in 1862 individuals of known PS genotype, and we compared the activity of wildtype and heterozygous individuals. Within this population, 1828 subjects harbored the wildtype allele while 34 were heterozygous for the K196E mutation. No individuals were homozygous for the mutant E-allele. Within the total population, the mean ± SD PS activity was 87.6% ± 19.9%.

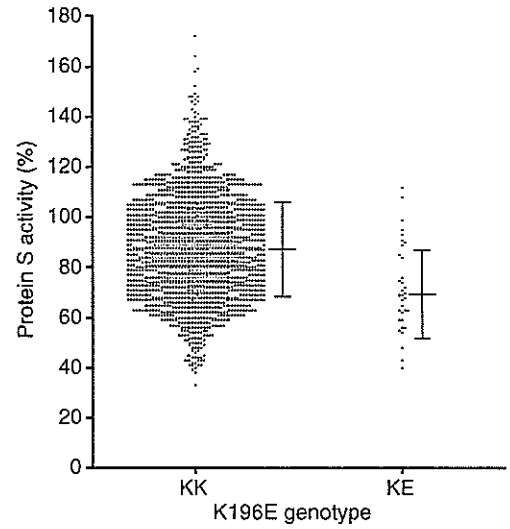

Fig. 1. Protein S (PS) activity in wild-type and K196E heterozygous individuals. Mean ± SD PS activity in heterozygous and wild-type individuals was 71.9% ± 17.6% (n = 34) and 87.9% ± 19.8% (n = 1828) (P < 0.0001), respectively.

Individuals heterozygous for the K196E mutation had reduced plasma PS activity compared to individuals with the KK genotype (mean ± SD, 71.9% ± 17.6%, n = 34 vs. 87.9% ± 19.8%, n = 1828, P < 0.0001) (Fig. 1). However, several heterozygous individuals with the mutant E-allele (n = 8) had measured PS activity greater than the total population average, while 26 wildtype subjects had PS activity at least 2SD less than the population mean (47.8%). Thus, PS activity does not appear to be a useful surrogate marker for PS genotype.

To determine whether an individual's genotype for any coagulation related protein could be determined by measuring the activity of the respective factor, we further examined the genotype and plasma activity of plasminogen in 3295 subjects. We identified 92 individuals heterozygous for the plasminogen A620T mutation, and the plasma plasminogen activity of these individuals was significantly less than wildtype individuals. Furthermore, there was little to no overlap between the measured plasminogen activities of wildtype and heterozygous individuals. Thus, the concept we originally wished to test was validated (Fig. 2).

There are well-documented gender- and age-related differences in PS activity [14], and this was true for our study population as reported [11] (Fig. 3A). When we examined the relationship between PS activity, genotype, and age, we observed decreased PS activity across all ages for individuals with the KE-genotype (Fig. 3B).

Discussion

DVT is a multi-factorial disease caused by the interaction of environmental and genetic factors. In Caucasian populations,

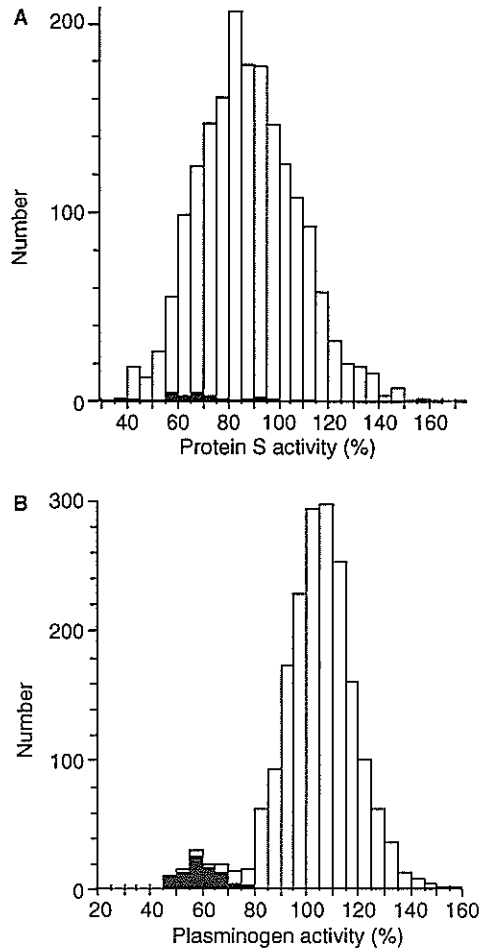

Fig. 2. Histogram representation of protein S (PS) (A) and plasminogen (B) activity in wildtype and heterozygous individuals. PS activity was measured in 1862 individuals, and plasminogen activity was measured in 3295 individuals. Activity was divided into groups by 5% increments, and mutation carriers are shown in closed bars.

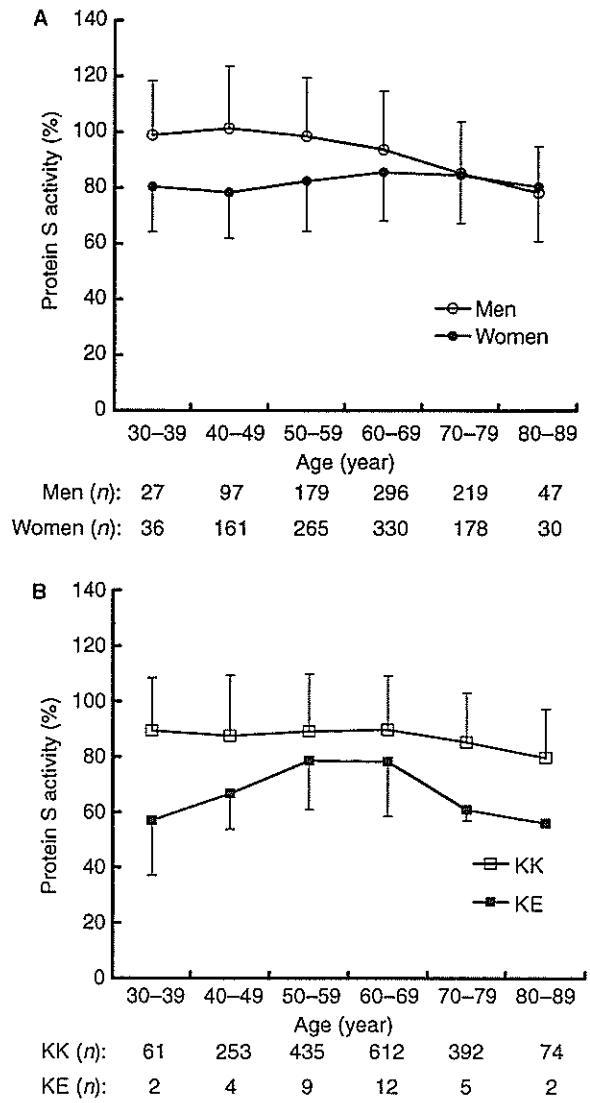

Fig. 3. Protein S (PS) activity divided in sex, age, and genotype. Open circles and closed circles in (A) show the mean PS activity in men and women, respectively. Open squares and closed squares in (B) show the mean PS activity in wild-type (KK-genotype) and heterozygote (KE-genotype). Error bars represent SD.

the FV Leiden (FVL) mutation, R506Q mutation in FV, is an important risk factor for the development of DVT. FVL carriers can be readily identified using the APC resistance test [15]. A FVL carrier will exhibit a prolonged clotting time in an activated thromboplastin time assay following the addition of APC. The incidence of this particular mutation varies in different ethnic populations [16,17] and is not observed in the Japanese [18]. In contrast, the PS K196E mutation present in the Japanese population is a genetic risk factor for DVT [4,5]. Therefore, a plasma assay for detecting PS 196E carriers should be developed. To understand the relation of the PS activity with the K196E mutation, we examined the PS activity and the K196E genotype in the Japanese population enrolled in the Suita Study.

The plasma PS activity in individuals with the PS K196E mutation remained controversial [6,9,10]. In one report, four members in a family who carried this mutation showed the PS activity with 37%, 72%, 101%, and 77%, respectively [10]. In a second family in this report, two members carried this mutation with the PS activity with 87% and 92%. On the basis of these results, the authors suggested this mutation as a phenotypically neutral polymorphism. In contrast, another study identified the same mutation correlated with low PS activity [6,9]. In this study, the authors identified this mutation in three patients with DVT. In addition, four individuals who did not show history of thrombosis were carriers of this mutation. All of these carriers showed low PS activity (mean ± SD, 43.1% ± 9.1%). Thus, so far, the relationship between the plasma PS activity and K196E mutation has not been settled. To address this issue, we have measured the PS activity and determined the genotype in the general Japanese population. As the results, we found that individuals heterozygous for the PS K196E mutation had reduced plasma PS activity compared to wildtype subjects, but this difference was relatively small and did not sufficiently differentiate between the two genotypes. In contrast, plasma

plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Regular Article

*Diverse Structures of Chimeric CYP-REP7/6-Containing CYP2D6 and a Novel Defective CYP2D6 Haplotype Harboring Single-type *36 and CYP-REP7/6 in Japanese*

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Summary: Chimeric REP7/6 has been used as a marker of *CYP2D6* deletion, such as for *CYP2D6*5*. However, the *CYP2D6*10D* (**10D*) haplotype found in a Japanese population consist of *CYP2D6*10B*, *CYP2D7P*-derived 3′-flanking region, and a chimeric repetitive sequence, CYP-REP7/6 (REP7/6) (Ishiguro *et al.* Clin. Chim. Acta. 2004: 347, 217–221). From our analysis, REP7/6 was found in 26 out of 254 Japanese subjects. Thus, the REP7/6-containing *CYP2D6* genes (*2D6*-REP7/6) were analyzed in detail. In order to specifically detect the *2D6*-REP7/6 structure, primers were designed in *CYP2D6* intron 6 and the REP7/6 3′-flanking region. Among 26 subjects analyzed by PCR, 5 had *2D6*-REP7/6. The other 21 subjects were confirmed to have **5* by another **5*-specific primer set. Three out of five subjects with *2D6*-REP7/6 had the **10D* structure. However, further analysis by PCR and sequencing revealed that their haplotypes were further divided into tandem-type **36*-**10D* (n=2) and single-type **10D* (n=1). The remaining two subjects had a novel type of a **36*-containing defective structure that consists of *CYP2D6*36* and 3′-flanking REP7/6 (single-type **36*-REP7/6). Then, REP7/6 sequences in **5*, **10D*, **36*-**10D*, and single-type **36* were determined and classified into 5 types: types A to D for **5*, type E for **10D* and **36*-**10D*, and type F for **36*. These findings could be useful for accurate determination of **5* and REP7/6-harboring aberrant *CYP2D6* haplotypes.

Key words: REP7/6 with *CYP2D6*; *CYP2D6*10D*; tandem-type *36-*10D*; single-type *CYP2D6*36*; CYP-REP diversity

Introduction

Cytochrome P450 (CYP) 2D6 is extremely important in drug therapy. It is involved in the metabolism of numerous drugs such as anti-arrythmics, psychiatrics, anti-histamines, and anti-depressants as well as endogenous substances.[1] Subjects administered these compounds can be divided into poor, intermediate, extensive, and ultrarapid metabolizers based on CYP2D6 phenotypes. In Caucasian populations, the frequency of poor metabolizers (PM) with defective alleles such as **3*, **4* and **5*, is approximately 7%.[2,3] In African populations, the PM frequency is approximately 1.9–8.7%.[4-7] In contrast, PM frequencies in Chinese and Japanese were reported as low as 0–0.7%.[8-12] However, the **10* allele, which confers a partially reduced enzymatic activity, has been detected at much higher frequencies in Japanese (37%),[13] Chinese (50.7%)[14] and Koreans (51%)[15] than in Caucasians (2.6%).[8]

The key single nucleotide polymorphism (SNP) of *CYP2D6*10* was reported as 100C>T (P34S) in exon 1 in Japanese.[12] Recently, a novel **10*-related haplotype, designated *CYP2D6*10D* (**10D*), was found by Ishiguro *et al.*[16] Its allelic frequency is approximately 0.3% in Japanese.[16,17] The **10D* haplotype is character-

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