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Original Article

Components of Metabolic Syndrome and their Combinations as Predictors of Cardiovascular Disease in Japanese Patients with Type 2 Diabetes. Implications for Improved Definition. Analysis from Japan Diabetes Complications Study (JDCS)

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Aim: The prognostic power of metabolic syndrome (MetS) in patients with diabetes has been studied with inconsistent results depending on the definition of MetS. To clarify the best combination of MetS components to predict future cardiovascular disease (CVD) events, we estimated CVD risk in Japanese patients with type 2 diabetes according to MetS components.
Methods: Patients were categorized according to the presence three MetS components in addition to hyperglycemia. hypertension, dyslipidemia and excess waist circumference (WC) (according to either Japanese or Asian cut-off values). Hazard ratios for CVD events were compared in patients with various categories of MetS components.
Results: At least two components of MetS were required for a significantly elevated risk for CVD; however, component combinations with significantly increased risk differed depending on gender or the WC cut-off value. Any two among 1) excess WC (men ≥90 cm, women ≥80 cm); 2) hypertension (systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or use of an antihypertensive agent); and 3) dyslipidemia (triglycerides ≥150 mg/dL or HDL-cholesterol <40 mg/dL or use of drug treatment) could be used to identify significantly higher risk (approximately twice) for CVD regardless of gender.
Conclusions: The results suggest that the current MetS criteria should be modified when applied to patients with type 2 diabetes.

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Key words; Diabetic macroangiopathy, Cardiovascular risk factors, Hypertension, Dyslipidemia

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Introduction

Patients with diabetes are at greater risk for cardiovascular disease (CVD) than non-diabetic subjects, and metabolic syndrome (MetS), a constellation of multiple cardiometabolic risk factors, is strongly asso-

ciated with increased risk of CVD events; however, previously, we found that the diagnosis of MetS had only a limited prognostic value for future CVD events in Japanese patients with type 2 diabetes[1-3]. Since that time many reports have also addressed the issue of whether a diagnosis of MetS is predictive in patients with diabetes, reflecting the need to identify diabetic patients at very high risk for CVD events in clinical settings; unfortunately, the results have been inconsistent[4-13]. Some studies[5-7, 10] revealed that the clinical relevance of a diagnosis of MetS as a predictor of CVD morbidity and mortality differs markedly among diabetic patients, depending on the definition of MetS. Moreover, the contribution of each MetS component to cardiovascular risk was shown to significantly vary in the general population[14].

These findings strongly suggest that various combinations of individual components of MetS could have substantially different contributions to CVD risk in diabetic patients. In fact, a recent cross-sectional study of 4020 German patients with type 2 diabetes[15] demonstrated considerably diverse odds ratios for established CVD according to heterogeneous clusters of traits; however, prospective studies evaluating the impact of specific combinations of MetS components on CVD risk in diabetic populations are scarce, although such a study in the general population has been published recently[16]. Such information would be useful for screening patients at extremely high risk of CVD as well as for improving the definition of MetS for a diabetic subgroup. For this purpose, we determined the prevalence of various combinations of MetS components among Japanese patients with type 2 diabetes and estimated the risks of CVD presented by these components in this patient group.

Methods

The Japan Diabetes Complications Study (JDCS) is a nationwide multi-center prospective study of type 2 diabetic patients[17]. In 1996, 2205 patients aged 40–70 years with previously diagnosed type 2 diabetes but no CVD were registered. The detailed protocol of the JDCS has been described previously[17]. Of the 2205 patients, 1424 (771 men and 653 women, mean age; 58.4±7.4 years) with a complete set of data, including the parameters necessary to satisfy the World Health Organization (WHO)[18] and the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII)[19] criteria for the definition of MetS at baseline, were prospectively followed for 8 years for fatal/non-fatal coronary heart disease (CHD) and stroke events. CHD events consisted of angina pectoris and fatal/non-fatal acute myocardial infarction. A detailed definition of CHD and stroke events was previously described[1]. CHD and stroke events (hereafter referred to as CVD) identified during follow-up were confirmed by at least two members of the experts committee who were blinded as to risk factor status and the other member's diagnosis. The JDCS protocol was conducted according to the Declaration of Helsinki and received approval from the institutional review board. All participants gave written informed consent.

Thresholds for individual risk factors were adopted from the Japanese definition of MetS[20], which is similar to that of IDF[21] with the exception that hypertriglyceridemia and low HDL-cholesterolemia are combined as one component, i.e, 'dyslipidemia'. Since all subjects in this study had diabetes mellitus, 3 criteria other than an elevated fasting plasma glucose level (>110 mg/dL) were used: (i) excess WC (male ≥85 cm, female ≥90 cm), (ii) hypertension (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg), and (iii) dyslipidemia (triglyceride >150 mg/dL and/or HDL-cholesterol <40 mg/dL). MetS is defined as the presence of excess WC and two of the following three parameters: hypertension, dyslipidemia and elevated fasting plasma glucose[20]. Subjects using agents for hypertension or hyperlipidemia were considered to have either hypertension or hyperlipidemia according to the recent MetS criteria[19, 21]. The alternative WC cut-off values for general Asians, as decided by the WHO and the International Diabetes Federation (IDF) definition (male ≥90 cm, female ≥80 cm)[19, 21], were used for additional evaluation.

Data are presented as the means ± SD or as a proportion unless otherwise specified. WC in each group was assessed by Wilcoxon's rank sum test. Cox regression analysis was used to calculate the age-adjusted hazard ratio and 95% confidence intervals (CI) of risk factors for CVD. The SAS software package (Version 9.0, Cary, NC) was used for all analyses. $P<0.05$ was considered significant.

Results

Distribution of Patients According to Status of Risk Factor Clustering

Baseline characteristics of the study patients are shown in **Table 1**. Distribution of patients categorized by risk factor status employing either the Japanese or Asian WC cut-off is shown in **Fig. 1**. Approximately 60–70%, 30–0% or 20–25% of all diabetic patients, including both males and females, had hypertension,

Table 1. Baseline characteristics of patients analyzed

	Men	Women
Number of Patients (%)	771	653
Age (y)	58.2 ± 7.4	58.7 ± 7.4
Diabetes duration (y)	10.9 ± 7.6	10.1 ± 6.7
BMI (kg/m^2)	22.9 ± 2.6	23.4 ± 3.3
Waist circumference (cm)	82.3 ± 7.7	76.5 ± 9.8
Waist/Hip ratio	0.89 ± 0.07	0.83 ± 0.08
Blood pressure (mmHg)	132 ± 16/78 ± 10	132 ± 17/76 ± 10
HbA$_{1c}$ (%)	7.61 ± 1.36	8.05 ± 1.45
Fasting plasma glucose* (mmol/L)	8.3 (7.2, 10.0)	8.6 (7.3, 10.2)
Fasting plasma insulin* (pmol/L)#	6.2 (0.5, 1.9)	7.1 (0.5, 1.9)
Serum LDL cholesterol (mmol/L)	3.03 ± 0.86	3.38 ± 0.82
Serum HDL cholesterol (mmol/L)	1.34 ± 0.39	1.47 ± 0.44
Serum triglycerides** (mmol/L)	1.39 (0.75)	1.29 (0.72)
Current smoker (%)	43.9	8.7
OHA (without insulin) use (%)	72	77
Insulin (with or without OHA) use (%)	16	20
Medication for hypertension (%)	22	29
Medication for hyperlipidemia (%)	15	35

mean ± SD, *median (IQR) or **geometric mean (1SD), #patients on insulin therapy were excluded
OHA, oral hypoglycemic reagents

dyslipidemia or both, respectively. When the Japanese WC cut-off value (male ≥ 85 cm, female ≥ 90 cm) was applied, the proportion of female patients with excess WC was much lower than that of male patients. Among all diabetic patients, the proportion of patients having all 3 risk factors (i.e. excess WC, hypertension and dyslipidemia) was 13% among men but only 3% among women. When the Asian WC cut-off value (male ≥ 90 cm, female ≥ 80 cm) was used instead of the Japanese cut-off value, the proportion of female patients with excess WC increased nearly 4 times (approx. from 10 to 37%) while the proportion of male patients decreased by half (approx. from 37 to 18%).

CVD Risk of Patients in Individual and Combined Risk Category

Table 2 shows hazard ratios for CVD (i.e. CHD and/or stroke) events in patients in the individual and combined risk categories indicated in **Fig. 1** compared to those not in these areas. For example, patients in area (b + c) were compared to patients in other areas. Analysis was performed using either Japanese or Asian WC cut-off values. In general, especially in female patients, substantially greater risk assessment accuracy was achieved when using the Asian WC cut-off value than the Japanese valve, since a relatively large number of categories with significantly elevated hazard ratios were obtained when the Asian cut-off value was used. Moreover, hazard ratio values were generally higher when using the Asian WC cut-off value.

When the risk for patients included in an individual category (i.e., a b, c, d, e, f, or g) was calculated separately from risks for patients not included in that particular area, male patients with all three MetS components (i.e. area c) had a significantly increased risk, regardless of the WC threshold. Male patients in area f also had a significantly elevated risk, but only when the Asian WC cut-off value was applied; however, in female patients, none of the individual categories represented a significantly increased risk.

When risks in male patients included in combinations of two areas (i.e., (b + c), (c + d) or (c + f)) were assessed and compared with those not included in such combinations of those areas, only men in areas (c + d) and (c + f) had a significantly elevated hazard ratio.

Similarly, when men in areas (b + c + d), (b + c + f) or (c + d + f) were assessed against those in the complement set of each area, men in areas (b + c + f) and (c + d + f) had a significantly elevated risk. In female patients, a substantially different risk profile was obtained in combinations of two or three areas. For example, the hazard ratios for categories (b + c), (b + c + d) and (b + c + f) were significantly elevated to approximately twice that in those in the comple-

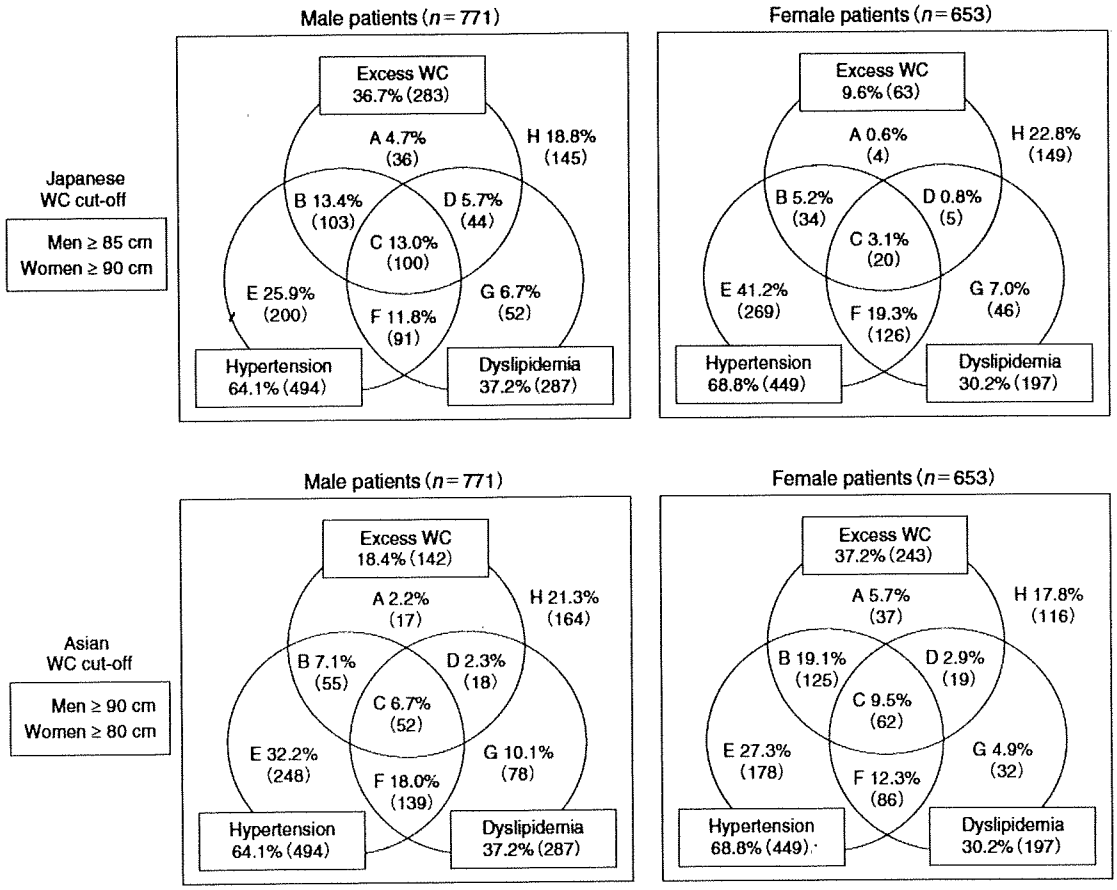

Fig. 1. Distribution of Japanese patients with type 2 diabetes categorized by baseline status of risk factor(s) (excess waist circumference (WC), hypertension and dyslipidemia).

ment set of each area. The current Japanese criteria of MetS[20] (corresponding to patients in area (b + c + d)) were predictive of CVD events only in female patients when the Asian WC cut-off value was applied.

When risks in patients included in a combination of four areas (b + c + d + f) were assessed, both male and female patients had significantly elevated hazard ratios when using the Asian cut-off value. The hazard ratio values were similar to those with combinations of two or three areas.

Discussion

The present analysis prospectively demonstrated in Japanese patients with type 2 diabetes that 1) at least two components of MetS in addition to hyperglycemia are required to have a significantly elevated CVD risk; 2) the combination of MetS components associated with a significantly elevated CVD risk is markedly different depending on gender and the WC cut-off value and 3) the combinations of MetS components with high hazard ratios for CVD did not completely agree with the current definitions of MetS. These findings imply that the current MetS criteria need to be modified when applied to patients with type 2 diabetes.

Although the clinical relevance in diagnosing MetS in diabetic subjects is still under debate[22], a simple assessment tool for cardiovascular risk in patients with diabetes but without an elevated LDL cholesterol level or who do not smoke is greatly needed in clinical settings, as demonstrated by the numerous recent studies that compared the hazard ratio for CVD between diabetic patients with and without MetS[1, 2, 4-13]; unfortunately, the results were inconsistent.

Some of these studies concluded that MetS diagnosed by the current definitions has a considerable role in the increased CVD risk in patients with type 2 diabetes[5, 6, 9, 12, 13], and that the impact of diabetes itself on CVD risk is relatively limited without coex-

Table 2. Hazard ratio of CVD events (CHD and/or stroke) in diabetic patients according to individual and combined risk categories indicated in Fig. 1 compared to those not in these categories

Threshold	Japanese cut-off value				Asian cut-off value			
	Men ≥85 cm		Women ≥90 cm		Men ≥90 cm		Women ≥80 cm	
Patient category	Hazard ratio (95% C.I.)	*p* value[§]	Hazard ratio (95% C.I.)	*p* value[§]	Hazard ratio (95% C.I.)	*p* value[§]	Hazard ratio (95% C.I.)	*p* value[§]
a	0.54 (0.13-2.21)	0.39	n/a[¶]		n/a[¶]		n/a[¶]	
b	0.68 (0.31-1.48)	0.33	2.27 (0.90-5.75)	0.08	0.94 (0.38-2.33)	0.89	1.71 (0.90-3.26)	0.10
c	2.05 (1.18-3.57)	0.01	n/a[¶]		2.25 (1.12-4.54)	0.02	1.91 (0.85-4.28)	0.12
d	1.39 (0.56-3.45)	0.48	n/a[¶]		1.33 (0.33-5.42)	0.69	1.88 (0.46-7.78)	0.38
e	0.80 (0.46-1.39)	0.43	0.74 (0.40-1.36)	0.34	0.69 (0.40-1.17)	0.17	0.54 (0.25-1.15)	0.11
f	1.64 (0.88-3.05)	0.12	1.90 (1.00-3.61)	0.05	1.73 (1.03-2.93)	0.04	1.11 (0.47-2.61)	0.82
g	0.61 (0.19-1.94)	0.40	1.65 (0.65-4.17)	0.29	0.85 (0.37-1.97)	0.71	1.27 (0.39-4.10)	0.69
b+c	1.34 (0.82-2.20)	0.24	1.33 (0.52-3.37)	0.55	1.57 (0.88-2.82)	0.13	2.06 (1.14-3.71)	0.02
c+d	1.97 (1.18-3.27)	0.01	-	0.99	2.06 (1.08-3.91)	0.03	1.98 (0.95-4.11)	0.07
c+f	2.12 (1.32-3.41)	0.00	1.53 (0.80-2.91)	0.20	2.12 (1.32-3.41)	0.00	1.53 (0.80-2.91)	0.20
b+c+d*	1.42 (0.88-2.28)	0.15	1.22 (0.48-3.09)	0.67	1.57 (0.90-2.73)	0.11	2.19 (1.22-3.93)	0.01
b+c+f	1.63 (1.03-2.58)	0.04	1.87 (1.03-3.40)	0.04	1.93 (1.22-3.07)	0.01	2.04 (1.13-3.68)	0.02
c+d+f	2.16 (1.36-3.43)	0.00	1.47 (0.77-2.79)	0.24	2.12 (1.33-3.39)	0.00	1.63 (0.88-3.04)	0.12
b+c+d+f	1.74 (1.01-2.77)	0.02	1.81 (1.00-3.29)	0.05	1.96 (1.23-3.11)	0.00	2.22 (1.21-4.05)	0.01

e.g., patients in area (b + c) were compared to patients in an area other than (b + c).
*corresponds to Japanese criteria of metabolic syndrome, [§]ANOVA, [¶]could not calculate because of no events

isting MetS or its components[23]. In contrast, in Finnish women[11] and Singaporean men[24], MetS diagnosed by existing criteria does not present a further risk of CVD in addition to that presented by type 2 diabetes per se. Likewise, combinations of any two MetS components were not significantly associated with higher mortality in Italian patients with type 2 diabetes[7, 25] and a single component of MetS was a more powerful predictor than the overall syndrome in Type 1 diabetic patients[26]. Similarly, a recent report of the United Kingdom Prospective Diabetes Study[4] also questioned the clinical value of diagnosing MetS for CVD risk stratification in patients newly diagnosed with type 2 diabetes.

These inconsistencies in previous prospective studies along with our current results suggest that the established definitions of MetS leave room for improvement when applied to diabetic patients. It also implies that specific combinations of MetS components that increase CVD risk in diabetic patients differ depending on the ethnic group; therefore, an ethnicity-specific definition of MetS might be necessary.

The current results also revealed gender differences in combinations of MetS components associated with higher CVD risks. In male patients, dyslipidemia had a relatively large prognostic value since area [c + (d and/or f)] indicated a significantly elevated risk for CVD events. On the other hand, in female patients, hypertension was important, which was similar to the result reported in Chinese patients with type 2 diabetes[10]. A large gender difference was also seen in other cohorts[11, 24, 27]. Most studies did not stratify results by gender in their analysis, which is considered to be a of the low prognostic power of the established definition of MetS; however, the gender difference became insignificant when area (b + c + d + f) in **Fig. 1** is considered.

The current results also indicated that the Asian WC cut-off value is more appropriate than the Japanese cut-off value, even for Japanese diabetic patients, for discriminating patients at high risk; however, WC per se was not indispensable for predicting CVD events in our patients despite the worldwide definition of IDF[21] as well as Japanese[20] definitions of MetS, as we reported previously[2]. The poor prognostic power of the IDF definition in diabetic subjects has also been reported for other ethnic groups, such as Hong Kong Chinese[5], Native Americans[6] and Italians[7]. Lack of a rationale for excess WC as a mandatory component of MetS was also shown in recent studies of non-diabetic subjects[14, 28].

Table 3 shows the suggested definitions of MetS for Japanese patients with type 2 diabetes based on our current and previous results. This criteria is similar

Table 3. Suggested definition of MetS for Japanese patients with type 2 diabetes for predicting future CVD events

Patients with two or more of the following
1) Excess WC: men ≥90 cm, women ≥80 cm
2) Hypertension: systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg or use of an agent for this condition
3) Dyslipidemia: triglyceride ≥150 mg/dL and/or HDL-cholesterol <40 mg/dL or use of an agent for this condition

to that of the American Heart Association/National Heart Lung and Blood Institute (AHA/NHLBI)[19] (revised version of NCEP-ATPIII) but differs in terms of dyslipidemia. that is, an elevated triglyceride level and decreased HDL cholesterol level were treated as individual components in the AHA/NHLBI definition. We previously determined that hazard ratios of hypertriglyceridemia alone and hypertriglyceridemia or low HDL cholesterol were almost identical, but the latter covers more patients. These two indices are known to be frequently correlated and so could have greater prognostic power when combined.

Monami and colleagues[8] suggested that a MetS diagnosis based only on the unweighted number of components present in each patient, without considering each specific combination, could be inadequate to predict the risk level because a different risk profile is determined by different combinations of metabolic alterations. Although our current results principally support their conclusion, we still consider that using area (b+c+d+f) in **Fig. 1** as a definition of MetS has merit in clinical settings because 1) the hazard ratios are similar (or even higher in female patients) to other significant combinations, 2) it can be used regardless of patient gender, 3) it covers more subjects than combinations of two or three areas among b, c, d and f, and 4) it is simple and easy to remember.

The current study has several strengths and limitations. The strengths include the nationwide multicentered setting and prospective design, which enabled us to assess the predictability of a CVD event. In addition, all institutes that participated are university or large general hospitals; therefore, the quality of risk evaluation and the accuracy of CVD diagnosis were excellent. A limitation is that the results may only be applicable to Japanese patients with type 2 diabetes. As described above, ethnicity can be considered an important factor for determining CVD risk in diabetic subjects as well as in the general population, so the clinical significance of MetS should be determined separately in each ethnic group. In addition, we do not have sufficient data on mortality, which needs to be determined in the future. We did not determine different cut-off values for blood pressure and serum lipids since their cut-off values have been well-established in many guidelines, unlike WC.

In conclusion, Japanese diabetic patients with two or more features of MetS with excess WC according to the Asian cut-off value (male ≥90 cm, female ≥80 cm), hypertension and dyslipidemia have a significantly elevated CVD event risk. Nevertheless, the rationale was weak for including WC as a mandatory component when evaluating CVD risk despite existing definitions of MetS. The definition of MetS should be modified to provide better prognostic value in clinical settings of diabetes management.

Appendix

The Japan Diabetes Complications Study (JDCS) Group:

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Waist Circumference as a Cardiovascular and Metabolic Risk in Japanese Patients With Type 2 Diabetes

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Excess waist circumference (WC) is a frequently used indicator of abdominal obesity and/or cardiovascular disease (CVD) risk. Nonetheless, search of the literature revealed no prospective studies on the association between WC and CVD events in diabetic patients. In this study, the clinical significance and implications of WC as a cardiovascular and metabolic risk indicator was prospectively investigated in Japanese patients with type 2 diabetes. For this purpose, baseline data on WC, hypertension, and dyslipidemia were collected and subsequent CVD (coronary heart disease and stroke) events during the following 8 years were studied in 1,424 Japanese type 2 diabetic patients, and the cross-sectional/longitudinal associations between WC and CVD risk factors/events were analyzed. Mean WC levels were significantly increased according to the number of coexisting risk factors. However, no significant difference in mean WC between subgroups with and without CVD events was noted, and excess WC alone was not predictive of subsequent CVD events either in male or female subjects even after adjustment for age, smoking, hypertension, and dyslipidemia. In female patients, excess WC (≥80 cm) was predictive of CVD events only with the coexistence of hypertension. In Japanese diabetic patients, excess WC alone, although a good marker for clustering of CVD risk factors, did not raise the risk of CVD events unless accompanied by hypertension in female patients. Further investigations are necessary before WC as a risk factor can be utilized in clinical settings for the management of diabetes in this population.

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INTRODUCTION

Waist circumference (WC) is a simple and widely used indicator of abdominal adiposity and the risk of cardiovascular disease (CVD) (1), and, thus, has been adopted in most definitions of metabolic syndrome (MetS) (2–4). However, prospective studies of the relationship between WC and CVD events *per se* have been performed mostly in white populations (5–10). Moreover, we are not aware of studies that are specific to diabetic subjects.

The incidence and characteristics of CVD are known to be quite different between Asians and whites (11) or between diabetic and nondiabetic subjects. In particular, the importance of central obesity in the diabetic population has hardly been examined. Therefore, a prospective study is needed on the clinical significance of WC in relation to CVD in Asian diabetic subjects, who comprise more than one-third of the global diabetic population (12).

In previous studies (13,14), we examined the diagnosis of MetS as a predictor of CVD and found that MetS, as defined by the International Diabetes Federation (IDF) (2), has lower predictability for future CVD events than that according to the World Health Organization (WHO) (15) and National Cholesterol Education Program/Adult Treatment Panel III (16) in Japanese patients with type 2 diabetes. Since IDF criteria (2) include excess WC as a mandatory component for the diagnosis of MetS and the criteria of the other two organizations do not, it is possible that the lower prognostic power of IDF criteria is derived from mandatory inclusion of WC. In

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this study, we undertook a detailed evaluation of the significance of WC in cardiovascular and metabolic risk in Japanese patients with type 2 diabetes.

METHODS AND PROCEDURES

The Japan Diabetes Complications Study is a multicenter prospective study of Japanese patients with type 2 diabetes (17–20). In 1996, 2,205 patients aged 40–70 years with previously diagnosed type 2 diabetes and HbA_{1c} levels of >6.5% were recruited from 59 institutes specializing in diabetes care. The eligibility criteria for participating patients have been described previously (17). Of the 2,205 patients, the present study focused on the 1,424 patients (771 men and 653 women) analyzed previously (13,14), who had no history of CVD but had a complete set of data including those parameters necessary to satisfy the WHO (15) and National Cholesterol Education Program (16) criteria for the definition of MetS at baseline. Baseline characteristics of the patients analyzed are shown in **Table 1**. In terms of representativeness of the 1,424 patients among the 2,205 patients, comparison between the 1,424 patients and the remaining patients (i.e., 781 patients) showed that only two of the parameters listed in **Table 1** differed significantly between these two groups as determined by the *t*-test. These two exceptions were fasting plasma glucose and HbA_{1c}, which were slightly but significantly higher in the 1,424 patients than in the 781 patients (159 ± 44 mg/dl vs. 154 ± 40 mg/dl, $P = 0.006$, and 7.8 ± 1.4 vs. 7.6 ± 1.2%, $P = 0.006$, respectively). The protocol of the Japan Diabetes Complications Study received ethical approval from the institutional review boards of all of the participating institutes and all of the study participants gave written informed consent.

Baseline WC was analyzed with baseline presence of hypertension and dyslipidemia. Also evaluated was the association of baseline WC with future CVD events (fatal/nonfatal coronary heart disease (CHD) and stroke) during an 8-year period. Thresholds for individual risk factors were adopted from the Japanese definition of MetS (3), which was close to that of IDF (2). Since all of the subjects in this study had diabetes mellitus, three criteria other than an elevated fasting plasma glucose level (>110 mg/dl) were used: (i) excess WC (male ≥85 cm, female ≥90 cm), (ii) hypertension (systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg), and (iii) dyslipidemia (triglyceride ≥150 mg/dl and/or high-density lipoprotein cholesterol <40 mg/dl). Subjects using agents for hypertension and hyperlipidemia were considered to have those risk factors. The alternative WC cutoff values for Asians in general as decided by the WHO (male ≥90 cm, female ≥80 cm) (2,4) were also used for additional evaluation.

Patients were assessed for CHD and stroke at baseline and yearly thereafter. In all subjects, a 12-lead electrocardiogram was recorded at each assessment. Both fatal/nonfatal CHD and stroke events identified during follow-up were certified by at least two members of the experts' committee who were masked as to risk factor status of the patient and the other member's diagnosis. In terms of CHD, myocardial infarction was defined in accordance with the criteria of the WHO Monitoring of Trends and Determinants in Cardiovascular Disease (21), and angina pectoris was defined as typical effort-dependent chest pain or oppression relieved at rest or by use of nitroglycerine as validated by exercise-positive electrocardiogram and/or angiography.

Stroke events were defined as a constellation of focal or global neurological deficits that were sudden or rapid in onset and for which there was no apparent cause other than a vascular accident on the basis of a detailed history, neurologic examination, and ancillary diagnostic procedures such as computed tomography, magnetic resonance imaging, cerebral angiography, and lumbar puncture. Stroke events were classified as cerebral infarction (including embolus), intracranial hemorrhage (including subarachnoid hemorrhage), transient ischemic attack, or stroke of undetermined type in accordance with WHO criteria (22). No cases of asymptomatic lesions detected by brain imaging (i.e., silent infarction) were included. Only "first-ever" CHD or stroke events during the study period were counted for the analysis and if a patient had both CHD and stroke events, then each event was counted separately.

Measurement of WC was at the level of the umbilicus. Information regarding cigarette smoking was collected using a standardized questionnaire. All laboratory tests were undertaken using the standard methods of each participating institute, apart from the HbA_{1c} assays that used a common standard, with 5.8% as the upper normal limit. Plasma low-density lipoprotein cholesterol was calculated using Friedwald's equation, except where triglycerides exceeded 400 mg/dl, in which case the low-density lipoprotein cholesterol data were treated as "missing." Data are presented as means ± s.d. or as a proportion unless otherwise specified. WC in each group was assessed by Wilcoxon's rank sum test. Cox regression analysis was used to calculate the age-adjusted hazard ratio and 95% confidence intervals of risk factors for CVD. The SAS software package (version 9.0; SAS Institute, Cary, NC) was used for all analyses. $P < 0.05$ was considered to be significant.

RESULTS

WC was associated with a number of CVD risk factors but not with future CVD events

We first determined mean WC values with 95% confidence intervals in groups of patients stratified according to the number of risk factors at baseline (**Table 2**). In both men and women, WC significantly increased in a stepwise manner beginning with those with no risk factors to those with two risk factors. Differences in mean WC levels did not differ significantly between groups with 0 vs. 1 or 2 risk factors, as well as groups with 0 or 1 vs. 2 risk factors. Then, we compared

Table 1 Baseline characteristics of patients analyzed

	Men	Women
Number of patients (%)	771	653
Age (years)	58.2 ± 7.4	58.7 ± 7.4
Diabetes duration (years)	10.9 ± 7.6	10.1 ± 6.7
BMI (kg/m^2)	22.9 ± 2.6	23.4 ± 3.3
Waist circumference (cm)	82.3 ± 7.7	76.5 ± 9.8
Waist-to-hip ratio	0.89 ± 0.07	0.83 ± 0.08
Blood pressure (mm Hg)	132 ± 16/78 ± 10	132 ± 17/76 ± 10
HbA_{1c} (%)	7.61 ± 1.36	8.05 ± 1.45
Fasting plasma glucose[a] (mmol/l)	8.3 (7.2, 10.0)	8.6 (7.3, 10.2)
Fasting plasma insulin[a] (pmol/l)[c]	6.2 (0.5, 1.9)	7.1 (0.5, 1.9)
Serum LDL cholesterol (mmol/l)	3.03 ± 0.86	3.38 ± 0.82
Serum HDL cholesterol (mmol/l)	1.34 ± 0.39	1.47 ± 0.44
Serum triglycerides[b] (mmol/l)	1.39 (0.75)	1.29 (0.72)
Current smoker (%)	43.9	8.7
OHA (without insulin) use (%)	72	77
Insulin (with or without OHA) use (%)	16	20
Medication for hypertension (%)	22	29
Medication for hyperlipidemia (%)	15	35

Data are presented as mean ± s.d.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; OHA, oral hypoglycemic agents.
[a]Median (IQR). [b]Geometric mean (1 s.d.). [c]Patients on insulin therapy were excluded.

Table 2 Mean waist circumference (WC) with 95% confidence intervals (CI) in Japanese patients with type 2 diabetes stratified according to the number of risk factors (i.e., hypertension and/or dyslipidemia) at baseline

				Model 1		Model 2	
	Number of CVD risk factors		No. patients	Mean WC (95% CI)	*P* value	Mean WC (95% CI)	*P* value
Men	0 vs. 1 vs. 2	0	181	78.8 (77.7–79.8)*	<0.0001	78.7 (77.6–79.8)*	<0.0001
		1	399	82.5 (81.7–83.2)*		82.4 (81.6–83.2)*	
		2	191	85.2 (84.1–86.2)*		85.5 (84.4–86.6)*	
	0 vs. ≥1	0	181	78.8 (77.7–79.9)	<0.0001	78.7 (77.6–79.8)	<0.0001
		≥1	590	83.3 (82.7–83.9)		83.4 (82.8–84.0)	
	1≤ vs. 2	1≤	580	81.3 (80.7–81.9)	<0.0001	81.2 (80.6–81.9)	<0.0001
		2	191	85.2 (84.1–86.2)		85.5 (84.4–86.6)	
Women	0 vs. 1 vs. 2	0	153	72.9 (71.4–74.5)**	<0.0001	72.1 (70.1–74.0)**	<0.0001
		1	354	77.0 (76.0–78.0)**		76.5 (75.0–78.1)**	
		2	146	78.9 (77.4–80.5)**		78.4 (76.5–80.3)**	
	0 vs. ≥1	0	153	72.9 (71.4–74.5)	<0.0001	72.0 (70.2–74.2)	<0.0001
		≥1	500	77.6 (76.7–78.4)		77.2 (75.8–78.6)	
	1≤ vs. 2	1≤	507	75.8 (74.9–76.6)	0.0008	75.2 (73.8–76.7)	0.0013
		2	146	78.9 (77.3–80.5)		78.4 (76.5–80.3)	

Model 1, adjusted by age; model 2, adjusted by age and smoking status; *P* values were calculated by ANOVA.
CVD, cardiovascular disease.
*Significant differences existed between all three groups, **significant differences existed between 0 vs. 1 and 0 vs. 2 by Tukey's multiple comparison test. Significant trend ($P < 0.0001$) also existed for both men* and women**.

Table 3 Mean waist circumference (WC) with 95% confidence intervals (CI) in Japanese patients with type 2 diabetes stratified according to CVD events during follow-up

				Model 1		Model 2		Model 3	
	CVD event		No. patients	Mean WC (95% CI)	*P* value	Mean WC (95% CI)	*P* value	Mean WC (95% CI)	*P* value
Men	CHD	–	703	82.2 (81.6–82.8)	0.616	82.2 (81.6–82.8)	0.609	82.4 (81.7–83.0)	0.777
		+	42	82.7 (80.9–84.5)		82.7 (80.9–84.5)		82.1 (80.4–83.8)	
	Stroke	–	738	82.3 (81.7–82.8)	0.962	82.3 (81.7–82.8)	0.936	82.4 (81.8–83.0)	0.619
		+	33	82.2 (79.6–84.8)		82.1 (79.5–84.8)		81.7 (79.1–84.3)	
	CHD and/or stroke	–	673	82.2 (81.7–82.8)	0.523	82.2 (81.6–82.8)	0.513	82.3 (81.7–83.0)	0.956
		+	72	83.0 (80.7–85.3)		83.0 (80.7–85.4)		82.3 (80.0–84.5)	
Women	CHD	–	618	76.3 (75.5–77.1)	0.131	76.0 (74.6–77.4)	0.197	75.6 (74.2–77.1)	0.269
		+	20	78.6 (75.7–81.4)		78.0 (74.9–81.2)		77.4 (74.3–80.5)	
	Stroke	–	627	76.4 (75.6–77.1)	0.237	76.0 (74.6–77.4)	0.297	75.7 (74.3–77.1)	0.400
		+	26	78.7 (74.9–82.5)		78.2 (74.1–82.2)		77.4 (73.4–81.4)	
	CHD and/or stroke	–	593	76.4 (75.6–77.2)	0.523	76.1 (74.7–77.5)	0.611	75.8 (74.4–77.2)	0.696
		+	45	77.8 (73.5–82.1)		77.3 (72.6–82.0)		76.7 (72.1–81.2)	

Model 1, adjusted by age; model 2, adjusted by age and smoking status; model 3, adjusted by age, smoking status, hypertension, and dyslipidemia. *P* values were calculated by ANOVA.
CHD, coronary heart disease; CVD, cardiovascular disease.

mean WC in groups stratified according to whether CVD events (CHD and/or stroke) occurred during the 8-year follow-up period (**Table 3**). However, unlike groups stratified by baseline risk factors (**Table 2**), mean WC values did not differ significantly between either male or female groups with and without CVD events (**Table 3**). These relationships between WC and the number of risk factors or between WC and CVD events were not altered even after adjustment for age, smoking, (**Tables 2** and **3**) and existence of hypertension and dyslipidemia (**Table 3**). The relationships between WC and CHD events were not changed regardless of inclusion or exclusion of subjects with asymptomatic myocardial infarction, which accounted for 5 of 42 men and 3 of 20 women (data not shown).

Table 4 Hazard ratio (HR) of those who have each risk factor compared to those who do not (as a categorical variable)

		Model 1		Model 2	
	(Yes vs. no, no = reference)	HR	*P* value	HR	*P* value
Men	Hypertension	1.34 (0.79–2.27)	0.28	1.30 (0.76–2.22)	0.35
	Dyslipidemia	**1.93 (1.21–3.07)**	**0.006**	**1.90 (1.18–3.07)**	**0.009**
	High WC (by Japanese cutoff, i.e., > 85 cm)	1.32 (0.83–2.12)	0.24	1.07 (0.65–1.74)	0.80
	High WC (by Asian cutoff, i.e., >90 cm)	1.32 (0.75–2.31)	0.33	1.09 (0.62–1.93)	0.76
Women	Hypertension	1.06 (0.53–2.14)	0.86	0.96 (0.47–1.96)	0.91
	Dyslipidemia	1.58 (0.84–2.96)	0.16	1.47 (0.77–2.79)	0.24
	High WC (by Japanese cutoff, i.e.,. > 90 cm)	0.92 (0.33–2.60)	0.88	0.84 (0.29–2.38)	0.74
	High WC (by Asian cutoff, i.e., >80 cm)	1.68 (0.91–3.11)	0.099	1.52 (0.81–2.85)	0.19

HR of each of the various risk factors for cardiovascular disease events (coronary heart disease and/or stroke) in Japanese patients with type 2 diabetes calculated by the Cox regression analysis. Statistically significant ($P < 0.05$) values are shown in boldface. Model 1, adjusted by age and smoking status; model 2, adjusted by age, smoking status, hypertension, and dyslipidemia. Adjustment by hypertension or dyslipidemia was performed in cases when either or both of these two parameters were not used (e.g., when calculating odds ratio of hypertension, only age, smoking status, and dyslipidemia were used for adjustment).
WC, waist circumference.

Table 5 Hazard ratio (HR) of having every 1 s.d. higher value of each risk factor (ss continuous variable)

		Model 1		Model 2	
	(Per 1 s.d. increase of each value)	HR	*P* value	HR	*P* value
Men	Systolic blood pressure	1.26 (0.99–1.60)	0.051	1.25 (0.98–1.59)	0.077
	Diastolic blood pressure	**1.28 (1.00–1.64)**	**0.046**	1.25 (097–1.61)	0.091
	Triglycerides	**1.32 (1.08–1.61)**	**0.007**	**1.29 (1.05–1.59)**	**0.015**
	HDL cholesterol	0.43 (0.18–1.05)	0.065	0.43 (0.18–1.07)	0.069
	HbA_{1c}	**1.37 (1.12–1.67)**	**0.002**	**1.37 (1.11–1.68)**	**0.004**
	WC	1.09 (0.86–1.37)	0.49	0.94 (0.73–1.21)	0.62
Women	Systolic blood pressure	**1.40 (1.02–1.92)**	**0.038**	1.37 (1.00–1.89)	0.051
	Diastolic blood pressure	1.18 (0.89–1.58)	0.26	1.14 (0.85–1.53)	0.40
	Triglycerides	1.09 (0.82–1.43)	0.55	1.02 (0.77–1.37)	0.88
	HDL cholesterol	0.55 (0.17–1.71)	0.30	0.61 (0.19–2.00)	0.41
	HbA_{1c}	1.29 (0.97–1.72)	0.086	1.32 (0.98–1.77)	0.072
	WC	1.21 (0.90–1.63)	0.21	1.15 (0.84–1.57)	0.40

HR of each of the various risk factors for cardiovascular disease events (coronary heart disease and/or stroke) in Japanese patients with type 2 diabetes calculated by the Cox regression analysis. Statistically significant ($P < 0.05$) values are shown in boldface. Model 1, adjusted by age and smoking status; model 2, adjusted by age, smoking status, hypertension, and dyslipidemia. Adjustment by hypertension or dyslipidemia was performed in cases when either or both of these two parameters were not used (e.g., when calculating odds ratio of hypertension, only age, smoking status, and dyslipidemia were used for adjustment).
HDL, high-density lipoprotein; WC, waist circumference.

WC was not an independent risk factor for CVD in diabetic patients

The above results led us to further investigate the individual hazard ratio for WC in comparison with other risk factors when expressed as categorical (**Table 4**) or continuous (**Table 5**) variables. Although hypertension, dyslipidemia, and glycemia in men and hypertension in women had a significantly elevated hazard ratio, the hazard ratio for WC, either as a categorical (**Table 4**) or as a continuous (**Table 5**) variable, was not significantly elevated. Then, we determined whether WC could have a potential interaction between hypertension and dyslipidemia, or both by calculating the hazard ratio adjusted by either of these potential confounders, replacing each with the other. However, we did not find any significantly elevated hazard ratio even after that calculation (data not shown).

Results of subgroup analysis of participants categorized according to the presence of hypertension and/or dyslipidemia are shown in **Table 4**. Only in female patients did those with hypertension demonstrate a significantly elevated hazard ratio in accordance with excess WC as defined by the Asian cutoff (i.e., 80 cm) or per 1 s.d. Hazard ratios were not significantly elevated in male subjects in any category regardless of whether they had hypertension and/or dyslipidemia.

DISCUSSION

The current results shown in **Table 2** are partially concordant with results of a cross-sectional study by Tseng (23) showing that excess WC is strongly associated with clustering of cardiovascular risk factors in East Asian patients with type 2 diabetes. Nevertheless, according to our current results shown

in **Table 3**, this positive relationship could not be used for the prediction of future CVD events. Although to the best of our knowledge no study has evaluated WC and its association with the baseline presence of CVD risk factors and future incidence of CVD in the same cohort, the results shown in this study clearly demonstrate a vast discrepancy between cross-sectional and longitudinal results for this topic. These discrepancies are somewhat understandable as the majority of the current WC cutoff values, including the Japanese (24) and Asian (25) values, were not determined based on prospective data on CVD events (26) but on the presence of cardiovascular risk factor(s), which are only surrogate markers for CVD events.

However, it was also reported that clustering of cardiovascular risk factors increased the risk of events even in subjects with diabetes (27). A possible explanation for the apparent contradictory results shown here that WC was not a predictor of CVD events, despite being a good marker for risk factor clustering, is that excess WC alone might not be predictive in these patients without the presence of other risk factors. This hypothesis is partially supported by our results shown in **Table 6** that excess WC was predictive only with coexisting hypertension in women with diabetes. Another interpretation is that the risk factors that tend to be clustered with an enlarged WC might be insufficient to significantly raise the risk of CVD events. Although we mostly used only the CVD risk factors adopted in the MetS definitions in this study, existing definitions of MetS have been shown to be quite poor predictors of future CVD events in patients with type 2 diagnosis, as recently reported by us (13,14) as well as by the United Kingdom Prospective Diabetes Study (28). Therefore, other risk factors such as diabetes duration, status of glycemic control or low-density lipoprotein- cholesterol level, smoking habits or the presence of atrial fibrillation, which do not have close associations with WC *per se*, also have potent effects (29,30). Actually, the status of glycemic control as expressed by HbA_{1c} values was a significant risk factor in men (**Table 5**).

Another possible explanation for the contradiction is that a longer period of observation than ours might be necessary for CVD events to occur or that diabetes *per se* might impact on body composition. Finally, another possibility is that WC might not be a good indicator of visceral adiposity in diabetic patients and that other methods of assessment such as computed tomography or magnetic resonance imaging might be better. In addition, it should be considered whether this result was obtained by chance; therefore, further investigations are necessary.

This is a primary prevention study of macrovascular complications of diabetes and our patients had no baseline CVD even though the mean duration of diabetes was 10 years. Although such patients might be considered quite unusual in Europe or in the United States, patients with diabetes of a rather long duration but without CVD are common in Japan because the incidence of CVD is markedly lower in East Asian countries than in Europe or in the United States (31). In fact, only ~5% of patients with a 10-year history of type 2 diabetes in Japan have CHD in their history (32). Moreover, although the mean WC in our patients is much lower than in European or American patients with type 2 diabetes, the mean WC in our cohort is almost identical to that reported in the Japanese general population (33,34). In fact, as we have reported previously (18), Japanese diabetic patients, in general, are not obese compared to the general population, which is an important characteristic of patients with diabetes in Japan. In addition, thresholds of WC appropriate for diagnosis of MetS are also reportedly much lower in Japanese compared with white patients (35);

Table 6 Hazard ratio (HR) of having larger waist circumference (WC; as a categorical or a continuous variable) for cardiovascular disease events (coronary heart disease and/or stroke) according to patients subgrouped by existence of hypertension and/or dyslipidemia

	Hypertension		Dyslipidemia	No. patients	(1) WC ≥ 85 cm (vs. WC < 85 cm)	*P* value	(2) WC ≥ 90 cm (vs. WC < 90 cm)	*P* value	(3) per 1 s.d. increase in WC	*P* value
Men	–		–	181	0.74 (0.16–3.33)	0.69	NA		0.88 (0.51–1.50)	0.63
	+		–	303	0.86 (0.35–2.08)	0.73	1.21 (0.45–3.25)	0.71	0.93 (0.61–1.40)	0.72
	–		+	96	2.31 (0.55–9.68)	0.25	1.51 (0.30–7.54)	0.62	1.02 (0.48–2.16)	0.96
	+	and	+	191	1.27 (0.60–2.72)	0.53	1.42 (0.64–3.14)	0.39	1.18 (0.77–1.80)	0.44
	+	or	+	590	1.34 (0.80–2.24)	0.26	1.44 (0.81–2.55)	0.22	0.88 (0.51–1.50)	0.63
	Hypertension		**Dyslipidemia**	**No. patients**	**(1) WC ≥ 90 cm (vs. WC < 90 cm)**	***P* value**	**(2) WC ≥ 80 cm (vs. WC < 80 cm)**	***P* value**	**(3) per 1 s.d. increase in WC**	***P* value**
Women	–		–	153	1.10 (0.84–1.44)	0.47	NA		0.64 (0.22–1.91)	0.43
	+		–	303	2.49 (0.90–6.85)	0.078	**2.41 (0.99–5.82)**	**0.050**	**1.56 (1.04–2.32)**	**0.031**
	–		+	51	NA		1.57 (0.25–9.86)	0.63	0.87 (0.30–2.52)	0.79
	+	and	+	146	NA		1.43 (0.47–4.39)	0.53	0.94 (0.54–1.62)	0.82
	+	or	+	500	NA		**1.93 (1.02–3.64)**	**0.042**	1.26 (0.94–1.70)	0.13

HRs were calculated in three ways, i.e., HR of those whose WC was equal to or greater than cutoffs by (1) Japanese or (2) Asian criteria, compared to those whose WC was less than those cutoffs (as a categorical variable); or (3) HR of having every 1 s.d. larger WC (as a continuous variable). Statistically significant ($P < 0.05$) values are shown in boldface.
NA, could not analyze due to small numbers of events.

however, even a small increase in WC is predictive of a substantial increase in the risk for CVD in other East Asian countries (36).

It is widely accepted that WC cutoff values should be modified in consideration of ethnicity (26,37,38). However, even using ethnic-specific (either for Japanese or Asians) cutoffs, WC alone was not a significant risk factor for CVD events in both male and female patients with type 2 diabetes (13,14), which is inconsistent with previously reported prospective results in the general population or in mainly nondiabetic populations (5–10,39,40). It is speculated that diabetes itself could greatly enhance the CVD risk, thus mask the influence of coexisting excess WC. If so, clinical significance of excess WC in predicting CVD might need to be modified according to diabetes status.

The current study has several strengths and limitations. Strengths include the multicentered setting and prospective design, which enabled us to assess the predictability of a CVD event *per se*. A limitation is that the results may only be applicable to Japanese patients with type 2 diabetes. Further investigations are necessary in non-Asian diabetic patients. In addition, the combination of WC plus other indices of obesity such as BMI (41,42) should be examined in the future in diabetic subjects because the significance of those combinations differs between studies in chiefly nondiabetic subjects (43–46). However, there is increasing evidence that the significance of BMI alone is relatively limited (47).

In conclusion, despite the fact that WC is a good marker for clustering of CVD risk factors, a high WC value alone is not sufficient to raise the risk of CVD events significantly and is not an independent risk factor in Japanese diabetic patients. Further investigations are necessary before WC as a risk factor can be utilized in clinical settings for the management of diabetes in this population.

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DISCLOSURE

The authors declared no conflict of interest.

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Clinical factors such as B-type natriuretic peptide link to factor VII, endothelial NO synthase and estrogen receptor α polymorphism in elderly women

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ABSTRACT

Aims: This study evaluated the presence of genetic mutations in relation to thrombosis or atherosclerosis in elderly women.

Main methods: This is an observational study of 93 Japanese women with a mean age of 80.9 years recruited from outpatient clinics of Nagoya University and its related hospitals. Ten single nucleotide polymorphisms (SNPs) were studied. Each gene studied acts in or is related to either blood coagulation (factor V Leiden, prothrombin G20210A, factor XIII Val34Leu, factor VII Arg353Gln, MTHFR C677T, β-fibrinogen G-455A, PAI-1 4G/5G), metabolic syndrome-related pathways (PPARα Leu162Val), or endothelium/estrogen system (eNOS Glu298Asp, ERα IVS1-401). SNPs were analyzed for their relation to clinical values including lipids, B-type natriuretic peptide (BNP), fasting plasma glucose, tumor necrosis factor-α, interleukin-6, cyclic GMP, and nitric oxide metabolites.

Key findings: Comparisons between the distributions of different genotypes and clinical values showed three relationships. First, factor VII Arg353Gln and HDL-cholesterol (HDL-C) were linked to Arg/Arg carriers at higher levels ($P=.049$). The HDL-C to LDL-cholesterol ratio supported this link ($P=.027$). Second, eNOS Glu298Asp and triglycerides were linked to Glu/Glu carriers at higher levels ($P=.031$). Third, ERα IVS1-401 and BNP were related to CC genotype at lower levels ($P=.031$). Additionally, the last two relations showed that genotype does not influence the demarcation line of biomarkers, but the plasma/serum levels of biomarkers instead.

Significance: Correlations of factor VII Arg353Gln with HDL-C and eNOS Glu298Asp with triglycerides are new findings. Polymorphisms in the endothelium/estrogen system and the heart failure marker BNP are also correlated, with ERα IVS1-401 being the first identified marker. SNPs may be helpful for understanding the pathophysiology of atherosclerotic diseases in elderly women.

Introduction

A thrombogenic state is an important risk factor for atherosclerotic diseases such as myocardial infarction and stroke. Thrombosis has been suggested to be an adverse effect associated with postmenopausal hormone-replacement therapy (HRT) (Fisher et al. 1998; Ettinger et al. 1999; Rosendaal et al. 2002).

Genetic mutation factors have been associated with an elevated risk associated with thrombosis. For example, factor V Leiden (also known as Arg506Gln, R506Q, or G1691A) has been associated with a 6.69-fold enhancement of the risk posed by thrombosis (Cushman et al. 2004). This single nucleotide polymorphism (SNP) is a guanine (G) to adenine (A) transition at the second base of the codon for amino acid position 506 in exon 10 of the factor V (symbol; F5) gene. In total, 5% of Caucasians are carriers of this SNP, which is known to make the protein resistant to inactivation by activated protein C (Bertina et al. 1994). This SNP is found in 20% of venous thrombosis patients (Rosendaal et al. 1995). Regional differences exist; East Asians do not carry factor V Leiden (Rees et al. 1995), which could be one of the reasons for the lower incidence of thrombosis in East Asians, although this remains unclear. Another noted SNP seen in Caucasians is prothrombin G20210A, which is a G to A transition at position 20210 of the prothrombin (symbol; F2) gene. It is observed among 6% of

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venous thrombosis patients (Poort et al. 1996). For these two blood clotting factors, a meta-analysis has found a moderate association with coronary disease (Ye et al. 2006), implying that, for these and other clotting factors, a genetic study should be performed.

Several SNPs related to thrombosis and atherosclerotic risk have been proposed. Factor XIII Val34Leu shows a protective effect against venous thromboembolism (Mikkola et al. 1994; Wells et al. 2006). Factor VII Arg353Gln (R353Q or G10976A) leads to a reduction of its protein levels with a lower risk of cardiovascular disease (Green et al. 1991; Lane et al. 1992; Hunault et al. 1997; Girelli et al. 2000). MTHFR C677T leads to higher homocysteine levels and is thus a risk factor for coronary artery diseases (Frosst et al. 1995; Ma et al. 1996). β-fibrinogen G-455A (HaeIII) is associated with higher plasma fibrinogen levels (Thomas et al. 1991; Iso et al. 1995; van' t Hooft et al. 1999). PAI-1 4G/5G, which is the 4G allele related to elevation of circulating PAI-1, is associated with cardiovascular disease (Dawson et al. 1991; Eriksson et al. 1995; Hoekstra et al. 2004). PPARα Leu162Val (L162V) is associated with increased transcriptional activation of itself and the elevation of serum lipid levels (Vohl et al. 2000; Sapone et al. 2000). eNOS Glu298Asp (E298D) is associated with the possibility of impaired endothelial function (Philip et al. 1999; Guzik et al. 2001). Lastly, ERα IVS1-401 (IVS1-397 or PvuII), which is a transition in the intervening sequence 1 at position-401, is associated with cardiovascular disease (Herrington et al. 2002b; Nordström et al. 2003).

In atherosclerotic lesions, lipid accumulation in the plaque intima (Kramsch et al. 1971), increased cytokine levels (Rus et al. 1991, 1996), and an increased BNP level are all detected (Casco et al. 2002). It is believed that nitrite and nitrate, which are metabolites of nitric oxide (NOx) in vascular disease, are important factors that should be assessed (Palmer et al. 1987). BNP and NO elevates intracellular cGMP level (Palmer et al. 1987; Chinkers et al. 1989), indicating the importance of this second messenger. From a clinical point of view, it is important to identify biomarkers that can be used to predict both disease and longevity (Nomura et al. 2002; Hayashi et al. 2007). However, the use of SNPs as biomarkers is not well understood to date. Our aim is to evaluate genetic factors in elderly women in order to identify biomarkers that correlate with SNPs. The experimental design of this report was restricted to an East Asian population that does not possess factor V Leiden, because it is an easier model for screening candidate factors associated with thrombosis or atherosclerotic risk. In addition, analyses focusing on postmenopausal elderly women are rarely performed, and thus may result in an improved fundamental understanding of older people.

Material and methods

Subjects

We enrolled 104 Japanese female subjects over 60 years of age who were admitted to the Department of Geriatrics in an outpatient clinic of Nagoya University Hospital, Nagoya City (Japan), between May 2004 and January 2005. All subjects gave written informed consent. Proper authorization for the study was obtained from the Ethics Committee of the Nagoya University Graduate School of Medicine. As the data of serum or plasma collection or amount of assay for DNA analyses were insufficient in 11 patients, we finally analyzed the data of 93 patients.

Serum or plasma collection and measurement

Serum or plasma was collected from fasting blood samples after centrifugation at 3000 rpm at 4 °C, and stocked at −30 °C until measurement. BNP and cGMP concentrations were derived from the blood sample analysis (SRL Laboratories, Japan) as measured by a specific immunoassay. Tumor necrosis factor-α and interleukin-6 were measured using the Quantikine HS Kit (R & D Systems, USA). NOx was determined by high-performance liquid chromatography (Hayashi et al. 2007). Other clinical biochemical factors, such as LDL-C, HDL-C, and triglyceride levels, were also assessed.

DNA isolation and genotyping

DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Düsseldorf, Germany) and genotyped with the Mutector Dual Well Test Kit (TrimGen, Maryland, USA) according to the manufacturer's instructions. The Mutector kit was designed for mutation detection among known nucleotide substitutions using a 96-well strip plate, and can confirm all three genotypes (wild, mutant homozygous, and heterozygous types). Both positive and negative controls were provided by the manufacturer. In brief, a complimentary detection primer is designed and immobilized on the wells whose 3' end terminates just before the target base (i.e. order made by offering the specified sequence information). The polymerase chain reaction product from the preceding step is added to wells together with labeled nucleotides and extension primer for either mutant or wild strand. Primer for mutant strand makes extension when the target base is mutant type but not wild, and vice versa. As a result, labeled nucleotides are incorporated and a colorimetric reaction is observed and measured by 405 nm on a microplate reader.

Selection of SNPs

All ten analyzed SNPs have referential SNP cluster identification numbers (RefSNP ID) provided by the NCBI (National Center for Biotechnology Information, Maryland). They are: factor V Leiden (rs6025), prothrombin G20210A (rs1799963), factor XIII Val34Leu (rs3024472), factor VII Arg353Gln (rs6046), MTHFR C677T (rs1801133), β-fibrinogen G-455A (rs1800790), PAI-1 (-675) 4G/5G (rs1799889), PPARα Leu162-Val (rs1800206), eNOS Asp298Glu (rs1799983), and ERα IVS1-401 (rs2234693).

Statistical analyses

The association of genotype distribution with clinical factors, represented as mean ± SEM, was analyzed using Microsoft Excel enhanced software with either an unpaired Student's *t*-test or a Mann–Whitney *U*-test, depending on histogram distribution. A chi-square test was used to describe the effect of genotype on biomarker levels

Table 1
All variables are presented as mean ± SEM.

Characteristic		On registration [$n=93$]
Age, years	60–64 [$n=4$]	80.95 ± 0.90
	65–74 [$n=16$]	
	75–84 [$n=36$]	
	85–94 [$n=36$]	
	95–99 [$n=1$]	
Total cholesterol, mg/dL		206.13 ± 4.62
LDL-C, mg/dL		120.73 ± 4.28
HDL-C, mg/dL		57.89 ± 2.05
Triglycerides, mg/dL		113.37 ± 5.60
Creatinine, mg/dL		0.84 ± 0.03
BNP, pg/mL		77.67 ± 8.17
Glucose, mg/dL		99.29 ± 3.52
TNF-α, pg/mL		3.96 ± 0.31
IL-6, pg/mL		7.08 ± 2.04
cGMP, pmol/mL		7.28 ± 0.40
NOx, μmol/L		55.38 ± 4.00
Hemoglobin, g/dL		11.89 ± 0.20

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BNP, B-type natriuretic peptide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; cGMP, cyclic guanosine 5'-monophosphate; NOx, nitric oxide metabolites.