Original Article

Central blood pressure relates more strongly to retinal arteriolar narrowing than brachial blood pressure: the Nagahama Study

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Objectives: Although central blood pressure (BP) is considered to be more closely associated with large arterial remodeling and cardiovascular outcomes than brachial BP, few studies have investigated these associations with changes in small arteries. As morphological changes in retinal vessels might be associated with cardiovascular outcomes, we conducted a cross-sectional study to investigate the association of central BP with retinal vessel caliber.

Methods: The study included 8054 Japanese participants. Central BP was estimated by the radial arterial waveform by calibrating brachial BP. Central retinal arteriolar equivalent (CRAE) was computationally measured using fundus photography.

Results: CRAE was most strongly associated with central SBP ($r=-0.324$, $P<0.001$), followed by DBP ($r=-0.292$, $P<0.001$) and central pulse pressure (PP; $r=-0.226$, $P<0.001$). The correlation coefficient between SBP and CRAE was significantly greater in central SBP than in brachial SBP ($r=-0.300$, $P<0.001$). After adjustment for possible covariates, brachial SBP ($\beta=-0.221$, $P<0.001$) and central SBP ($\beta=-0.239$, $P<0.001$) were independently associated with CRAE. Further, higher brachial SBP ($\beta=-0.226$, $P<0.001$) and smaller PP amplification ($\beta=0.092$, $P<0.001$) were identified as independent determinants of narrowing of CRAE in the same equation, which indicated the superiority of central BP. Central BP-determined hypertensive individuals had a significantly narrower CRAE independent of brachial BP (central/brachial: hypertension/hypertension 121.4 ± 11.5, hypertension/normotension 120.9 ± 11.2, normotension/hypertension 125.1 ± 11.9, normotension/normotension 128.1 ± 11.5 μm).

Conclusion: Central BP was more closely associated with the narrowing of CRAE than brachial BP. Slight increases in central BP might be involved in the morphological changes in small retinal arteries, even in individuals with optimal brachial BP.

Keywords: central blood pressure, retinal vessel caliber, small artery narrowing

Abbreviations: AIx, augmentation index; BP, blood pressure; baPWV, brachial-to-ankle pulse-wave velocity; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; MRRM, Meng–Rosenthal–Rubin method; SBP2, late SBP

INTRODUCTION

Central aortic blood pressure (BP) directly reflects the BP load on target organs and is therefore considered to be more closely associated with the cardiovascular outcomes than brachial BP. Compared with brachial BP, central BP estimated via radial arterial waveform or measured via carotid tonometry is more effective in showing the degree of association with intima–media thickness of the carotid artery in hypertensive individuals [1], the severity of coronary stenosis in patients with coronary artery diseases [2], the incidence of cardiovascular disease in the general population [3], and all-cause mortality in patients with end-stage renal disease [4]. One reason for these discrepancies is the difference in pulse pressure (PP) between the brachial artery and central aorta (PP amplification), which is largely dependent on the velocity of the reflection pressure wave. Increased pulse-wave velocity

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(PWV) augments overlapping of the reflection pressure wave on the forward pressure wave, which strengthens the forward wave and in turn increases central aortic BP.

High BP load is also a causative factor for small-vessel diseases such as silent cerebral infarction [5]. Retinal vessels are the only visible arterioles and venules whose caliber can be easily measured by fundus photography. Retinal vessel signs, that is, the narrowing of retinal arteriolar caliber and widening of venular caliber, have been associated with cardiovascular risk factors [6,7], systemic inflammation [8], and decreased renal function [9]. Further, because of the similar anatomic features and physiological properties of retinal vessels and cerebral microvessels [10], retinal vessel caliber has been suggested to predict stroke incidence [11,12] and stroke death [13]. Recently, Ott *et al.* [14] assessed the retinal arteriolar wall-to-lumen ratio in 135 nondiabetic individuals and reported that central PP was significantly associated with retinal arteriolar remodeling, though the superiority of central BP to brachial BP was not evaluated. Muiesan *et al.* [15] also reported a significant correlation between central BP and media-to-lumen ratio of subcutaneous small resistance arteries, but again did not evaluate the superiority of central BP. Although it remains unclear whether changes in retinal vessel caliber represent structural changes in small arteries and arterioles, a strong correlation between arterial diameter and medial cross-sectional area has been observed in the subcutaneous small arteries [16]. Further, by considering a substantial number of studies that reported a clinical and prognostic significance of retinal vascular caliber measurements [17], it is promising that retinal vascular calibers represent the vascular disease risks in various kinds of populations. Given these backgrounds, it was speculated that central BP might also be more closely correlated with the pathophysiological changes in retinal vessel calibers than brachial BP, whereas a paradoxical result was reported [18].

Here, we conducted a large-scale cross-sectional study in a general population to clarify the possibility of a superior association of central BP with not only large arterial diseases, but also small-vessel properties by measuring retinal arteriolar and venular calibers. Given that the prognostic significance of retinal vessel properties on the cardiovascular and cerebrovascular outcomes has been suggested, our results might be of clinical and epidemiological significance to the possible addition of central BP measurement to conventional brachial measurement in the assessment of small arterial disease risks.

METHODS

Study participants

Participants consisted of 8054 apparently healthy middle-aged to elderly citizens who were participants of the Nagahama Prospective Cohort for Comprehensive Human Bioscience (the Nagahama Study). The Nagahama Study cohort was recruited from 2008 to 2010 from the general population living in Nagahama City, a largely suburban city of 125 000 inhabitants located in central Japan. Community residents aged 30–74 years, living independently in the community and with no physical impairment or dysfunction, were recruited for the Nagahama cohort. Of a total of 9804 participants, those meeting any of the following conditions were excluded from this study, which are as follows: unsuccessful measurement of retinal vascular caliber ($n=1521$), presence of retinal vein occlusion or collateral vessels in either eye ($n=78$), unsuccessful assessment of retinopathy ($n=45$) or clinical parameters required for this study ($n=57$), extreme deviation of renal function [estimated glomerular filtration rate $194 \times \text{creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ (if women)] less than 30 ml/min per m^2 ($n=7$), and women who were pregnant ($n=42$). All study procedures were approved by the ethics committee of Kyoto University Graduate School of Medicine and the Nagahama Municipal Review Board. Written informed consent was obtained from all the participants.

Blood pressure measurements

Radial arterial waveform and brachial BP were measured simultaneously (HEM-9000AI; Omron Healthcare, Kyoto, Japan) after 5 min rest in the sitting position. Measurements were taken twice, and the mean value was used in the analysis. Absolute pressure of the late systolic peak (SBP2) of the radial arterial waveform was considered the central SBP. The radial augmentation index (AIx) was calculated from the waveform as the ratio of the late systolic peak to the first systolic peak, and the BP measurements are briefly described in the Supplemental Methods. PP amplification was calculated by subtracting central PP from brachial PP and expressed in the absolute values (mmHg). Mean BP (MBP) was calculated from SBP and DBP using the following formula: $(\text{SBP}-\text{DBP})/3+\text{DBP}$. Hypertension was defined as any or all of the following: use of antihypertensive medication, DBP greater than 90 mmHg, or brachial SBP greater than 140 mmHg or central SBP greater than 130 mmHg according to a previous report [19] that estimated central BP using the SphygmoCor system. SBP2 measured by the HEM-9000AI was almost identical to central SBP measured by the SphygmoCor system [20].

Retinal vessel caliber measurements

Fundus photographs of both eyes were taken in a shaded area using a 45-degree digital nonmydriatic camera (CR-DG10; Canon, Tokyo, Japan) at a 5-degree angle from the nasal side of the macula. A fundus photograph of the right eye was used for retinal caliber measurements. Retinal vascular caliber was measured using a semi-automated computer-based program (IVAN; University of Wisconsin, Madison, Wisconsin, USA). The measurement of central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE) are briefly described in the Supplemental Methods. Intragrader and intergrader intraclass correlation coefficients for the retinal arteriolar measurements were 0.80 ± 0.06 and 0.75 ± 0.06, and for the venular caliber measurements were 0.88 ± 0.13 and 0.86 ± 0.01, respectively.

Assessment of retinopathy

Retinopathy of both eyes was independently assessed in a masked fashion by two ophthalmologists, with a third

ophthalmologist making a final decision in cases of disagreement. Retinopathy was defined by the Early Treatment Diabetic Retinopathy Study Severity Scale [21] by the presence of any of the following characteristic lesions: microaneurysms, retinal hemorrhages, cotton wool spots, hard exudates, intraretinal microvascular abnormalities, venous beading, vitreous hemorrhages, or neovascularization. Individuals having at least one characteristic lesion in either eye were diagnosed as having retinopathy.

Basic clinical parameters

Basic clinical parameters, including plasma markers, were measured at baseline. Age was calculated from the year of birth to the year of baseline measurements. Smoking, alcohol consumption, and a history of cardiovascular disease, namely symptomatic stroke or ischemic heart disease, were determined using a structured self-administered questionnaire. Daily alcohol consumption was determined using the standard Japanese alcohol unit (1 unit corresponds to 22.9 g of ethanol). Brachial-to-ankle pulse-wave velocity (baPWV) was measured as an index of arterial stiffness. The method of baPWV measurement is detailed in the Supplemental Methods. Collinearity of baPWV with a carotid-to-femoral PWV, a standard measure of arterial stiffness, has been reported elsewhere [22].

Statistical analysis

Comparison of overlapping correlation coefficients was performed using the Meng–Rosenthal–Rubin method (MRRM). Identification of the factors independently associated with retinal vessel caliber, and the assessment of differences in CRAE by hypertension status was performed using multiple regression analysis. Statistical analysis was performed using JMP 9.0.2 software (SAS Institute, Cary, North Carolina, USA) and R software. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Clinical characteristics of the study participants are shown in Table 1. Mean age was 52 ± 13 years old. There were approximately two times more female than male participants. We excluded 1750 individuals from the analysis, mostly because of the unsuccessful measurement of retinal vessel caliber. Although the excluded participants were significantly older (Figure S1), BP levels of these excluded individuals and roughly measured retinal vessel calibers in a part of the individuals ($n = 229$) were not different from those of the remaining study participants (Table S1, http://links.lww.com/HJH/A416).

Distribution of CRAE and CRVE is shown in Figure S2, http://links.lww.com/HJH/A416. There was a moderate interrelationship between CRAE and CRVE ($r = 0.352$, $P < 0.001$). CRVE was significantly larger in men than in women (183.8 ± 15.9 vs. 178.7 ± 15.1 μm, $P < 0.001$). In contrast, CRAE was only slightly different between men and women (125.7 ± 12.0 vs. 126.3 ± 11.8 μm, $P = 0.016$). Basic factors that were significantly associated with retinal vessel caliber included age (CRAE, $r = -0.210$, $P < 0.001$; CRVE, $r = -0.204$, $P < 0.001$), habitual smoking (CRAE: current smoker 128.7 ± 11.7 μm, nonsmoker 125.7 ± 11.9 μm, $P < 0.001$; CRVE: 187.3 ± 15.8, 179.2 ± 15.1 μm, $P < 0.001$), and drinking (CRAE: habitual drinker 125.2 ± 11.9 μm, nondrinker 126.6 ± 11.9 μm, $P < 0.001$; CRVE: 182.0 ± 15.8, 179.6 ± 15.3 μm, $P < 0.001$). Figure 1 shows the age-related changes in BP, baPWV, and retinal vessel caliber. Changes in CRAE and CRVE were symmetrical to those in MBP, and were predominant in middle age, whereas the progression of large arterial stiffness evaluated by baPWV was greater in older age.

TABLE 1. Participants characteristics ($n = 8054$)

Age (years old)	52 ± 13
Sex (male, %)	32.5
Body height (cm)	160.4 ± 8.4
Body weight (kg)	57.4 ± 11.0
BMI (kg/m^2)	22.2 ± 3.3
Waist circumference (cm)	80.0 ± 9.2
Smoking (current/past/never, %)	15.1/20.1/64.8
Alcohol drinking (habitual/occasional/never, %)	22.4/10.4/67.2
Daily alcohol consumption (Japanese alcohol unit)	0.60 ± 0.99
History of cardiovascular disease (%)	2.3
Brachial SBP (mmHg)	122 ± 17
Central SBP (mmHg)	113 ± 18
Brachial PP (mmHg)	47 ± 11
Central PP (mmHg)	37 ± 11
PP amplification (mmHg)	10 ± 6
DBP (mmHg)	76 ± 11
Radial AIx (%)	80 ± 14
Heart rate (beats/min)	69 ± 10
Antihypertensive medication (%)	14.6
baPWV (cm/s)	1245 ± 218
CRAE (μm)	126.1 ± 11.9
CRVE (μm)	180.4 ± 15.5
Retinopathy (%)	5.8
Axial length (mm)	24.0 ± 1.3

Values are mean ± standard deviation. Cardiovascular disease includes symptomatic stroke or ischemic heart disease. AIx, augmentation index; baPWV, brachial-to-ankle pulse-wave velocity; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; PP, pulse pressure.

The correlations of BP with CRAE and CRVE are summarized in Table 2. CRAE was strongly associated with SBP, followed by DBP, PP, radial AIx, and PP amplification. Results of MRRM analysis indicated that the correlation coefficient between BPs and CRAE was significantly larger in central BP than brachial BP even after applying the Bonferroni correction ($P = 0.05/16 = 0.003$). Approximately 6% of individuals were diagnosed with retinopathy (Table 1). These individuals were significantly older, and had higher brachial and central SBP (Table S2, http://links.lww.com/HJH/A416). However, the results of a sensitivity analysis (Table S3, http://links.lww.com/HJH/A416) indicated that the superiority of central BP in association with CRAE was independent of retinopathy (model B), as well as of antihypertensive treatment (model C), history of cardiovascular diseases (model D), and metabolic syndrome (model E).

Table 3 shows the results of multiple linear regression analysis for CRAE. After adjustment for possible covariates, brachial SBP (model 1) and central SBP (model 2) were independently associated with CRAE. Further, in the equation that included both brachial SBP and PP amplification, higher brachial SBP and smaller PP amplification were independently associated with the narrowing of CRAE

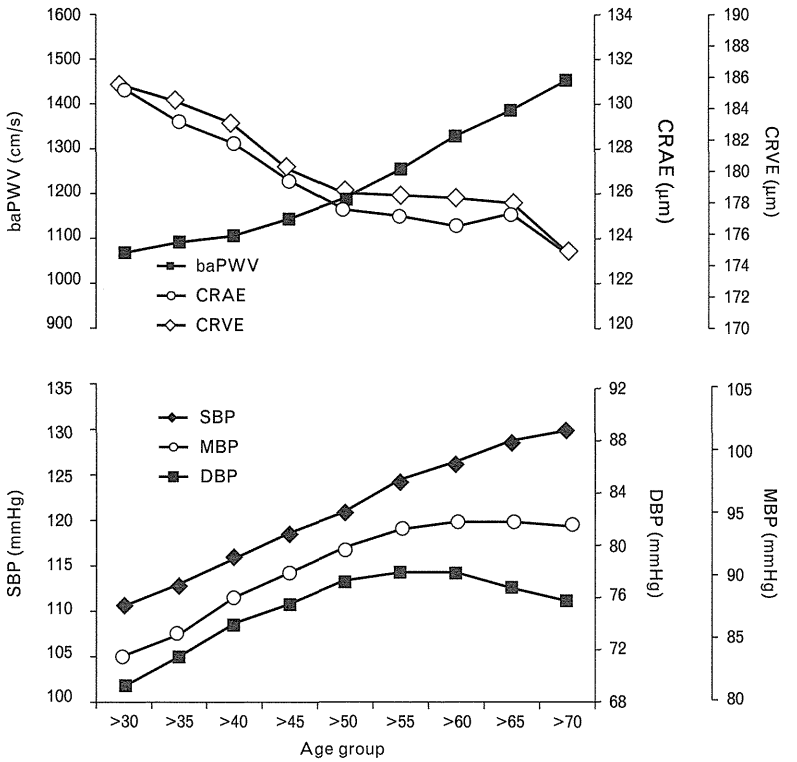

FIGURE 1 Age-related changes in retinal vessel calibers and arterial parameters. Each symbol represents the mean of individuals not taking antihypertensive drugs ($n=6877$). baPWV, brachial-to-ankle pulse-wave velocity; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent.

(model 3), suggesting that a relatively high central PP is a risk for the narrowing of CRAE. Radial AIx was significantly associated with CRAE in a model that included brachial SBP (model 4), but not central SBP (model 5). In contrast, central SBP was not an important determinant for CRVE (central SBP, $\beta=-0.031$, $P=0.026$).

Multiple linear regression analysis for retinal vessel caliber (Table 4) indicated that CRAE was strongly associated with BP. In contrast, CRVE was affected by various factors, namely body fluid parameters including hematocrit and white blood cell count, body weight, and smoking, and had only a weak association with MBP. Metabolic and hematological characteristics of the study participants are summarized in Tables S4 and S5, http://links.lww.com/HJH/A416.

Figure 2a shows the differences in mean CRAE by hypertension status defined by central and brachial BP. Central BP-determined hypertensive individuals had a significantly narrower CRAE that was independent of brachial BP. Further, CRAE was linearly decreased with increasing central BP even within the same brachial BP levels (Fig. 2b). By a simple correlation analysis, central SBP corresponded to an approximately 10 mmHg lower brachial SBP (central SBP $=-7.15+0.98\times$ brachial SBP). Individuals exhibiting a relatively lower central SBP, that is, whose central SBP was more than 10 mmHg lower than brachial SBP, had a wider CRAE than those whose central SBP was at a similar level to brachial SBP. In contrast, individuals exhibiting a relatively higher central SBP showed a narrower CRAE.

TABLE 2. Correlation between BP and retinal vessel caliber

	CRAE				CRVE			
	Simple correlation		MRRM		Simple correlation		MRRM	
	r	*P*	*z*	*P*	*r*	*P*	*z*	*P*
Brachial SBP (mmHg)	−0.300	<0.001	6.85	<0.001	−0.107	<0.001	11.65	<0.001
Central SBP (mmHg)	−0.324	<0.001			−0.149	<0.001		
Brachial PP (mmHg)	−0.181	<0.001	7.87	<0.001	−0.108	<0.001	11.65	<0.001
Central PP (mmHg)	−0.226	<0.001			−0.176	<0.001		
DBP (mmHg)	−0.292	<0.001			−0.064	<0.001		
Radial AIx (%)	−0.163	<0.001			−0.175	<0.001		
PP amplification (mmHg)	−0.105	<0.001			0.142	<0.001		

Overlapping correlation coefficients were compared with the Meng–Rosenthal–Rubin method (MRRM). AIx, augmentation index; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; PP, pulse pressure.

TABLE 3. Multiple linear regression analysis for CRAE

	Model 1		Model 2		Model 3		Model 4		Model 5	
	β (VIF)	P	β (VIF)	P	β (VIF)	P	β (VIF)	P	β (VIF)	P
Brachial SBP (mmHg)	−0.221 (1.90)	<0.001			−0.226 (1.91)	<0.001	−0.206 (1.98)	<0.001		
Central SBP (mmHg)			−0.239 (1.84)	<0.001					−0.244 (2.46)	<0.001
PP amplification (mmHg)					0.092 (1.50)	<0.001				
AIx (%)							−0.072 (1.86)	<0.001	0.009 (2.40)	0.545

Adjusted factors were as follows: age, sex, body height, body weight, current smoking, daily alcohol consumption, history of cardiovascular diseases, antihypertensive medication, heart rate, brachial-to-ankle pulse-wave velocity, axial length, retinopathy, and fellow retinal vessel caliber. β, standardized regression coefficient; AIx, augmentation index; CRAE, central retinal arteriolar equivalent; PP, pulse pressure; VIF, variance inflation factor.

DISCUSSION

In this study of an apparently healthy general population, we observed that central BP was more closely associated with the narrowing of CRAE than brachial BP. Even in individuals diagnosed as normotensive by brachial BP, slight increases in central SBP might be a risk factor for the narrowing of CRAE.

We clarified the stronger association of central SBP with the morphological changes in small arterioles, though close associations between central BPs and pathophysiological changes in large arteries [1,2] and cardiovascular morbidity [3] have already been demonstrated. In contrast, central SBP was not a major determinant for venular caliber. Given the strong correlation between BPs and CRAE but not CRVE, changes in arteriolar caliber might more accurately reflect the pressure load of the central aorta. Previous cross-sectional population-based studies have suggested that the associations of retinal vessel caliber with the cardiovascular risk factors largely differs between CRAE and CRVE, with most observing a strong association between brachial BP and the narrowing of CRAE rather than the widening of CRVE [6,7,23,24]. The wide range of covariates for CRVE (Table 4) might also be a reason for the weak association between BPs and CRVE.

Correlation coefficient between central SBP and CRAE was stronger than that of the brachial SBP (Table 2). As a result of the strong collinearity between brachial and central SBP, we could not directly compare the superiority by including both SBPs in a same regression model. However, in the model that included brachial SBP and PP amplification (Table 3, model 3), both the parameters were identified as independent determinant for CRAE. As central SBP is a function of brachial SBP and PP amplification, the results indirectly support the superiority of central SBP in association with CRAE. Further, radial AIx was independently associated with CRAE in a model that included brachial SBP (Table 3, model 4). However, when central SBP was exchanged for brachial SBP (model 5), the association between AIx and CRAE became insignificant. These results suggest that the absolute value of central aortic pressure rather than the ratio of forward and reflection pressure waves may be important for retinal arteriolar narrowing.

A recent longitudinal study of cardiovascular mortality [19] reported that a central BP of 130/90 mmHg has the best discriminatory power in the prediction of cardiovascular outcomes. In addition, a cross-sectional study based on 10 756 Japanese participants reported a central SBP of 129 mmHg as a reference value of normal BP [25]. Here, we found a significantly narrower CRAE in individuals whose central SBP exceeded 130 mmHg independent of the brachial BP levels. Further, the narrowing of CRAE was observed in cases with even lower central SBP. Small arteries may be adversely impacted by even minor increases in central BP load, even in those within the normal limits.

Results from the Strong Heart Study of Native Americans showed that PP measured at the central aorta or brachial artery was more strongly associated than SBP at any artery with large arterial remodeling, namely increased carotid intima–media thickness, vascular mass, and plaque score

TABLE 4. Multiple linear regression analysis for retinal vessel caliber

	CRAE		CRVE	
	β	P	β	P
Body height (cm)	0.079	<0.001	−0.023	0.184
Body weight (kg)	−0.017	0.255	0.136	<0.001
Currently smoking	0.048	<0.001	0.068	<0.001
Hyperglycemia	0.012	0.226	−0.017	0.106
Dyslipidemia	0.010	0.360	0.018	0.103
White blood cell count ($\times 10^2/\mu l$)	−0.008	0.436	0.073	<0.001
Hematocrit (%)	−0.042	0.001	0.115	<0.001
Mean BP (mmHg)	−0.249	<0.001	−0.056	<0.001
baPWV (cm/s)	−0.017	0.259	−0.008	0.596

Metabolic and hematological characteristics of patients are shown in Tables S4 and S5, http://links.lww.com/HJH/A416, respectively. Hyperglycemia was defined as either or both of plasma glucose greater than 126 mg/dl (fasting) or 200 mg/dl (nonfasting), or the use of hypoglycemic treatment, including insulin therapy. Individuals who met any of the following criteria were diagnosed with dyslipidemia: LDL-cholesterol greater than 140 mg/dl, HDL-cholesterol lower than 40 mg/dl, triglyceride greater than 150 mg/dl, or the use of lipid-lowering drugs. Adjustment factors were as follows: age, sex, daily alcohol consumption, history of cardiovascular disease, antihypertensive medication, heart rate, axial length, retinopathy, fellow retinal vessel caliber, and fasting time. β, standardized regression coefficient; baPWV, brachial-to-ankle pulse-wave velocity; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent.

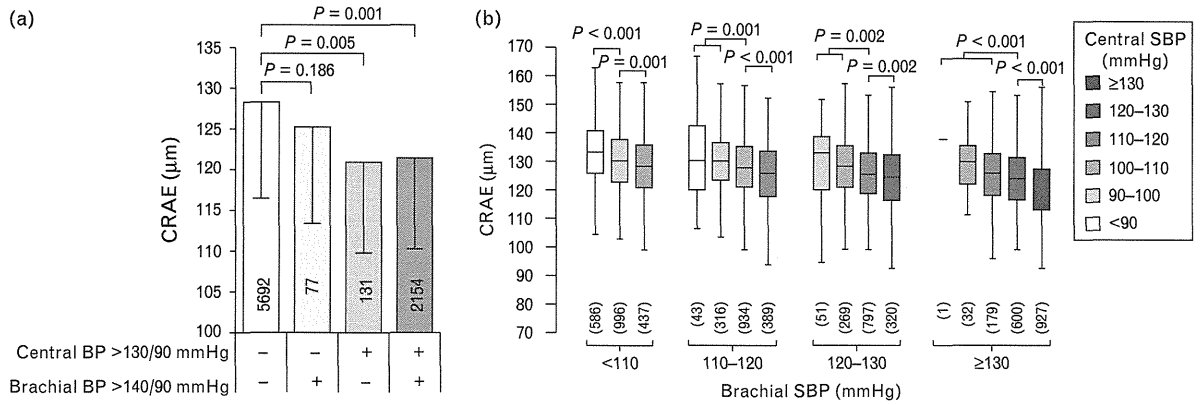

FIGURE 2 Differences in mean CRAE by central and brachial BP levels. (a) Values are mean ± standard deviation. Participants were classified into four groups according to the hypertension status as defined by central BP (any or all of central SBP ≥130 mmHg, DBP ≥90 mmHg, or use of antihypertensive medications) or brachial BP (any or all of brachial SBP ≥140 mmHg, DBP ≥90 mmHg, or use of antihypertensive medications). (b) Box plot of mean CRAE in patients not prescribed antihypertensive drugs ($n = 6877$) by various brachial and central BP levels. Statistical significance was assessed by a multiple linear regression analysis adjusted for age, sex, body height, body weight, current smoking, daily alcohol consumption, history of cardiovascular diseases, heart rate, brachial-to-ankle pulse-wave velocity, axial length, retinopathy, and CRVE. The number of patients in each subgroup is shown in the figure. CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent.

[3]. The superiority of PP in prognosis for all-cause mortality was also reported from a longitudinal study based on patients with end-stage renal disease [4]. In contrast, we observed a close association between CRAE and SBPs rather than PPs. Remodeling of large arteries decreases the Windkessel function of the aorta, which increases the SBP, decreases DBP, and consequently increases PP. In contrast, systemic remodeling of small arteries increases both SBP and DBP. The different pathophysiological features of large and small arterial remodeling in association with BPs might be a factor that explains the stronger association of SBP with the narrowing of CRAEs.

Several limitations of our study warrant mention. First, we estimated central SBP using radial arterial waveform analysis, that is, late systolic peak of the radial arterial waveform (SBP2) was considered equivalent to central SBP. SBP2 was recently reported to not always represent central SBP accurately, particularly in cases with a type C aortic pressure waveform, in which peak SBP precedes an inflection point [26]. The type C waveform is observed in young individuals [27]. Therefore, misestimation of central SBP, if any, might have had no substantial impact on the present findings which were obtained from individuals aged 30 years or older. Second, we excluded a considerable number of potential individuals from analysis, mostly because of the unsuccessful measurement of retinal vessel caliber as a result of an increased frequency of cataracts, small pupils, and difficulties with ocular fixation. However, our ungradable rate 15.5% (1521 of 9804) was not too high compared with that of other large-scale epidemiological studies using the same semi-automated computer system to measure retinal vascular calibers: Atherosclerosis Risk In Communities study, 19% [28]; Beaver Dam Eye Study, 13.8% [29]; and Rotterdam study, 16.3% [30]. Further, as BP level and retinal vessel caliber of the excluded individuals did not differ from those of the included participants, the findings are unlikely confounded by the selection bias. Third, as this study was a cross-sectional setting, further longitudinal studies are needed to clarify the prognostic significance of central hemodynamics in retinal vessel morphological change.

In summary, we have clarified for the first time that central BP is strongly associated with the narrowing of retinal arteriolar caliber in a large-scale general population. As narrowing of the retinal artery is suggested to represent a subclinical cardiovascular and cerebrovascular risk and has been associated with poor prognosis, our study supports the importance of evaluating central BP in the assessment of small arterial disease risks.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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Reviewers' Summary Evaluations

Reviewer 2

In a cross-sectional analysis of over 8000 middle-aged to older Japanese adults, the authors report that central SBP as measured by radial artery waveform is more strongly related to retinal arteriolar narrowing than brachial artery SBP. The cohort size is impressive and the findings are seemingly novel in relating retinal arteriolar narrowing to central pressures as opposed to brachial pressures, as has been in prior studies. Study limitations include its observational design and exclusion of large number of enrolled subjects (>1500) because of having been unable to successfully measure retinal arteriolar diameter.

Reviewer 3

In this large cohort study central blood pressure has been found to be associated with retinal arteriolar narrowing independently of brachial blood pressure. The paper focuses the attention on a topic of marked interest employing an outstanding methodology.

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A main contribution of DRB1*04:05 among shared epitope and involvement of 57th DRB1 amino acid in association with joint destruction in ACPA(+) RA

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HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

Abstract

Objectives: The shared epitope (SE) is associated with increased joint destruction in rheumatoid arthritis (RA) as well as RA susceptibility and the production of anti-citrullinated peptide antibody (ACPA). However, previous studies addressing whether the association of SE with joint destruction is independent of ACPA have reported different results in different populations. Different allelic distributions in SE may explain this ethic-heterogeneity. We aimed to assess the associations of the SE and HLA-DRB1*04:05, the most common SE allele in Japanese, on joint destruction in patients with ACPA-positive RA.

Methods: A total of 861 patients with ACPA-positive RA who had not received any biological agents were recruited from three different sets. Joint destruction was assessed using the modified total Sharp score (SHS). The associations of SE, HLA-DRB1*04:05 and other SE allele group on the SHS were analyzed in a linear regression analysis. Amino acid variations associated with SHS were also analyzed.

Results: The SE was significantly associated with increased SHS ($p=0.0017$). Although HLA-DRB1*04:05 was significantly associated with increased SHS ($p=2.7\times10^{-5}$), the group of other SE alleles, including HLA-DRB1*01:01, did not show an association with SHS in spite of sufficient power ($p=0.67$). HLA-DRB1*04:05 was associated with joint destruction in a dose-dependent manner. Analyses of amino acid associations of HLA-DRB1 revealed that serine at position of 57, recently shown its susceptibility effect on ACPA-positive RA in Asian, showed a significant association ($p=5.0\times10^{-6}$).

Conclusions: HLA-DRB1*04:05, characterized by the 57th serine, accounts for the detrimental association between the SE and SHS in Japanese RA patients with ACPA.

Keywords: rheumatoid arthritis, genetics, HLA

HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

Introduction

Rheumatoid arthritis (RA) is the most common cause of chronic autoimmune arthritis all over the world. Chronic synovitis in RA leads to joint destruction, which can result in joint deformity[1]. Genetic components are associated with joint destruction in RA patients as well as its onset[2, 3]. Of all susceptibility loci for RA, HLA-DRB1 exhibits the strongest association with RA and its progression[4]. The shared epitope (SE), a common amino acid sequence of the HLA-DRβ chain, is a widely accepted concept to explain important HLA-DRB1 alleles for RA[5]. Originally, SE extends positions 72 to 74 of HLA-DRB1. Modifications have been proposed including positions 70 and 71[6, 7]. Recent studies suggest that amino acid combinations in HLA-DRB1 would explain the association between HLA-DRB1 and RA better than SE[8]. Previous studies have shown that joint destruction is associated with SE and positivity for anti-citrullinated peptide antibody (ACPA), a highly specific marker of RA[9]. SE is also associated with ACPA production[10]; however, it remains unclear whether SE has further associations with joint destruction that are independent of ACPA positivity. European studies in which RA patients were stratified according to ACPA positivity found that SE does not contribute to joint destruction[11, 12]. On the other hand, a Japanese study showed that the SE is associated with the progression of joint destruction that is independent of ACPA positivity[9]. As the distribution of HLA-DRB1 alleles varies between different populations[13,

14], the associations between the SE and joint destruction which are independent of ACPA might also differ between populations due to different allelic frequencies. A study based on large amount of data about joint destruction in patients with ACPA-positive RA would elucidate allele-specific effects of the SE on bony destruction. Furthermore, allele-specific effects would reveal contribution of specific amino acid residues on joint destruction.

Here, we focused on HLA-DRB1*04:05, the most common SE allele in Japanese population and less frequent in European population, and analyzed its effect on joint destruction to uncover the possible reason for the ethnic-different associations.

Materials and Methods.

This study was approved by the local ethical committees. Written informed consent was obtained from each participant in the current study.

Study Population

A total of 350 and 362 ACPA-positive RA with or without experience of methotrexate (MTX) treatment, respectively, were recruited at the IORRA cohort[15] as the 1st and 2nd sets. These populations were used in the previous study[9]. 149 RA patients were recruited from the KURAMA cohort[1]. All of the patients fulfilled ACR revised criteria for RA in 1987 or ACR

and EULAR classification criteria for RA in 2010[16]. 84% of the subjects in set 1 developed RA before 1999 when MTX was introduced to RA treatment in Japan. 77% of the subjects in the current study developed RA before 2003 when the first biologic agent appeared in the Japanese market and none of the patients had ever received biological agents before they underwent the X-ray examinations described below.

SHS score

X-rays of the patients' joints, which were obtained at five years after onset of RA for sets 1 and 2 and at four to six years after onset for set 3, were assessed using the modified Sharp score (SHS)[17]. The X-rays of the 1st and 2nd sets were evaluated by a trained rheumatologist (K.Y), resulting in an intra-observer agreement of 0.95. Another trained rheumatologist (M.F) assessed the X-rays of the 3rd set with intra-observer agreement of 0.93. The two rheumatologists who evaluated the X-rays were blinded to the purposes of the study and the patients' genetic information. Since foot X-rays were not available for about half of the patients, the scores for the hand X-rays were used in the subsequent analyses.

Quantification of ACPA

The MESACUP CCP ELISA kit (Medical and Biological Laboratories Co., Ltd) was used to

HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

detect 2nd generation ACPA. A cut-off value of 4.5 U/ml was used to define ACPA positivity according to the manufacturer's instructions.

HLA Genotyping

The WAKFlow system (Wakunaga) or the AlleleSEQR HLA-DRB1 typing kit (Abbott) was used for the HLA-DRB1 typing, as previously described[18]. Of the HLA-DRB1 alleles detected in the current study, the following were classified as SE based on the 70th to 74th amino acid residues according to our previous studies[14, 18]: DRB1*01:01, *04:01, *04:04, *04:05, *04:10, *10:01, *14:02, and *14:06. Modified definition and classification of SE by du Montcel et al[7] were also applied to assess the association of HLA-DRB1*04:05.

Association study for stratified subjects

We finely defined a total of six subjects' groups based on positivity of DRB1*04:05, SE other than DRB1*04:05 (SEx) and non-SE alleles. We combined the group with SEx/SEx and that with SEx/non-SE as one group to assess the effect of SEx because the number of the subjects carrying SEx/SEx was limited. We put the group with non-SE/non-SE as reference to assess joint destruction in the other groups.

HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

Association study for amino acid variations

To analyze which amino acid variation is associated with joint destruction in patients with ACPA-(+) RA, we performed association studies of amino acid variations in HLA-DRB1 according to the method previously described[19]. Briefly, variations of amino acid in HLA-DRB1 protein were searched across all the *HLA-DRB1* alleles in the current study population based on amino acid sequences for four digits resolution from IMGT database (http://www.ebi.ac.uk/ipd/imgt/hla/). The 96 amino acids over 45 positions were incorporated in analysis.

Statistical Analysis

The patients' SHS scores were log-transformed to fit normality and used as dependent variables ($\log_{10}$(SHS+1)). Multiple linear regression analyses were performed to assess the effects of HLA-DRB1 alleles on SHS with sex, age at onset and usage of bucillamine, salazosulfapyridine, tacrolimus and leflunomide, representative DMARDs in Japan, as covariates. In the 3rd set, disease duration and a history of MTX use were also used as covariates. The inverse-variance method assuming fixed-effects was employed to combine all of the results for the three sets. Heterogeneity across different sets was evaluated by I^2 statistics based on Cochran Q test. After confirming the effects of the SE, *04:05 and SE other than *04:05, the effect of each SE were

analyzed. For analysis of amino acid residues, the 96 amino acids over 45 positions were used as independent variables in linear regression analysis using the same covariates as the allelic association study. Omnibus test[8, 20] in the combined data set adjusted by study sets and the same covariates was also applied to the current linear regression analysis framework to assess an association of each amino acid position of HLA-DRB1 (for details, see Supplementary Note). Statistical significance was evaluated in the meta-analysis or combined analysis. P-value less than 0.05 was regarded as significant except for analyses of each HLA-DRB1 allele and amino acid residues. As for analyses of each HLA-DRB1 allele and amino acid variations and positions, a stringent significant level was set based on Bonferroni's correction (0.05/96). Power calculation was performed by using 'GeneticsDesign' package in R software.

Results

We focused on HLA-DRB1*04:05 among the SE because this is the most common SE allele among the Japanese population and rarely seen in European population. We evaluated the effects of the SE, DRB1*04:05 and other SE alleles using three independent sets by inverse-variance method assuming fix-effects (Table 1).

As a result, we confirmed the association between the SE and SHS across the three sets (overall p=0.0017, Figure 1A). In addition, a significant association between DRB1*04:05 and

SHS was observed (overall p=0.000027, Figure 1). However, SE alleles other than DRB1*04:05 did not show a significant association with SHS in all of the three sets (overall p=0.67,Figure 1A). The detailed statistics were shown in Supplementary Table 1. We also confirmed that the ACPA levels were not associated with SHS (p=0.53, data not shown).

Next, we assessed the dose-dependency of the association of DRB1*04:05 with SHS. Patients with one and two copy of DRB1*04:05 were separately analyzed in reference to patients without DRB1*04:05. Meta-analysis using inverse-variance method indicated that DRB1*04:05 is dose-dependently associated with increased SHS (beta=0.10 and 0.29, p=0.0036 and 0.00036 for one and two copies of DRB1*04:05, respectively, Figure 1B). Conversely, we did not detect any significant associations between SHS and other SE alleles (beta<0.0061, p>0.20, Figure 1B). To confirm this association pattern, we separated the subjects into closely defined subgroups based on their positivity for DRB1*04:05 and the other SE alleles. While the subjects who carried one copy of DRB1*04:05 without other SE alleles exhibited significantly increased SHS compared with the subjects without SE (beta=0.11, p=0.0097), no such effect was detected in the subjects without DRB1*04:05 who carried at least one copy of another SE allele (beta=0.012, p=0.82, Supplementary Figure 1). Furthermore, while the subjects who were homozygous for DRB1*04:05 exhibited significantly increased SHS compared with the

DRB1*04:05/non-SE allele subjects (beta=0.21, p=0.0052), the DRB1*04:05/non-04:05 SE allele subjects did not (beta=-0.0055, p=0.94, Supplementary Figure 1). These results confirmed that DRB1*04:05 is associated with increased SHS in a dose-dependent manner, but the other SE alleles do not have any association with SHS. We also evaluated the association of each SE allele other than DRB1*04:05 with SHS. The associations of the SE alleles were highly variable (Supplementary Table 2). Although DRB1*04:10 exhibited trends suggesting that they were associated with increase of SHS, we could not conclusively determine their associations due to the low frequencies. DRB1*01:01 and *04:01 did not display any positive associations with SHS.

We further analyzed the association between SHS and SE with modification proposed by du Montcel et al in which HLA-DRB1 alleles were classified into X, S1, S2, S3D or S3P[7]. As a result, S3P which contained DRB1*04:05 showed a significant association with increased SHS (p=0.0013, Supplementary Table 3) but S2, the other allelic group associated with RA susceptibility in the original study[7], did not (beta=-0.061, p=0.35, Supplementary Table 3). When we excluded DRB1*04:05 from S3P, S3P alleles other than DRB1*04:05 did not show a significant association with increased SHS (beta=-0.040, p=0.30, Supplementary Table 3).

HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

Since a recent study showed that RA susceptibility associations of the HLA locus could be explained by combination of amino acid residues including HLA-DRB1 which partly differed among different populations[8, 20], we performed association studies between SHS and amino acid residues of HLA-DRB1. We aligned HLA-DRB1 alleles in the current study and found 96 amino acid variations over 45 positions. Linear regression analysis revealed that serine at position 57 in HLA-DRB1 protein was the only amino acid variation showing significant p-value ($p=5.0x10^{-6}$, Figure 2A and Supplementary Table 4). This amino acid position was recently shown to be involved with RA susceptibility in Asian population[20]. Amino acid variations with p-value smaller than 0.005 and HLA-DRB1 alleles with or without serine at position 57 were shown in Supplementary Table 4 and 5, respectively. In addition to association of each amino acid variation, we also calculated omnibus p-value of each amino acid position to assess the entire association of position 57. As a result, position 57 was the only position showing significant omnibus p-value among the 45 positions (p=0.00021, Figure 2B). Amino acid positions different between DRB1*04:05 and other SE alleles were summarized in Supplementary Table 6 with omnibus and amino acids' p-values.

Discussion.

We showed that the association between the SE and SHS that was previously detected in

HLA-DRB1*04:05 and 57th amino acid in association with joint destruction in ACPA(+) RA

Japanese ACPA-positive RA patients can be largely explained by DRB1*04:05, the most common SE allele among the Japanese population. The main association of DRB1*04:05 seems explain the lack of the association between joint destruction and the SE in European population which is independent of ACPA because DRB1*04:05 is a rare allele in European population.

We did not distinguish between each SE allele during the first part of the study in order to avoid type II statistical errors. As the use of biological agents can greatly influence SHS, we mainly recruited patients who developed RA before introduction of biologic agents to Japanese market. Since most of the participants in set 1 developed RA before 1999, the DMARDs other than MTX were much more frequently used in the subjects of set 1 than those of set 2 (data not shown). Since this big difference of DMARDs usage may result in different associations of genetic components, we separately analyzed set 1 and set 2. Since 77% of the study participants develop RA before introduction of biologic agents and prevalence of biologic agents was not so rapid in Japan, we assume that selection bias of study participants in the current study is limited. Since the previous study showed gender and age at onset were associated with SHS[9], we incorporated these two factors as covariates as well as usage of each DMARD. Since joint X-rays were taken at five years from onset of RA in sets 1 and 2, we did not include disease duration as a covariate in sets 1 and 2. To avoid heterogeneity, we used in the set 3 the data whose X-rays were taken from four to six years after onset, very similar disease duration to the

sets 1 and 2. We confirmed that the same conclusions were obtained when we set yearly progression of SHS as outcome value (data not shown).

Multiple linear regression analysis demonstrated that DRB1*04:05 was associated with increased SHS, but the other SE alleles did not display similar associations. It should be noted that DRB1*01:01, the most common SE allele in European population, did not exhibit any significant association. The association of DRB1*04:05 was demonstrated to be dose-dependent. Detailed classifications of subjects according to combination of the SE alleles supported these findings. The current results indicate that the detrimental association of SE with SHS can be largely attributed to DRB1*04:05. Since power analysis indicated that this study had a power of 0.74 or more for detecting the same beta coefficients as DRB1*04:05 with p-values of 0.05 for DRB*01:01, and the SE alleles other than *04:05, it is not very likely that the lack of significant results for these allele and allelic group was due to their frequencies. However, since this study does not have enough power to detect significant effects of alleles with small allele frequency, it is not deniable that other SE alleles have similar effects to DRB1*04:05. Increasing the number of subjects may aid further characterization of detailed allelic associations among SE other than *04:05 and *01:01. The fact that previous European studies have failed to detect a non-ACPA-related association of SE with SHS might be explained by the low frequency of