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ORIGINAL ARTICLE

Reverse white-coat effect as an independent risk for left ventricular concentric hypertrophy in patients with treated essential hypertension

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Recent studies have shown that the converse phenomenon of white-coat hypertension called 'reverse white-coat hypertension' or 'masked hypertension' is associated with poor cardiovascular prognosis. We assessed the hypothesis that this phenomenon may specifically influence left ventricular (LV) structure in treated hypertensive patients. A total of 272 outpatients (mean age, 65 years) with chronically treated essential hypertension and without remarkable white-coat effect were enrolled. Patients were classified into two groups according to office and daytime ambulatory systolic blood pressure (SBP); that is subjects without (Group 1: office SBP $\geqslant$daytime SBP, $n=149$) and with reverse white-coat effect (Group 2: office SBP$<$daytime SBP, $n=123$). LV mass index and relative wall thickness were echocardiographically determined. In all subjects, LV mass index and relative wall thickness were positively correlated with daytime and 24-h SBP, but not with office SBP. In addition, these two indices were inversely correlated with office – daytime SBP difference. LV mass index (136±31 and 115±28 g/m^2, mean±s.d.) and relative wall thickness (0.49±0.09 and 0.46±0.07) were significantly greater in Group 2 than in Group 1. As for LV geometric patterns, Group 2 had a significantly higher rate of concentric hypertrophy compared with Group 1 (48 and 28%). Multivariate analyses revealed that the presence of reverse white-coat effect was a predictor for LV concentric hypertrophy, independent of age, sex, hypertension duration, antihypertensive treatment and ambulatory blood pressure levels. Our findings demonstrate that reverse white-coat effect is an independent risk factor for LV hypertrophy, especially concentric hypertrophy, in treated hypertensive patients.

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Introduction

Ambulatory blood pressure (BP) is an important determinant of target organ damage and a significant predictor for cardiovascular morbidity and mortality in hypertensive patients.[1–6] There is often a discrepancy between office and ambulatory BPs, such as white-coat hypertension, a normal ambulatory but elevated office BP. On the other hand, the converse phenomenon of white-coat hypertension called 'reverse white-coat hypertension' or 'masked hypertension', that is, a high ambulatory but normal (or well-controlled) office BP, has received little attention.[7] Whereas, some studies have revealed that the proportion of subjects with reverse white-coat condition reaches 20–40% of the general population and hypertensives.[8,9] In treated hypertensive patients with this phenomenon, particularly, the chance of active and sufficient antihypertensive treatment may be lost by an apparent well-controlled BP in the office. Recent studies suggested that an elevated ambulatory or home BP despite a well-controlled office BP is associated with poor cardiovascular prognosis in treated hypertensive patients.[10,11] However, it remains unclear what mechanism is involved in the association of reverse white-coat phenomenon with cardiovascular prognosis.

Left ventricular hypertrophy (LVH), which is a common cardiac consequence of hypertension, is well known to be an independent risk factor for cardiovascular complications and death.[12,13] In addition, left ventricular (LV) morphologic

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alteration in hypertensive patients is not uniform, and concentric hypertrophy among various LV geometric patterns is shown to be most closely related to poor cardiovascular prognosis.[13]

Thus, we hypothesized that the presence of reverse white-coat effect may promote LV hypertrophy, especially concentric hypertrophy, in treated hypertension. To assess the hypothesis, the present study investigated the influence of reverse white-coat effect on LV mass and geometry in treated hypertensive patients.

Methods

Subjects

From consecutive patients with essential hypertension who were chronically treated and underwent a 24-h ambulatory BP monitoring at an outpatient clinic of our hospital between May 2000 and December 2003, 272 subjects (142 men and 130 women; mean age, 65 years) in whom satisfactory echocardiographic data were simultaneously obtained were enrolled in the present study. Patients with secondary hypertension, stroke, ischaemic heart disease including myocardial infarction, congestive heart failure, renal failure (serum creatinine $\geqslant 160\,\mu$mol/l) or poorly controlled (haemoglobin A1c $\geqslant 8.0\%$) or insulin-treated diabetes mellitus were excluded from this study. Individuals with a remarkable white-coat effect (described below) were also excluded. Diabetes mellitus was diagnosed according to the American Diabetes Association criteria, such as a fasting plasma glucose of $\geqslant 7.0$ mmol/l and/or a plasma glucose level at 2 h after a 75-g oral glucose load of $\geqslant 11.1$ mmol/l, or when medication was taken for treatment of hyperglycaemia. A diagnosis of hyperlipidemia required a serum total cholesterol level of $\geqslant 5.69$ mmol/l and/or a serum triglyceride level of $\geqslant 1.69$ mmol/l or the use of lipid-lowering drugs, according to the Japan Atherosclerosis Society guidelines.[14]

All patients had taken antihypertensive drugs for at least 1 year (average, 12 years). One hundred and ninety-five patients (72%) were treated with Ca channel blockers, 140 (51%) with renin angiotensin system inhibitors (i.e., angiotensin II receptor blockers and angiotensin converting enzyme inhibitors), 82 (30%) with β-blockers, 53 (19%) with diuretics and 29 (11%) with other classes of agents. All subjects gave their informed consent to participate in the present study. All procedures of the present study were carried out in accordance with institutional and national ethical guidelines for human studies.

Measurement of BP

In each visit, office BP was measured twice by a physician in a hospital outpatient clinic with the patient in a sitting position after over 20 min of rest, using an appropriate-size cuff on the left arm and mercury sphygmomanometer. The first and fifth Korotkoff sounds were used to identify systolic and diastolic values, respectively, and measurements were taken to the nearest 2 mm Hg. Office BP was determined by averaging six measurements taken on three separate occasions during a 3-month period.

In the same study period, all subjects underwent 24-h ambulatory BP monitoring. BP was measured every 30 min during the day and night by the oscillometric method using an automatic monitoring device (TM-2421, A&D Co Ltd, Tokyo, Japan).[15] The accuracy and performance of this device have been demonstrated previously.[16] The patients were instructed to carry on with their normal daily activities during measurements and note their activity and location in a diary. According to the diary, daytime and night time were determined as the waking and sleeping periods of the patient, respectively, and mean values of daytime, night time and 24-h BP (systolic and diastolic) were calculated. Nocturnal BP dipping was determined as 100 × (daytime BP−night time BP)/daytime BP.

In the present study, all subjects were classified into two groups by the difference between office and daytime ambulatory systolic BP levels; that is, subjects without reverse white-coat effect (Group 1: office systolic BP $\geqslant$ daytime systolic BP, and office systolic BP−daytime systolic BP $<$ 20 mm Hg) and with reverse white-coat effect (Group 2: office systolic BP $<$ daytime systolic BP). Subjects with a remarkable white-coat effect (office systolic BP−daytime systolic BP $\geqslant$ 20 mm Hg) were excluded from the study.

Echocardiography

A comprehensive 2-dimensional and M-mode echocardiography was performed using a cardiac ultrasound unit (Sonos 5500, Philips Medical Systems, Andover, MA, USA) as described previously.[17] Echocardiographic parameters were measured by the consensus of two experienced investigators who were blinded to the clinical data including office and ambulatory BP of the subjects. Interventricular septal thickness (IVSTd), posterior wall thickness (PWTd), LV diameter at end-diastole (LVDd), and LV diameter at end-systole (LVDs) were measured according to the American Society of Echocardiography recommendations.[18,19] Fractional shortening was calculated as 100 × (LVDd−LVDs)/LVDd. Relative wall thickness (RWT) was calculated as (IVSTd + PWTd)/LVDd. LV mass was estimated using the formula validated by Devereux and Reichek[20]: LV mass (g) = $1.04 \times \{(\text{IVSTd} + \text{PWTd} + \text{LVDd})^3 - \text{LVDd}^3\} - 13.6$. LV mass was normalized for body surface area and expressed as the LV mass index (LVMI). LVH was defined as a LVMI of $\geqslant 125$ g/m^2 in men and 110 g/m^2 in women.[21] The intra-observer and inter-observer coefficients of variation of LVMI were 6.7 and 9.8%, respectively.

The geometry of LV was stratified into four different patterns according to the values of LVMI (< or ⩾125/110 g/m^2, men/women) and RWT (< or ⩾0.44). Patients with increased LVMI and increased RWT were considered to have concentric hypertrophy, and those with increased LVMI and normal RWT were considered to have eccentric hypertrophy. Those with normal LVMI and increased or normal RWT were considered to have concentric remodelling or normal geometry, respectively.

Biochemical measurement

Blood samples were obtained in the morning after an overnight fast. Total cholesterol, triglycerides, fasting plasma glucose, haemoglobin A1c and serum creatinine levels were determined by standard laboratory measurements. Creatinine clearance was calculated from the Cockcroft-Gault formula.[22]

Statistical analysis

Statistical analysis was performed using StatView Version 5 Software (Abacus Concepts Inc., Berkeley, CA, USA). Values are expressed as the mean ± s.d. Simple correlations between variables were assessed using univariate linear regression analyses and Pearson's correlation coefficient. An unpaired Student's *t*-test was used for comparison between the two groups. The significance of differences among the three groups was evaluated by an unpaired ANOVA with subsequent Fisher's multiple comparison test. A multiple logistic regression analysis was performed to identify independent determinants of LV mass increase and concentric hypertrophy. A value of $P<0.05$ was accepted as statistically significant.

Results

Simple correlations of office and ambulatory BP levels with two indices of LV structural changes, LVMI and RWT, in all subjects are shown in Table 1. Office systolic or diastolic BP had no correlation with either LVMI or RWT. In contrast, LVMI and RWT were positively correlated with daytime and 24-h systolic BPs, and LVMI was also correlated with night time systolic BP. In addition, these two indices were significantly correlated with the difference between office BP and daytime BP. As shown in Figure 1, LVMI had a close negative correlation with office–daytime systolic BP difference ($r=-0.377$, $P<0.001$). RWT were also inversely correlated with office–daytime systolic BP difference ($r=-0.170$, $P=0.005$). These results suggested that reverse white-coat effect was significantly associated with increases in LVMI and RWT.

Clinical characteristics of the two subject groups classified according to the difference between office and daytime ambulatory systolic BP levels are summarized in Table 2. One hundred and twenty-three (45%) patients were identified as having reverse white-coat effect (Group 2), and the other 149 (55%) patients belonged to Group 1. The proportion of men and the rate of habitual drinkers

Table 1 Correlation of office and ambulatory blood pressure with left ventricular structure in all subjects

	LVMI		*RWT*	
	r	P	r	P
Office systolic BP	0.039	0.526	0.014	0.816
Office diastolic BP	−0.124	0.053	−0.040	0.508
Daytime systolic BP	0.290	<0.001	0.173	0.004
Daytime diastolic BP	0.020	0.742	0.100	0.099
Night time systolic BP	0.318	<0.001	0.113	0.062
Night time diastolic BP	0.099	0.104	0.078	0.198
24-h systolic BP	0.325	<0.001	0.158	0.009
24-h diastolic BP	0.051	0.398	0.096	0.113
(Office − daytime) systolic BP	−0.377	<0.001	−0.170	0.005
(Office − daytime) diastolic BP	−0.211	<0.001	−0.147	0.015

Abbreviations: BP, blood pressure; LVMI, left ventricular mass index; RWT, relative wall thickness.

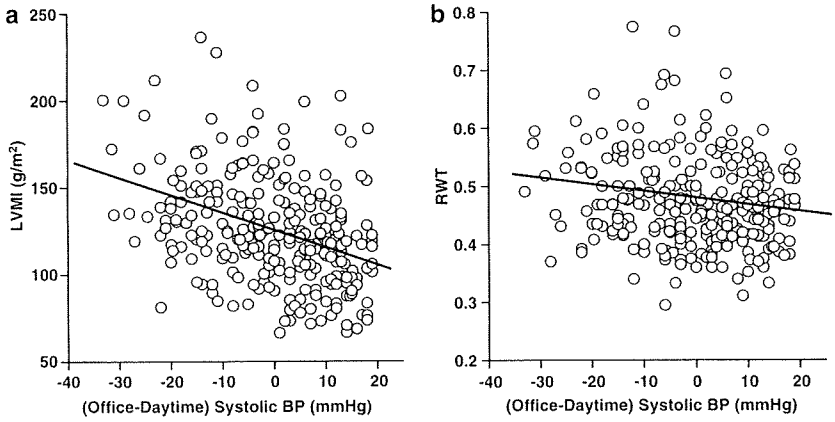

Figure 1 Correlation of the difference between office and daytime systolic BP levels with LVMI (**a**, $r=-0.377$, $P<0.001$) and RWT (**b**, $r=-0.170$, $P=0.005$) in all subjects.

Table 2 Clinical characteristics of two study groups

	Group 1 (n = 149)	*Group 2 (n = 123)*	P
Age (years)	65.8 ± 9.1	65.1 ± 11.0	0.593
Sex (male) (%)	41.6	65.0	<0.001
Body mass index (kg/m^2)	24.2 ± 2.9	24.7 ± 4.0	0.182
Duration of hypertension (years)	17.6 ± 10.8	17.8 ± 11.0	0.850
Diabetes mellitus (%)	19.5	23.6	0.412
Hyperlipidemia (%)	64.9	66.1	0.831
Current smoking (%)	15.6	21.1	0.235
Habitual drinking (%)	50.7	63.6	0.033
Creatinine clearance (ml/min)	81.5 ± 24.8	85.1 ± 32.8	0.297
Fasting plasma glucose (mmol/l)	5.7 ± 1.2	5.8 ± 1.1	0.486
Hemoglobin A1c (%)	5.6 ± 0.8	5.7 ± 0.7	0.257
Total cholesterol (mmol/l)	5.3 ± 0.8	5.2 ± 0.7	0.576
Triglycerides (mmol/l)	1.4 ± 0.7	1.5 ± 0.8	0.126
Antihypertensive treatment			
Period of medication (years)	12.4 ± 9.3	11.7 ± 9.1	0.497
Ca channel blockers (%)	71.8	71.5	0.961
RAS inhibitors (%)	49.7	53.7	0.514
β-Blockers (%)	28.2	32.5	0.440
Diuretics (%)	16.8	22.8	0.216
Others (%)	9.4	12.2	0.458
Total number of classes	1.8 ± 0.9	1.9 ± 0.9	0.141
Office systolic BP (mm Hg)	145.6 ± 12.7	133.8 ± 11.6	<0.001
Office diastolic BP (mm Hg)	83.4 ± 9.9	78.8 ± 10.0	<0.001
Daytime systolic BP (mm Hg)	136.5 ± 12.6	145.1 ± 11.9	<0.001
Daytime diastolic BP (mm Hg)	80.1 ± 9.3	84.8 ± 11.5	<0.001
Night time systolic BP (mm Hg)	126.8 ± 14.9	134.1 ± 15.8	<0.001
Night time diastolic BP (mm Hg)	73.1 ± 9.5	76.9 ± 11.1	0.002
24-h systolic BP (mm Hg)	134.0 ± 12.3	141.6 ± 12.2	<0.001
24-h diastolic BP (mm Hg)	78.2 ± 9.0	82.3 ± 10.5	<0.001
Nocturnal systolic BP dipping (%)	7.1 ± 8.0	7.6 ± 8.0	0.572
Nocturnal diastolic BP dipping (%)	8.5 ± 8.4	9.0 ± 8.5	0.671

Abbreviations: BP, blood pressure; RAS, renin angiotensin system.
RAS inhibitors represent angiotensin II receptor blockers and angiotensin converting enzyme inhibitors. Values are mean ± s.d. or percentage.

were significantly higher in Group 2 than in Group 1. Age, body mass index, hypertension duration, the prevalence of diabetes mellitus and hyperlipidemia, the rate of current smokers, renal function and glucose and lipid parameters did not differ between the two groups. In addition, there were no intergroup differences in the period of medication, the use of any class of antihypertensive agent and the total number of classes of antihypertensive drugs.

Office and ambulatory BP levels had clear differences between the two groups. That is, Group 2 had significantly lower office systolic and diastolic BPs than Group 1, but daytime, night time, and average 24-h ambulatory BPs in Group 2 were significantly elevated compared with those in Group 1. The degree of nocturnal BP dipping, an index of circadian BP variation, did not differ between the two groups.

The comparison of echocardiographic parameters between the two groups is shown in Table 3. Group 2 had a significantly greater LVMI than Group 1, resulting from more increased LV wall thickness and internal dimension. RWT was also significantly increased in Group 2 compared with Group 1. In addition, the prevalence of LVH, defined as an increased LVMI by sex, was significantly higher in Group 2. There was no difference in fractional shortening between the two groups.

Table 3 Comparison of ecchocardiographic parameters between the two groups

	Group 1 (n = 149)	*Group 2 (n = 123)*	P
IVSTd (mm)	10.3 ± 1.5	11.4 ± 1.9	<0.001
PWTd (mm)	10.3 ± 1.4	11.1 ± 1.5	<0.001
LVDd (mm)	44.8 ± 4.5	46.8 ± 4.2	<0.001
LVDs (mm)	26.5 ± 4.9	27.7 ± 4.6	0.037
Fractional shortening (%)	41.1 ± 7.4	41.0 ± 6.8	0.920
LVMI (g/m^2)	115.3 ± 28.3	136.4 ± 30.8	<0.001
RWT	0.46 ± 0.07	0.49 ± 0.09	0.010
Prevalence of LVH (%)	41.6	65.9	<0.001

Abbreviations: IVSTd, interventricular septal thickness at end-diastole; LVDd, left ventricular diameter at end-diastole; LVDs, left ventricular diameter at end-systole; LVMI, left ventricular mass index; LVH, left ventricular hypertrophy; PWTd, posterior wall thickness at end-diastole; RWT, relative wall thickness.
LVH is defined as LVMI of ⩾125 g/m^2 in men and 110 g/m^2 in women. Values are mean ± s.d. or percentage.

To assess the impact of reverse white-coat effect on LVH, Group 2 was divided into two sub-groups by the extent of its phenomenon. As shown in Figure 2, both LVMI and prevalence of LVH were

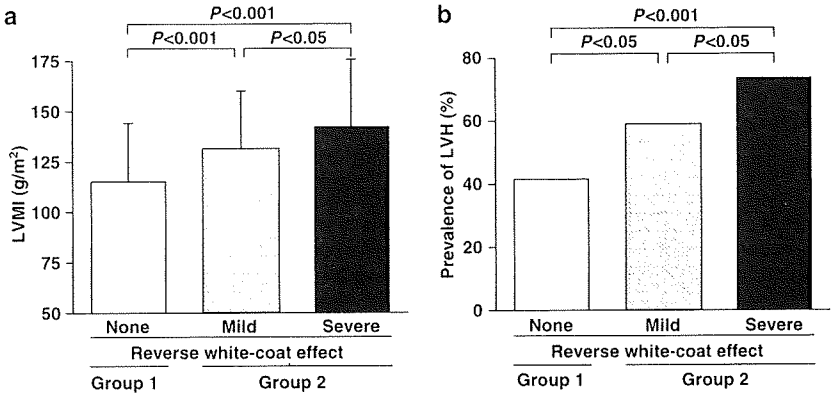

Figure 2 Comparison of LVMI (**a**) and prevalence of LVH (**b**) among the three groups classified by the extent of reverse white-coat effect. None, office systolic BP ⩾daytime systolic BP (i.e., Group 1, $n=149$); Mild, office systolic BP < daytime systolic BP, but daytime systolic BP−office systolic BP < 10 mm Hg ($n=63$); Severe, daytime systolic BP−office systolic BP ⩾10 mm Hg ($n=60$). LVH is defined as LVMI of ⩾125 g/m² in men and 110 g/m² in women. Values are given as the mean ± s.d. (**a**) or percentage (**b**).

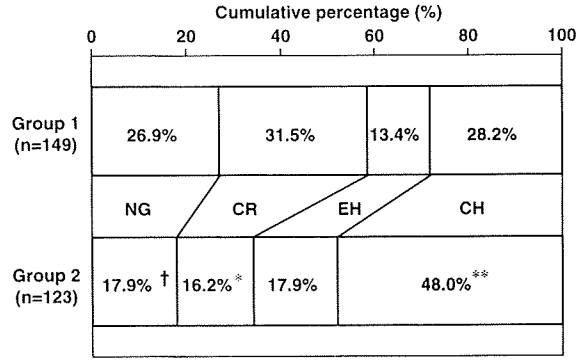

Figure 3 Comparison of LV geometric patterns between the two groups. NG, normal geometry (normal LVMI and RWT); CR, concentric remodelling (normal LVMI and increased RWT); EH, eccentric hypertrophy (increased LVMI and normal RWT); CH, concentric hypertrophy (increased LVMI and RWT). †$P<0.05$, *$P<0.01$, and **$P<0.001$ vs Group 1.

significantly greater in subjects with mild reverse white-coat effect (office systolic BP < daytime systolic BP, but daytime systolic BP−office systolic BP < 10 mm Hg) than in those without reverse white-coat effect (i.e., Group 1), and these values were further increased significantly in the sub-group with severe reverse white-coat effect (daytime systolic BP−office systolic BP ⩾10 mm Hg).

Figure 3 shows the comparison of LV geometric patterns between the two groups. Group 2 had a significantly higher rate of concentric hypertrophy compared with Group 1 (48 vs 28%, $P<0.001$). In contrast, the rates of patients with normal geometry and concentric remodelling were significantly lower in Group 2 than in Group 1.

To confirm whether the influence of reverse white-coat phenomenon on LV mass increase and specific geometric change was independent of various clinical parameters, we investigated possible predictive factors using a multiple logistic regression analysis in all subjects (Table 4). Although average 24-h systolic BP was the strongest predictor for both LVH and concentric hypertrophy, the presence of reverse white-coat effect (i.e., Group 2) was found to be a significant determinant for these LV structural changes, independent of age, sex, body mass index, hypertension duration, the use of any class of antihypertensive agent and 24-h systolic and diastolic BP levels (for LVH: odds ratio 2.42 vs Group 1, $P=0.005$; for concentric hypertrophy: odds ratio 1.89, $P=0.039$). The significant predictive value of reverse white-coat effect remained even when daytime systolic and diastolic BPs, instead of 24-h BPs, were adopted as independent predictors (data not shown).

Discussion

This study has demonstrated that the presence of reverse white-coat effect is one of the independent predictors for LVH, especially for LV concentric hypertrophy, in patients with treated essential hypertension. The new findings suggest that reverse white-coat phenomenon, independent of average ambulatory blood pressure levels, may have an unfavourable influence on left ventricular geometry in essential hypertension.

The present subjects with reverse white-coat effect (Group 2) had a controlled office BP in spite of elevated ambulatory BP, indicating that the group took on an aspect of masked hypertension. There have been a few studies reporting the possible association between masked hypertension and cardiac and carotid arterial structural changes in the general population. Liu *et al.*[23] found that LV mass and carotid wall thickness in patients with masked hypertension were significantly greater than those in true normotensive subjects and similar to those in patients with sustained hypertension. The data from the PAMELA Study also showed that LVMI was

Table 4 Independent predictors for left ventricular mass increase and concentric hypertrophy by multiple logistic regression analysis

	LVH		*Concentric hypertrophy*	
	OR (95% CI)	P	*OR (95% CI)*	P
Age (10 years)	0.88 (0.58–1.34)	0.544	0.70 (0.46–1.05)	0.087
Sex (male)	0.60 (0.30–1.16)	0.128	0.82 (0.42–1.60)	0.557
Body mass index (1 kg/m^2)	1.10 (1.00–1.23)	0.046	1.10 (1.00–1.22)	0.053
Hypertension duration (1 year)	1.02 (0.99–1.05)	0.123	1.03 (1.00–1.06)	0.028
Diabetes mellitus (yes)	1.04 (0.48–2.22)	0.929	1.02 (0.49–2.13)	0.959
Hyperlipidemia (yes)	1.49 (0.82–2.71)	0.186	1.35 (0.73–2.47)	0.339
Current smoking (yes)	0.99 (0.46–2.13)	0.978	1.25 (0.60–2.58)	0.553
Habitual drinking (yes)	1.09 (0.58–2.06)	0.783	1.22 (0.64–2.32)	0.541
Creatinine clearance (10 ml/min)	0.91 (0.77–1.06)	0.231	0.87 (0.74–1.02)	0.095
Ca channel blocker (yes)	1.36 (0.67–2.77)	0.402	1.09 (0.53–2.24)	0.808
RAS inhibitor (yes)	1.15 (0.60–2.21)	0.678	1.57 (0.82–2.99)	0.170
β-Blocker (yes)	1.89 (0.99–3.59)	0.052	1.36 (0.73–2.55)	0.337
Diuretic (yes)	1.04 (0.49–2.22)	0.921	0.72 (0.34–1.56)	0.407
24-h systolic BP (10 mm Hg)	2.35 (1.66–3.33)	<0.001	1.97 (1.45–2.68)	<0.001
24-h diastolic BP (10 mm Hg)	0.60 (0.40–0.90)	0.014	0.67 (0.45–0.98)	0.041
Reverse white-coat effect				
Absence (Group 1)	1 (reference)		1 (reference)	
Presence (Group 2)	2.42 (1.31–4.48)	0.005	1.89 (1.03–3.44)	0.039

Abbreviations: BP, blood pressure; CI, confidence interval; LVH, left ventricular hypertrophy; OR, odds ratio; RAS, renin angiotensin system. RAS inhibitor represents angiotensin II receptor blocker or angiotensin converting enzyme inhibitor. LVH is defined as LVMI of $\geqslant$125 g/m^2 in men and 110 g/m^2 in women. Concentric hypertrophy is defined as LVH combined with increased RWT ($\geqslant$0.44).

increased in untreated subjects with masked hypertension and sustained hypertension than in those with true normotension.[24] In addition, our recent study showed that masked hypertension was associated with advanced target organ damage in treated hypertensive patients, comparable to that in cases of sustained hypertension.[25] Furthermore, prospective studies have revealed that a high ambulatory or home BP is a powerful predictor for cardiovascular morbidity and mortality in the general population and treated hypertensive patients even when their office BP is normal or well controlled.[10,11,26–28] As for the association between LV geometry and cardiovascular prognosis, it was reported that hypertensive patients with concentric hypertrophy among four LV geometric patterns had the highest incidence of cardiovascular events and death.[13] Taken together, it is likely that advanced target organ changes including LV concentric hypertrophy in patients with masked hypertension or reverse white-coat condition are linked to poor cardiovascular prognosis in such patients.

A higher level of ambulatory BP is a major determinant of target organ damage in hypertensive patients.[1,2] In the present study, however, the presence of reverse white-coat effect was a significant predictor for LVH and concentric hypertrophy, independent of average 24-h ambulatory BP levels. Other factors than a higher ambulatory BP could contribute to target organ damage in reverse white-coat hypertension. Our study has not provided the specific mechanism by which reverse white-coat effect could promote LV concentric hypertrophy in patients with treated hypertension. Therefore, further investigations are required to clarify how reverse white-coat or masked hypertension has a specific unfavourable effect on the hypertensive target organ.

There were some limitations in our study. The present findings were derived from cross-sectional data on the basis of one-time examination of ambulatory BP monitoring and echocardiography. Our subjects were divided into subgroups based on office–daytime difference only in systolic BP, not considering diastolic BP difference. In addition, cardiac magnetic resonance imaging might be more adequate than echocardiography in evaluating LV mass exactly.

All patients in the present study had received antihypertensive medication. As another limitation of this study, therefore, we must consider the possibility that different classes of antihypertensive drugs may have differently affected the development of LVH, partly independently of their BP-lowering effects. Renin angiotensin system inhibitors, particularly, are known to have BP fall-independent protective effects on hypertensive target organ. However, the percentage of patients treated with angiotensin II receptor antagonists or angiotensin converting enzyme inhibitors did not differ between the two study groups. Our multivariate analysis also showed that the association of reverse white-coat effect with LVH and concentric hypertrophy was independent of the use of any class of antihypertensive agent.

In conclusion, the present study indicates that reverse white-coat effect is a significant determinant of LVH, especially concentric hypertrophy, in patients with treated essential hypertension, independent of average ambulatory BP levels and

various other clinical risk factors. Our findings suggest that the presence of this phenomenon may be an independent risk for the adverse LV geometric change in treated hypertensive patients and ambulatory BP monitoring seems to be necessary to unmask this latent risk that is not detectable by routine BP measuring in the office.

What is known about this topic

- Ambulatory blood pressure is an important determinant of target organ damage and a predictor for cardiovascular morbidity and mortality in hypertensive patients.
- The converse phenomenon of white-coat hypertension called 'reverse white-coat hypertension' or 'masked hypertension' is associated with poor cardiovascular prognosis.
- Left ventricular hypertrophy, especially concentric hypertrophy, is a significant risk factor for cardiovascular complications and death.

What this study adds

- Reverse white-coat effect was an independent predictor for left ventricular hypertrophy, especially for concentric hypertrophy, in treated hypertensive patients.
- The presence of reverse white-coat phenomenon, independent of average ambulatory blood pressure levels, may have an unfavourable influence on left ventricular geometry.

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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese.
Patients and methods: We sequenced the TM putative promoter, exon, and 3′-untranslated region in DVT patients (n=118). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms (−202G>A, 2487A>T, 2729A>C) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5′-UTR, 5′-untranslated region; 3′-UTR, 3′-untranslated region.

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with that in the age, body mass index-adjusted population-based controls.
Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio=2.76, 95% confidence interval=1.14–6.67; $p=0.02$). This mutation was in linkage disequilibrium (r-square>0.9) with A455V mutation.
Conclusions: TM mutations, especially those with a haplotype consisting of 2729A>C and A455V missense mutation, affect sTM levels, and may be associated with DVT in Japanese.

Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3′ -untranslated regions (3′ -UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

DVT for genetic variation. By genotyping three polymorphisms (−202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

Patients and methods

DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age: 52.3 ± 16.1 years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3′ -UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

Table 1 Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years ± S.D.	52.3 ± 16.1	Nephrotic syndrome, *n* (%)	0 (0.0)
Women, *n* (%)	59 (50.0)	Chronic heart failure, *n* (%)	17 (14.4)
BMI, kg/m^2, mean ± S.D.	23.7 ± 3.2	Diabetes Mellitus, *n* (%)	47 (39.8)
DVT family history, *n* (%)	8 (6.8)	Hyperlipidemia, *n* (%)	48 (40.7)
Previous DVT, *n* (%)	12 (10.2)	Autoimmue disease, *n* (%)	11 (9.3)
		Inflammatory bowel disease, *n* (%)	2 (1.7)
Pregnancy, *n* (%)	5 (4.2)	Estrogen use, *n* (%)	3 (2.5)
Stroke, *n* (%)	1 (1.5)	Steroid use, *n* (%)	9 (7.6)
Prolonged immobility, *n* (%)	14 (11.9)	Paralysis, *n* (%)	5 (4.2)
Malignancy, *n* (%)	16 (13.6)	Myeloproliferative disease, *n* (%)	1 (0.8)
Major surgery (abd, hip, leg), *n* (%)	21 (17.8)	Reduced plasminogen activity, *n* (%)	7 (5.9)
Trauma (pelvis, hip, leg), *n* (%)	3 (2.5)	Reduced antithrombin activity, *n* (%)	7 (5.9)
Stasis due to compression, *n* (%)	6 (5.1)	Reduced protein C activity, *n* (%)	8 (6.8)
Central venous catheter, *n* (%)	0 (0.0)	Reduced protein S antigen, *n* (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin,ACLb2), *n* (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication; Hypertension, systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Myeloproliferative disease, Plt. $>5\times10^5$ and Ht. >55%; Reduced plasminogen activity, plasminogen activity <70%; Reduced antithrombin activity, antithrombin activity <80%; Reduced protein C activity, protein C activity <70%; Reduced protein S antigen, protein S antigen <60%.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r-square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means ± S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t-test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was established at $p<0.05$. Linkage disequilibrium was calculated using SNPAlyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*−832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5′ UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5′ UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5′ UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaagggtg [g/t] acggtggcgaca	
1754C>T		Exon(3′ UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3′ UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3′ UTR)		99.6	0.4	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3′ UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3′ UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3′ UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3′ flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3′ flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r-square=0.84, group B r-square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women ($n=1215$)	Men ($n=1032$)	p
Age, years ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's t-test or X^2 analysis.

mutations were missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$). Four mutations within the TM promoter region and the 5′ -untranslated region (5′ -UTR) (−832C>A, −754G>C, −265C>A, −58G>C) were rare. Twenty-five patients were heterozygous carriers for the −202G>A mutation within the promoter region, which was reported as a −33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r-square>0.8) with 2005G>A in the 3′ -UTR. No patients were carriers for previously reported mutations in the lectin-like domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain ($n=53$ heterozygous, $n=14$ homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r-square>0.9) with the 2729A>C mutation within the 3′ -UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (−202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects. As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p=0.02$ and odds ratio = 2.98, 95% confidence interval = 0.21–7.33; $p=0.02$, respectively) (Table 6). This mutation was in linkage disequilibrium (r-square > 0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3′ -UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β-estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2, or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3′ -UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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Age- and gender-related differences of plasma prothrombin activity levels

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Dear Sir,

Advancing age is an important risk factor for venous or arterial thrombosis in both sexes (1–3). Moreover, gender is associated with differences in the prothrombotic state and in the progression of atherosclerosis that occurs with aging (4, 5). Prothrombin is one of the dominant factors influencing thrombin generation (6), and the prothrombin G20210A mutation accompanied by an increased level of prothrombin poses a risk factor for venous or arterial thrombosis (7, 8). However, gender differences in age-related changes in plasma prothrombin activity have not been investigated until now. In the present study, we measured prothrombin activity in 742 individuals derived from a general Japanese population which was supposed to be free of prothrombin G20210A mutation (9).

The study population was composed of samples randomly selected from the residents of Suita, a city located in the second largest urban area in Japan (the Suita Study) (4). All subjects had been visiting the National Cardiovascular Center every two years since 1989 for regular health checkups. Only subjects who provided written informed consent to have a blood examination were enrolled in this study. We excluded subjects treated with oral anticoagulant therapy. Finally, 742 subjects, aged 36 to 85 years (mean age: 64 years), were included in this study. Spearman correlation analysis was used to assess the association between aging and the level of prothrombin activity within a given gender. For comparison between the two gender groups, the Mann-Whitney U test was used. Differences with a value of $p<0.01$ for the Spearman correlation analysis and $p<0.05$ for the Mann-Whitney U test were considered to be significant. Statistical calculations were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL, USA). Prothrombin activity was measured according to a published method (10) with a modification. Briefly, 200 µl of 20 mM Tris-HCl, 0.14 M NaCl, pH 7.5 buffer containing 1 mg/ml of bovine serum albumin (TBSA) was added to 50 µl of plasma anticoagulated with 0.13% sodium citrate. Then, diluted plasma was incubated for 150 seconds at 37°C, and we detected ΔA/min at 405 nm after adding 50 µl of the reagent containing 6 mM $CaCl_2$, 0.5 mM Boc-Val-Pro-Arg-pNA as a thrombin substrate, 500 pM carinactivase-1 as a thrombin activator, and TBSA. Calibration was performed with a standard-human-plasma (Dade Behring GmbH, Marburg, Germany). The coefficient of intra-assay variation for prothrombin activity assay was 2.0%.

The mean ± SD of prothrombin activity level in men and women was 110.2 ± 17.0 (range: 54.5–158.5%) and 120.4 ± 17.4 (range: 57.5–194.4%), respectively. Figure 1 shows the age-related distribution (36–85 years) of prothrombin activity in 348 men (Fig. 1A) and 394 women (Fig. 1B). As a whole, a linear decrease of prothrombin activity level with age was observed in

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men (r=-0.34, p<0.0001), but not in women (r=-0.04, p=0.47). When prothrombin activity level was analyzed in 10-year age groups, significant decreases were observed in the men aged 46–55 years and 56–65 years (p<0.0001), aged 56–65 years and 76–85 years (p<0.05), and in the women aged 66–75 years and 76–85 years (p<0.0001). Levels of prothrombin activity were decreased in both sexes in the oldest age group (aged 76–85 years). With regards to gender-related change, the prothrombin activity level in the age group of 56–65 years, 66–75 years, and 76–85 years was significantly lower in men than in women.

In the present study, we showed the age-related decrease in the plasma prothrombin activity of men and gender-related change in the plasma prothrombin activity. These results contribute to the understanding of age-related hypercoagulability and to the practical institution of anticoagulant therapy in older patients. It has been established that thrombin generation increases with age in both sexes, evidenced by plasma prothrombin fragment F1+2 levels produced by the cleavage of prothrombin by factor Xa (11, 12). Age-related hypercoagulability does not likely stem from the prothrombin activity, because the prothrombin activity of men showed the age-related decrease, but it may result from some other mechanisms including decreased levels of anticoagulant proteins such as protein C and S (11, 13). We presented here the gender-related change of significantly lower prothrombin activity levels in men in the age of 56–85 years than in women. Men tend to develop thrombotic events including recurrent venous thrombosis (14), but this tendency was not related to the plasma level of prothrombin activity. Our work sheds further light on the point that, when considering relative hypercoagulability, gender-adjustment is necessary for the comparison of prothrombin activity levels.

With regards to anticoagulant therapy, the plasma levels of vitamin K-dependent coagulation factors decrease with increasing intensity of anticoagulation therapy (15). At the same time, the risks of major haemorrhage increase according to the intensity of anticoagulation therapy, especially in patients older than 80 years (16). Given our current study results, the markedly decreased prothrombin level in the age group of 76–85 years, especially in men, provides a potential mechanistic explanation for the increased rate of major haemorrhage observed in elderly patients receiving anticoagulant therapy.

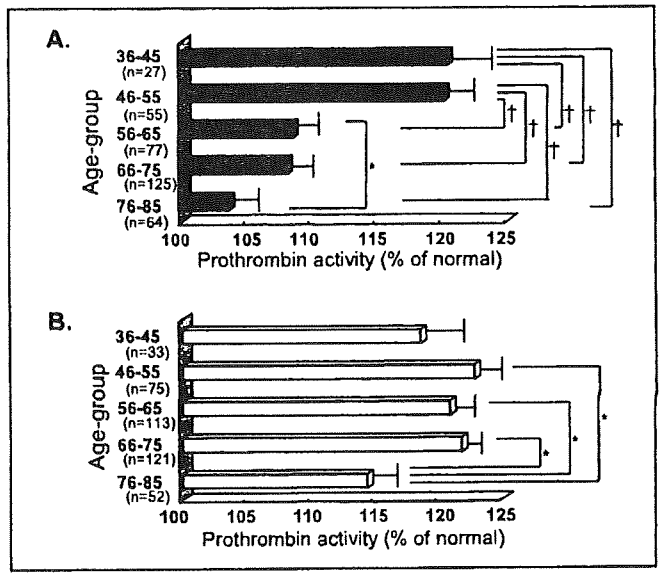

Figure 1: Age-related changes of plasma prothrombin activity levels according to gender (A: men, B: women). Populations aged from 36 to 85 years old were divided into five age groups by gender. Data are expressed as the mean ± SEM. *: P<0.05, †: P<0.0001, compared between two age groups of the same gender.

In conclusion, there are significant age- and gender-related differences in plasma prothrombin activity levels. In particular, the prothrombin activity level in men in the age group of 76–85 years was lower than that of any other age group in either gender.

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