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A common genetic variant of the chromogranin A-derived peptide catestatin is associated with atherogenesis and hypertension in a Japanese population

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Abstract. Chromogranin A (CHGA) is a major protein in the secretory granules of chromaffin cells. CHGA also gives rise to cardiovascular/metabolism regulatory peptides, such as catestatin (CST) and pancreastatin (PST). While CST is a potent inhibitor of catecholamine secretion, PST is a potent physiological inhibitor of glucose-induced insulin secretion. Recently, several SNPs were identified in the CST and PST domains of CHGA locus in different populations. Among the discovered SNPs, CST variant allele Ser-364 was associated with blood pressure alteration and PST variant allele Ser-297 was associated with significantly higher plasma glucose level. In this study, we examined whether these CST and PST variant alleles exist and influence cardiovascular and metabolic phenotypes in Japanese population. Our study comprised of 343 Japanese subjects aged 45-85 years (143 men and 200 women, mean age 66 ± 8 years). We determined the genotypes of CST and PST by PCR-direct sequencing method and carried out genotype-phenotype association analysis. In 343 participants, the minor allele frequency of CST variant Ser-364 was 6.10%. On the other hand, we did not detect the PST variant Ser-297 in this entire study population. The presence of Ser-364 allele was associated with increased in baPWV (an index of systemic arterial stiffness) that suggests an initiation and/or progression atherogenesis and hypertension. The Ser-364 allele was also associated with elevated systolic blood pressure and pulse pressure, consistent with increased baPWV. In conclusion, the CST Ser-364 allele may increase the risk for cardiovascular diseases in Japanese population.

Key words: Catestatin, Pancreastatin, Arterial stiffness, Blood pressure, Japanese

CHROMOGRANIN A (CHGA) is a well-known protein co-stored and co-released with catecholamines from secretory granules of chromaffin cells [1, 2]. CHGA plays a major role in the biogenesis of catecholamine storage vesicles [3, 4]. In addition, CHGA acts as a prohormone and undergoes proteolytic cleavage, resulting in the generation of several novel cardiovascular/metabolism regulatory peptides, such as vasostatin-I (human CHGA1–76), pancreastatin (PST; human CHGA250–301), catestatin (CST; human CHGA352–372), and serpinin (human CHGA411–43) [1, 2].

The CST peptide exerts potent catecholamine secre-

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Abbreviations: SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; PP, Pulse Pressure; BMI, Body Mass Index; HR, Heart Rate; baPWV, brachial-ankle Pulse Wave Velocity

tion-inhibitory effect *via* blockade of neuronal nicotinic acetylcholine receptor (nAChR) [5-9]. In addition, CST has been reported to act as a novel regulator of glucose/insulin homeostasis (*via* inhibition of gluconeogenesis) as well as lipogenesis, lipolysis, and fatty acid oxidation [10, 11].

Recently, several non-synonymous single nucleotide polymorphisms (SNPs) were identified in the CST domain in different ethnic populations [12, 13]. These CST variants showed significantly different potencies towards inhibition of nAChR-induced catecholamine secretion from chromaffin cells [12, 13]. Subsequent analysis demonstrated association of the CST SNP Gly364Ser (rs9658667) with several cardiovascular phenotypes [12, 13]. In particular, the CST Ser-364 allele was associated with reduced blood pressure in a Southern California population. Intriguingly, however, the Ser-364 allele tended to associate with increased blood pressure (but it did not reach statistical significance) in an Indian population. Additionally, in the same Indian population, the Ser-364 allele was associated with metabolic parameters such as elevated glucose and triglycerides. These results indicate that the Gly364Ser SNP could exert different effects, which may be dependent on ethnic/geographic background. However, it remains uncertain whether Gly364Ser could be associated with cardio-metabolic phenotypes in other populations outside these geographic locations.

Besides CST, functional genetic variations in the PST domain have also been studied in different populations due to its potent dysglycemic functions, including inhibition of glucose-induced insulin secretion [14], inhibition of glucose uptake in adipocytes and hepatocytes [15], activation of gluconeogenesis in liver [15]. Notably, the PST variant Gly297Ser peptide (PST-297Ser; rs9658664) displayed increased potency over the wild-type peptide (PST-WT) [15]. In agreement with this finding, the Ser-297 allele has been associated with significantly higher plasma glucose level in an Indian population [15].

In this first study on Japanese population, we aimed to detect genetic polymorphisms within the CST and PST domains at the CHGA locus. We also analyzed association of the CST Gly364Ser SNP with several cardiovascular and metabolic phenotypes. Significant association of the Ser-364 allele with increased in baPWV, SBP and PP were detected indicating that this common CST allele may increase the risk for cardiovascular diseases in Japanese people.

Methods

Subjects

Subjects were recruited through local newspaper advertisements from Ibaraki, Saitama, and Shizuoka prefectures in Japan; 143 men and 200 women aged 45-85 years (mean age 66 ± 8 years) were enrolled in the study. Data were collected between 1999 and 2004 [16]. Candidates having hypertension as well as other metabolic disorders (*i.e.*, hyperlipidemia and diabetes) were included in the study. The study subjects included 19 individuals with a current smoking status. All female participants were postmenopausal women. Hypertension was defined as use of anti-hypertensive medication or systolic or diastolic blood pressure ≥ 140/90 mmHg [17]. Diabetes was defined as anti-diabetics medication or fasting blood glucose ≥ 126 mg/dL [18]. Dyslipidemia was defined as anti-hyperlipidemic medication or triglyceride (TG) ≥ 150 mg/dL, HDL < 40 mg/dL in men and <50 mg/dL in women, which are some of the components of the criteria for metabolic syndrome proposed by the U.S. National Cholesterol Education Program-Adult Treatment Panel III, as described previously [19]. None of the subjects had apparent cardiovascular disease, as determined by reviewing the medical history and physical examination results.

The Institutional Review Board at the University of Tsukuba reviewed and approved this study (No.60-1). Each subject gave informed consent for use of his/her DNA for genetic analysis. All study procedures and potential risks were explained to the subjects, who provided written informed consent.

Study protocol

Brachial blood pressure, heart rate, and arterial stiffness (baPWV) were measured simultaneously, and blood samples were collected. All measurements were performed after abstinence from caffeine, alcohol and an overnight fast. Subjects were studied under supine resting conditions in a quiet, temperature-controlled room (24−25°C). All measurements were performed after a resting period of at least 15 minutes.

Detection of genetic polymorphisms at the CST and PST regions in CHGA locus

Genomic DNA was isolated from EDTA-anticoagulated blood samples using FlexiGene DNA kit (Qiagen, USA). The exon-7 region of *CHGA* was PCR amplified using Tks Gflex DNA Polymerase

(TaKaRa) and the following primers: forward, 5'-GAGTGGCAGAGACTGGGAAAATG-3'; reverse, 5'-ACAGAGCTGGCTCCCGCCC-3'. The detailed PCR protocol has been reported recently [20]. The PCR products were purified by using MonoFas DNA Purifucation Kit (GL Science) and sequenced by using an Applied Biosystems 3130 Genetic Analyzer (USA) and the forward primer (as mentioned above). Genetic variations were detected and manually confirmed from chromatograms. In case of any ambiguity about a polymorphism re-sequencing of the sample was carried out using the reverse primer (as mentioned above) to confirm the genotype.

Measurements of brachial-ankle PWV

baPWV (using formPWV/ABI; Colin Medical Technology, Komaki, Japan) was measured in triplicate as previously described [20, 21]. The baPWV value reflects the degree of stiffness in the central arteries, and baPWV correlates well with the aortic pulse wave velocity (PWV) measured using a catheter tip with pressure manometer [20].

Measurements of blood pressure and heart rate

Each subject's brachial arterial blood pressure (*via* volume plethysmography) and heart rate (*via* eletrocardoogram) were measured in triplicate by a semi-automated device (form PWV/ ABI; Colin Medical Technology, Komaki, Japan) after resting in supine position for at least 15 min, as previously described [20].

Estimation of biochemical parameters

The serum concentrations of cholesterol, triglycerides (TG), insulin and fasting plasma concentrations of glucose (FPG) were determined using standard biochemical techniques.

Statistical analysis

The minor allele frequency was calculated using a gene-counting method. Hardy-Weinberg equilibrium was confirmed using a χ^2 test. Unpaired Student's *t* test was used to evaluate a difference between the different genotype groups. Furthermore, the comparison of SBP or PP between the genotype groups was assessed by a covariance analysis (ANCOVA) that included gender, age, BMI, anti-hypertensive medication, diabetes, dyslipidemia and smoking as covariates. The comparison of baPWV between the genotype groups was assessed by ANCOVA that included gender, age, BMI, hypertension, diabetes, dyslipidemia and smoking as covariates. Unless indicated otherwise, a value of $p<0.05$ was considered statistically significant. Values are expressed as the means ± SEM. The data were analyzed using the SPSS statistical software (version 11.0J; SPSS Inc., Chicago, Illinois, USA).

Results

Discovery of genetic variations in the CST domain of CHGA in a Japanese population

To investigate genetic variations within the CST domain in a Japanese population, we sequenced the specific genomic region. In the 343 subjects, 301 (87.8%) had the wild-type GG (Gly/Gly) and 42 (12.2%) displayed the heterozygous GA (Gly/Ser) genotypes. However, we did not detect any homozygous AA (Ser/Ser) variant. Thus, the minor allele frequency (number of chromosomes in population) of this SNP was 6.1% and the genotypic frequencies followed Hardy-Weinberg equilibrium (χ^2=1.46, $p=0.227$). Representative sequencing data of wild-type GG (Gly/Gly), heterozygous G/A (Gly/Ser) individuals are shown in Fig. 1. Apart from the Gly364Ser, no other SNPs (that were previously reported in other populations) such as Pro370Leu, Arg374Gln and Gly367Val were detected in this study population.

Association of the CST Gly364Ser variant with phenotypic parameters

We investigated the association between the phenotypic characteristics and the CST Gly364Ser variant. Comparisons were made by unpaired Student's *t* test. Table 1 shows the characteristics of these subjects. Significant difference was observed in baPWV. Indeed, variant Gly/Ser individuals had 108.6 cm/sec higher values than those of wild-type Gly/Gly individuals ($p=0.040$), suggesting that Gly/Ser individuals may be at higher risk of initiation and/or progress of atherogenesis and hypertension as compared to the Gly/Gly individuals (Fig. 2-A). In agreement with this baPWV result, a significant difference was observed in PP (Fig. 2-B). Indeed, Gly/Ser individuals had 4.4 mmHg higher PP than those of Gly/Gly individuals ($p=0.030$). SBP for Gly/Ser individuals was 6.2 mmHg higher than those of Gly/Gly individuals, although the difference did not reach statistical significance ($p=0.055$) (Fig. 2-C). Other phenotypic parameters (including gender, age, BMI, HR, DBP, total

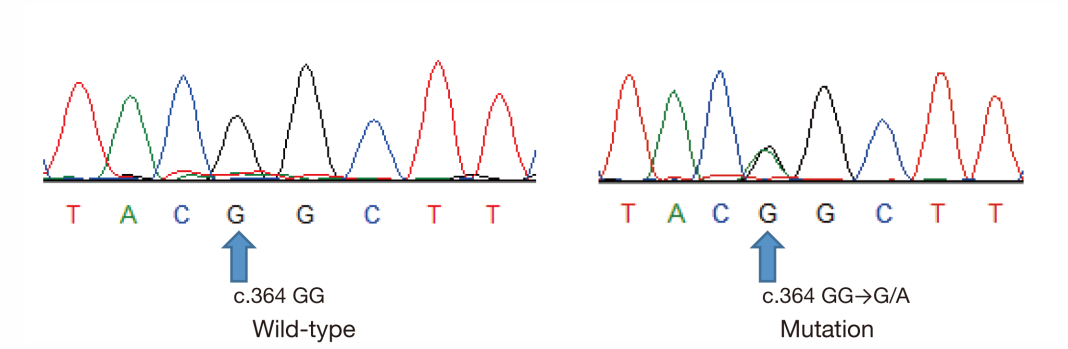

Fig. 1 Representative chromatograms from the direct sequencing of the catestatin domain
Wild-type GG (Gly/Gly) and heterozygous variant G/A (Gly/Ser) at the codon 364 are shown. The first nucleotide (indicated by an arrow) of the codon GGC (the wild-type) is changed to AGC (the variant) that may lead to the change of the amino acid glycine to serine.
Among the 343 participants, 301 individuals (87.8%) had wild-type GG, 42 subjects (12.2%) had heterozygous GA, and no (0%) homozygous AA genotype. Thus, the frequency of the minor allele (Ser-364) in this Japanese population is 6.1 %.

Table 1 Phenotypic parameters of the subjects stratified based on their genotypes.

Parameters	Gly/Gly	Gly/Ser	*p* value
Age (years)	66.2±0.39 (n=301)	65.4±1.14 (n=42)	*0.442*
Sex (male/female)	43%/57%	36%/64%	*0.409*
BM (kg/m^2)	23.4±0.16 (n=301)	23.4±0.42 (n=42)	*0.980*
baPWV (cm/sec)	**1588.4±17.79 (n=301)**	**1696.9±60.10 (n=42)**	***0.040***
SBP (mmHg)	**132.0±1.14 (n=301)**	**138.2±2.72 (n=42)**	***0.055***
DBP (mmHg)	80.3±0.60 (n=301)	82.0±1.37 (n=42)	*0.312*
MAP (mmHg)	100.7±0.86 (n=301)	104.5±1.97 (n=42)	*0.117*
PP (mmHg)	**51.7±0.72 (n=301)**	**56.1±1.92 (n=42)**	***0.030***
Heart Rate (beats/min)	61.5±0.55 (n=301)	63.2±1.59 (n=42)	*0.285*
Total Cholesterol (mg/dL)	210.1±1.92 (n=301)	215.5±6.88 (n=42)	*0.354*
HDL cholesterol (mg/dL)	60.1±0.82 (n=301)	58.5±2.19 (n=42)	*0.479*
LDL cholesterol (mg/dL)	125.8±1.66 (n=301)		*0.936*
Glucose (mg/dL)	103.9±1.26 (n=301)	104.5±3.05 (n=42)	*0.866*
Insulin (μU/mL)	6.3±0.25 (n=301)	7.2±0.99 (n=42)	*0.392*

Data are shown as Mean ± S.E. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high density lipoprotein; LDL, low density lipoprotein. The glucose values are fasting blood sugar levels.
Statistical significance between Gly/Ser and Gly/Gly genotype groups was determined by Student's *t* test for equality of means using SPSS software (SPSS Inc., Chicago, IL). A *p* value of < 0.05 was chosen for statistical significance between the groups. The significantly differing parameters were shown in bold.

cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, insulin, and plasma fasting glucose) did not differ significantly among the genotype groups (Table 1).

In addition, we investigated the association of CST Gly364Ser with prevalence of hypertension. The prevalence was 46.5% and 57.1% in Gly/Gly and Gly/Ser individuals, respectively. Thus, the frequency of Gly/Ser genotype was considerably higher (as compared to Gly/Gly genotype) among hypertensive subjects; the difference, however, between the two genotype groups did not reach statistical significance (χ^2 test, *p=0.248*).

Analysis of covariance (ANCOVA)

Followed by Student's *t* test, associations between Gly364Ser SNP and physiological parametres, such as baPWV, SBP, PP were tested with analysis of covariance (ANCOVA). The data are shown in Fig. 2. After including relevant covariates (gender, age, BMI, hypertension, diabetes, dyslipidemia and smoking), persistent significant differences were observed between Gly/Ser and Gly/Gly groups for baPWV and PP (*p=0.011, p=0.025*, respectively). Furthermore, significant differences were observed between Gly/Ser and Gly/Gly genotype groups for SBP (*p=0.040*) after including rel-

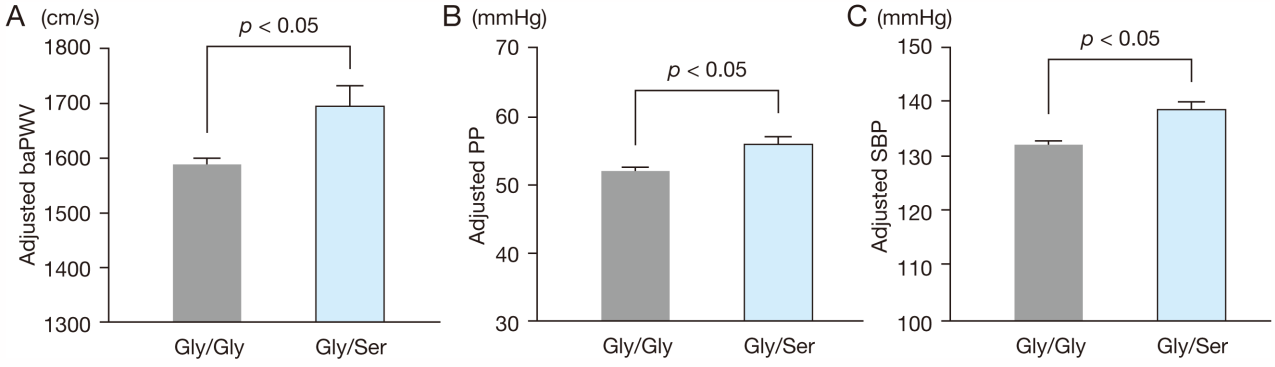

Fig. 2 Association between baPWV, PP, SBP and CST genotypes

A) Brachial-ankle pulse wave velocity (baPWV), an index of systemic arterial stiffness, in Gly364Ser and wild-type individuals. The baPWV value was measured according to the method described in experimental procedures. The baPWV values after adjustment for gender, age, BMI, hypertension, diabetes, dyslipidemia and smoking are expressed as mean ± SE.

B) PP (pulse pressure) in Gly364Ser and wild-type individuals. Brachial pulse pressure (PP) was calculated from SBP and DBP, both measured with volume plethysmography at the site of the brachial artery. PP values after adjustment for gender, age, BMI, anti-hypertensive medication, diabetes, dyslipidemia and smoking are expressed as mean ± SE.

C) SBP (systolic blood pressure) in Gly364Ser and wild-type individuals. SBP values after adjustment for gender, age, BMI, anti-hypertensive medication, diabetes, dyslipidemia and smoking are expressed as mean ± SE.

evant covariates (gender, age, BMI, anti-hypertensive medication, diabetes, dyslipidemia and smoking).

In addition, to exclude the effect of medication, we analyzed the genotype-phenotype association in the population without medication. In 224 subjects without medication, the presence of Ser-364 allele was indeed associated with increased SBP (by 4.4 mmHg, *p=0.044*) as well as baPWV (by 73.3 cm/sec, *p=0.016*) with ANCOVA, in agreement with the initial analysis (in which subjects with or without medication were included). A significant association was also observed with MAP (*p=0.032*) in this setting. However, the Ser-364 allele did not display an association with PP in the unmedicated subjects (*p=0.121*).

Investigation for genetic variations in the PST domain of CHGA in a Japanese population

To investigate genetic variations within the PST domain in this study population, targeted sequencing of the genomic DNA samples was carried out. We did not detect any genetic variants of PST in the 343 participants (data not shown). Of note, several genetic variations in the PST domain has previously been reported in other populations: Arg253Trp, Ala256Gly, Glu274Lys, Glu287Lys, Glu288Lys, Gly297Ser and Arg300Gln. Among these variants, the Gly297Ser was detected in several populations and people of Indian origin carried this variant at ~7% minor allele frequency [15].

Discussion

The CHGA-derived peptides CST and PST are emerging as important regulators of cardiovascular and metabolic functions [14, 22-24]. In corroboration, studies in different populations revealed association of CST and PST variants with cardio-metabolic disease states [12, 13, 15]. In view of these reports, we set out to investigate genetic variations within CST and PST domains in a Japanese population.

We observed that the frequency of the CST minor allele (Ser-364) in this Japanese population was ~6.1%, which is lower than that in an Indian population (~8.0%) and higher than a Southern California population (3.1%) [12, 13]. Interestingly, in this study population, apart from this CST variant (Ser-364), we did not detect any other CST SNP [13]. Thus, the CST domain of *CHGA* locus displayed genetic variations among different ethnic populations, with a relatively high frequency of the Ser-364 allele in a Japanese population.

Does this common Ser-364 variant contribute to pathogenesis of cardiovascular diseases in Japanese population? To address this question we carried out genotype-phenotype association analysis upon stratification of the subjects into two groups: wild-type Gly/Gly and heterozygous variant Gly/Ser. Analysis by Student's *t* test showed that the SBP was higher in Gly/Ser subjects, although the difference reached only marginal significance (*p=0.055).* However, analysis of

covariance (ANCOVA) by including relevant covariates (gender, age, BMI, anti-hypertensive medication, diabetes, dyslipidemia and smoking) displayed a significant association between Gly364Ser genotype and SBP (*p=0.040*). The SBP was 6.2 mmHg higher in the Gly/Ser individuals than the wild-type (Gly/Gly) individuals. It is also important to note that the frequency of individuals on antihypertensive drugs was significantly higher in the CST Gly/Ser group as compared to the wild-type Gly/Gly group (40.5% *vs* 24.9%). Therefore, the CST Ser-364 allele, in general, appears to be associated with higher SBP in Japanese population.

Of note, a previous study in a Southern California population reported association of this CST-Ser-364 allele with reduced blood pressure, especially in men [13]. However, this variant tended to associate with an increased blood pressure (but it did not reach statistical significance) in an Indian population [14]. In the present study, we demonstrated significant association with increased SBP in the Japanese population. These results indicate that the CST-Gly364Ser variant may exert differential effects dependent upon ethnic/geographic background of the subjects. Although the mechanism by which this Gly364Ser polymorphism might influence BP differently remains unclear, one possible reason for these differences among these three studies could be the different age of subjects. The mean age of the Southern California study population, the Indian study subjects and the Japanese study subjects were 59, 39 and 66 years, respectively. Another possible factor may be related to excess dietary salt intake in Japanese population, in comparison with those of Caucasian and Indian populations [25]. It is well-established that excess dietary salt (NaCl) causes hypertension through increased sympathetic nerve activity (SNA) *via* several mechanisms [26]. Considering that CST Ser-364 modulates catecholamine release differently than the wild-type CST peptide [9], it may be reasonable to speculate that this CST variation and excess dietary salt might alter SNA in a differential manner. However, our findings support the importance of this common CST variant in modulating blood pressure.

PWV is a reliable marker of aortic stiffness [27-31]. Elevated PWV represents initiation and/or progression of atherogenesis and hypertension [30]. In the present study, significantly higher baPWV (by ~109 cm/sec, ANCOVA *p=0.011*) was observed in CST Gly/Ser individuals than that in Gly/Gly individuals. Thus, the probability of initiation and/or progression of atherogenesis and hypertension is more likely in the Gly/Ser individuals as compared to the wild-type Gly/Gly individuals. Consistent with this elevated baPWV, variant Gly/Ser individuals displayed significantly higher PP that is known as indirect index of arterial stiffness [17] than those of wild-type Gly/Gly individuals (by 4.4 mmHg; ANCOVA *p=0.025*). However, the present study cannot evaluate whether the increased aortic PWV constitutes a triggering mechanism or a marker of morbid events in these subjects. Further replication study in Japanese population is needed to clarify this point.

To reinforce our current findings, we reanalyzed the genotype-phenotype associations in the population without medication. Consistent with original analysis (in which subjects with or without medication were included), the association between Ser-364 allele and increased SBP (*p=0.044*) as well as baPWV (*p=0.016*) prevailed in this setting. On the contrary, no association was observed with PP (*p=0.121*) in the un-medicated population. Comparison of the mean PP values in these two analyses showed that the effect size reduced from 4.4 mmHg to 1.6 mmHg. Thus, the associations of CST-364 Ser allele with SBP and baPWV seem to be robust and independent of medication status of the study subjects.

Another interesting observation in this study was absence of any pancreastatin (PST) variant in the entire study population. Of note, among the reported PST variants the Gly297Ser variant has previously been detected in various ethnic populations with the highest minor allele frequency in people of Indian origin [15]. It seems that even this variant is absent/extremely rare in Japanese population. Absence/extremely rare occurrence of PST-297Ser allele might be favorable regarding risk of developing diabetes in Japanese population beacuse PST is a potent inhibitor of insulin secretion, which in turn increases glycogenolysis in liver and reduces glucose uptake in adipocytes/hepatocytes, and the PST variant peptide (PST-297Ser) exerts enhanced dysglycemic effect than the wild-type peptide (PST-WT); indeed, carriers of 297Ser allele was associated with significantly higher plasma glucose level in an Indian population [15].

In conclusion, we discovered a common genetic variant of the physiological antihypertensive peptide CST (viz. CST-Gly364Ser) in a Japanese population. This CST variant displayed significant associations with increased baPWV, elevated SBP as well as PP and hence may alter the risk for development of cardiovas-

cular diseases in the Japanese.

Study limitations

A limitation of this study is the relatively small sample size. Therefore, we cannot exclude the possibility of different frequency of the CST-364Ser allele in different Japanese populations. Studies in larger Japanese populations may also lead to the identification of additional CST or PST genetic variations. Also, at present, it remains uncertain whether the CST Ser-364 allele has associations with morbidities of cardiovascular diseases. The problem of multiple comparisons also remains. We did not undertake a conservative analysis method such as the Bonferroni's correction; therefore, the type I error might occur. To resolve these problems, further replication study using large Japanese population is needed.

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