G-395A polymorphism in the promoter region of the *KLOTHO* gene and hypertension among elderly (90 years and older) Chinese individuals

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ABSTRACT. The aim of this study was to examine the possible associations between the *KLOTHO* G-395A gene polymorphism and hypertension in Chinese nonagenarians and centenarians. The G-395A (rs1207568) in the promoter region of the *KLOTHO* gene was genotyped using a standard TaqMan allelic discrimination assay. We included 710 participants aged 93.5 ± 3.2 years in the analyses. The expression of the A allele of the *KLOTHO* G-395A polymorphism was significantly downregulated in the hypertension group compared to the control group (0.137 *vs* 0.200, P < 0.001). The genotype distribution of the G-395A polymorphism between the hypertension and control groups was significantly different in women and smokers, and not in men or non-smokers. The mean systolic blood pressure, percentage of hypertension, and percentage of isolated systolic hypertension was significantly higher in the group with the GG genotype than in the group with the GA+AA genotype. Subjects expressing the GA+AA genotype demonstrated a significantly lower risk of hypertension even after adjusting for age, gender, and other relevant risk factors compared to the population expressing the GG genotype (odds ratio, 0.68; 95% confidence interval: 0.49 to 0.95).The -395A allele of the

KLOTHO gene may be a protective genetic factor for hypertension in the Chinese population.

Key words: KLOTHO gene; Hypertension; Single nucleotide polymorphism

INTRODUCTION

Hypertension, one of the most common causes of preventable death, is known to increase with advancing age (Zeglin et al., 2009; Virdis et al., 2011). Hypertension is known to be the result of interactions between genetic and environmental factors (Kunes and Zicha, 2009). Current evidence indicates that genetic components contribute approximately 30% to 50% to the etiology of hypertension (El Shamieh et al., 2012). Several recent studies have suggested that single nucleotide polymorphisms (SNPs) in some candidate genes may be related to hypertension (Delles et al., 2010).

Discovered by Kuro-o et al. (1995) during the development of hypertensive transgenic mice models, the *KLOTHO* gene, a new anti-aging gene (Wang and Sun, 2009), has recently attracted considerable attention from researchers around the world (Kuro-o, 2009). The identification of SNPs in the human *KLOTHO* gene is currently one of the most active areas of research. So far, more than 10 SNPs have been identified in the human *KLOTHO* gene, and linked to fasting glucose (Rhee et al., 2006b), coronary artery disease (Imamura et al., 2006; Rhee et al., 2006a), stroke (Kim et al., 2006), bone mineral density (Kawano et al., 2002), and kidney stone (Telci et al., 2011).

Among these, the G-395A polymorphism in the promoter region of the *KLOTHO* gene has been associated with blood pressure in several populations (Rhee et al., 2006b; Wang et al., 2010). For example, Rhee et al. (2006b) observed significantlyhigher systolic blood pressure levels in the A allele carriers of the G-395A polymorphism in Korean women. A more recentreport has suggested that the G-395A polymorphism might be associated with essential hypertension in the Chinese Han population (Wang et al., 2010). However, Shimoyama et al. (2009) reported the absence of any association between the mean systolic blood pressure (SBP) and the G-395A polymorphism in healthy Japanese people.

In 2005, we conducted a cross-sectional study of 870 elderly Chinese adults aged 90 years and older (Yue et al., 2010; Zhou et al., 2012), and built a relevant DNA specimen database. Most of the participants lived in their homeland for their entire lifetime and were never exposed to immigrants. The prevalence of hypertension was found to be extremely high (more than 60%) in this population; therefore, this population presented a unique opportunity to study the genetic factors of hypertension. Therefore, this study was performed to examine the possible associations between the *KLOTHO* G-395A SNP and hypertension in Chinese nonagenarians and centenarians.

MATERIAL AND METHODS

Study population

Data and DNA samples used in this study were from obtained from the Project of Longevity and Aging in Dujiangyan (PLAD), a previously reported cross-sectional study (Yue et al., 2010; Zhou et al., 2012). The PLAD study was conducted in the rural area of Dujiangyan, a small town in southwestern China, during April 2005. A total of 1115 adults aged 90 years and older dwelling

in this area were screened, among who, 870 subjects agreed to participate in this study. Trained interviewers visited all study participants at their homes or at community centers, and data was collected by conducting face-to-face interviews. Anthropometric measurements were performed and blood was collected by trained staff. The study protocol was approved by the Research Ethics Committee of Sichuan University. Informed consent was obtained from all of participants or their legal proxies.

Genotyping of polymorphisms in the *KLOTHO* gene

Genomic DNA was isolated from the whole blood samples drawn from the antecubital vein, using the TIANamp Blood DNA Midi Kit (DP332; Qiagen, Chatsworth, CA, USA) using standard procedures. The G-395A (rs1207568) polymorphism in the promoter region of the *KLOTHO* gene was genotyped using the TaqMan allelic discrimination assay, as described previously by Wang et al. (2010), using the following primers and probes: forward primer, 5'-TAGGGCCCGGCAGGAT-3'; reverse primer, 5'-CCTGGAGCGGCTTCGTC-3'; probe A 5'-(FAM) CCCCAAGTCGGGAAAAGTTG GTC(TAMRA)-3'; probe G 5'-(HEX) CCCCCAAGTCGGGGAAAGTTGGTC(TAMRA)-3' (TaKaRa Bio Inc., Dalian, China). The PCR was conducted in a 20 µL reaction volume containing 10 µL Premix Ex Taq, 1.5 µL of each primer, 0.5 µL probe A, 1 µL probe G, 1 µL of genomic DNA, and 4.25 µL sterile double distilled water. The reaction conditions were as follows: an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing for 30 s at 60°C. The PCR was performed and analyzed on a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.).

The genotypes of the rs1207568 polymorphism in the promoter of the *KLOTHO* gene were analyzed by random selection of 10% of the samples for forward and reverse sequencing. The results of the sequencing were identical to the results of the TaqMan allelic discrimination assay.

Diagnosis of hypertension

Qualified nurses or physicians measured the sitting blood pressure (BP) of all patients twice (interval of 10 min) to the nearest of 2 mmHg using a standard mercury sphygmomanometer (phase I and V of korotkoff sounds). The mean value of the two measurements was recorded. In this study, hypertension was defined according to the JNC VII criteria, as a systolic BP (SBP) of ≥140 mmHg and/or a diastolic BP (DBP) of ≥90 mmHg (Chobanian et al., 2003). Participants who met either of the following criteria were considered to have hypertension: the measured BP (during the interview) met the JNC VII criteria mentioned above,or a confirmed clinical diagnosis of hypertension based on formal medical records, and currently receiving anti-hypertension medication. In addition, isolated systolic hypertension (ISH) was defined as a SBP ≥ 140 mmHg and a DBP < 90 mmHg, based on the measured BP during the interview.

Measurement of covariates

The height and weight of all patients was measured using a wall-mounted stadiometer and a digital floor scale to the nearest 0.1 cm and 0.1 kg, respectively. Body mass index (BMI) was calculated by dividing the body weight in kilograms by the height in squared meters (kg/m^2). Laboratory analyses included the measurement of fasting plasma glucose (FPG), postprandial blood glucose (PBG), total cholesterol (TC), triglyceride content (TG), high density lipoprotein

cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C). Diabetes was diagnosed based on the criteria of the American Diabetes Association (ECDCDM, 2003), or if the subjects had a history of diabetes and were currently using antidiabetic agents. In addition, the following baseline information was included in the analyses: age, gender, and cigarette smoking status (smoker, defined ashaving smoked ≥ 100 cigarettes in his/her lifetime (Ryan et al., 2012), or nonsmoker).

Statistical analysis

The categorical variables are presented as percentages (%), whereas the continuous data are presented as the mean ± standard deviation (SD). A Chi-square test was used to evaluate the allelic and genotypic frequencies calculated from the observed genotypic counts. The Chi-square test was used for categorical variables and the unpaired Student *t*-test for continuous variables; the baseline characteristics were compared between those with or without hypertension and with or without the -395A allele. A previous study reported a significant association between the G-395A polymorphism and hypertension in females and nonsmokers (Wang et al., 2010); therefore, we performed subgroup analyses based on the gender and smoking status. Odd's ratio (OR) and 95% confidential intervals (CI) were determined for hypertension using binary logistic regression models. All statistical analyses were performed using the SPSS for Windows software package (v.11.5; SPSS Inc, Chicago, IL, USA). A two-tailed P <0.05 was considered to be statistically significant.

RESULTS

Characteristics of the study population

In this study, 160 participants were excluded because of the lack of DNA samples. Overall, 710 participants (224 men and 486 women) were included in the analyses. There were no significant differences between the included and excluded populations in terms of age (93.5 ± 3.2 *vs* 93.2 ± 2.8, P = 0.587), gender (women: 68.5 *vs* 65.0%, P = 0.325), or the percentage of hypertension (60.8 *vs* 62.5%, P = 0.368). All participants were Han Chinese.

Table 1 summarizes the characteristics of the included subjects with or without hypertension. The BMI of the subjects in the hypertension group was significantly lower than that of people in the control group (18.6 *vs* 19.6, P < 0.001). No significant difference was found with respect to age, gender, smoking, diabetes, TG, TC, HDL-C, and LDL-C between the hypertension and control groups.

Genotype distribution of *KLOTHO* G-395A

The genotype frequencies of the *KLOTHO* G-395A polymorphism in the study population were 1.7% for AA (N = 12), 29.0% for GA (N = 206), and 69.3% for GG (N = 492); this was in compliance with Hardy-Weinberg equilibrium (P = 0.069). The allele frequencies were 0.838 for the G allele and 0.162 for the A allele in the entire study population. Significant differences were identified in the genotype distribution of the G-395A polymorphism between the hypertension and control groups (Table 1). The allele frequencies were 0.863 for the G allele and 0.137 for the A allele in the hypertension group, whereas the corresponding frequencies were 0.800 and 0.200, respectively, for the control group (P < 0.001).

Table 1. Characteristics of the study subjects.

	Control (N = 278)	Hypertension (N = 432)	P value
Age (years)	93.4 ± 3.2	93.7 ± 3.3	0.331
Female (%)	69.4	67.1	0.534
Smoker (%)	64.0	62.0	0.592
BMI (kg/m^2)	19.6 ± 3.8	18.6 ± 3.3	<0.001
Diabetes (%)	8.3	5.6	0.155
TG (mM)	4.9 ± 1.6	4.0 ± 0.8	0.348
TC (mM)	1.3 ± 0.8	1.2 ± 0.5	0.132
HDL-C (mM)	1.6 ± 0.8	1.6 ± 0.6	0.425
LDL-C (mM)	2.3 ± 0.6	2.2 ± 0.6	0.075
Genotype distribution			
GG (%)	64.0	72.7	<0.001
GA (%)	32.0	27.1	
AA (%)	4.0	0.2	

All data represents the mean ± standard deviation (SD) unless otherwise indicated. BMI = body mass index; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; TC = total cholesterol; TG = triglycerides.

In order to compare the different genotypes with G-395A polymorphism, the subjects were divided by A allele into A allele carriers versus non-carriers (GA+AA *vs* GG). Table 2 summarizes the characteristics of the subjects included in the GG and GA+AA genotype groups. The mean SBP was significantly higher in the GG group than in the GA+AA genotype group; however, the DBP did not differ significantly between the two groups. The percentages of hypertension and ISH were significantly higher in the GG genotype group than in the GA+AA genotype group. In addition, no significant differences were found between the two groups with respect to age, gender, smoking status, BMI, diabetes, TG, TC, HDL-C, and LDL-C.

Subgroup analyses revealed that the genotype distribution of the G-395A polymorphism between the hypertension and control groups were significantly different in women and smokers, but not in men or non-smokers (Table 3).

Table 2. Characteristics of study subjects with respect to the G-395A polymorphism in the *KLOTHO* gene.

	GG (N = 492)	GA+AA (N = 218)	P value
Age (years)	93.6 ± 3.3	93.3 ± 3.0	0.241
Female (%)	67.3	72.1	0.312
Smoker (%)	63.4	61.5	0.621
SBP (mmHg)	143.6 ± 24.2	133.9 ± 19.7	<0.001
DBP (mmHg)	73.0 ± 12.1	71.9 ± 12.0	0.242
Hypertension (%)	63.8	54.1	0.015
ISH (%)	47.0	38.1	0.029
BMI (kg/m^2)	19.5 ± 3.4	19.1 ± 3.7	0.155
Diabetes (%)	7.9	6.1	0.401
TG (mM)	4.8 ± 3.7	4.1 ± 0.8	0.493
TC (mM)	1.3 ± 0.8	1.2 ± 0.5	0.327
HDL-C (mM)	1.6 ± 0.8	1.6 ± 0.7	0.503
LDL-C (mM)	2.3 ± 0.5	2.2 ± 0.6	0.675

All data represents the mean ± SD unless otherwise indicated. BMI = body mass index; DBP = diastolic blood pressure; HDL-C = high density lipoprotein cholesterol; ISH = isolated systolic hypertension; LDL-C = low density lipoprotein cholesterol; SBP = systolic blood pressure; TC = total cholesterol; TG = triglycerides.

Association between hypertension and the *KLOTHO* G-395A polymorphism

Table 4 shows the results of unadjusted and adjusted logistic regression models regarding the association between hypertension and the *KLOTHO* G-395A gene polymorphism. Compared

to subjects with the GG genotype, subjects with the GA+AA genotype showed a significantly lower risk of hypertension even after adjusting for age, gender, and other relevant risk factors (OR 0.68, 95%CI = 0.49 to 0.95).

Table 3. Subgroup analysis of the association between *KLOTHO* G-395A genotypes and hypertension with respect to the gender and smoking status.

		Hypertension	Control	P value
Gender				
Men (N = 224)	GG (%)	75.2	67.0	0.182
	GA+AA (%)	24.8	33.0	
Women (N = 486)	GG (%)	71.6	62.6	0.038
	GA+AA (%)	28.4	37.4	
Smoking status				
Smoker (N = 304)	GG (%)	73.5	64.6	0.045
	GA+AA (%)	26.5	35.4	
Nonsmoker (N = 401)	GG (%)	71.3	63.0	0.158
	GA+AA (%)	28.7	37.0	

Table 4. Estimate of the effect of the G-395A polymorphism on hypertension modeled via logistic regression.

	GG OR (95%CI)	GA+AA OR (95%CI)
Unadjusted	1 (Reference)	0.67 (0.48, 0.93)
Adjusted model 1†	1 (Reference)	0.66 (0.48, 0.91)
Adjusted model 2‡	1 (Reference)	0.68 (0.49, 0.95)

†Adjusted for age and gender. ‡Adjusted for age, gender, BMI, smoking, diabetes, use of anti-hypertensive drugs, TG, TC, HDL-C, and LDL-C. BMI = body mass index; CI = confidence interval; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; OR = odd's ratio; TC = total cholesterol; TG = triglycerides.

DISCUSSION

The results of our study indicated that the A allele carriers of the *KLOTHO* G-395A SNP were significantly less common in the hypertension group than in the control group. The *KLOTHO* G-395A SNP was associated with hypertension after adjusting for age, gender, and other relevant risk factors. These findings confirmed the results of a previous study that was also conducted in the Chinese Han population (Wang et al., 2010).

In our study, the frequency ofexpression of the A allele in the*KLOTHO* G-395A SNP was 0.162. In previous studies, the frequencies of the A allele in the G-395A SNP varied from 0.155 (Kim et al., 2008) to 0.171 (Rhee et al., 2006c) in Korean populations, 0.128 (Kawano et al., 2002) to 0.168 (Shimoyama et al., 2009) in Japanese populations, 0.191 in a Chinese Han population (Wang et al., 2010), and 0.227 in a Turkish population (Telci et al., 2011). Our results on the allele frequency were similar to those of studies conducted in East Asian populations, but not to that seen in the study conducted in the Turkish population. This finding suggests that the *KLOTHO* G-395A SNP may vary in different ethnic populations.

In this study, the mean SBP was significantly higher in the GG genotype group than in the GA+AA group; however, this finding was inconsistent with the results of previous studies (Rhee et al., 2006b; Shimoyama et al., 2009). Rhee et al. (2006b) found that the mean SBP was higher in -395A allele carriers compared to the non-carriers in healthy Korean women (Rhee et al., 2006b), whereas Shimoyama et al. (2009) reported no significant difference in the mean SBP between

-395A carriers and non-carriers in a healthy Japanese population. Notably, the two previous studies were both conducted in healthy populations, and the differences may be attributed to the lack of hypertension patients in the study population. Another possibility could be the differences in genetic backgrounds between different ethnic populations. Wang et al. (2010) did not report this outcome in a Chinese population; therefore, we could not compare their results with ours. However, the comparable difference in the association between *KLOTHO* G-395A SNP and coronary heart disease may support this hypothesis. For example, the -395 A allele of the *KLOTHO* gene was considered to be a protective factor for coronary heart disease in a Korean population (Rhee et al., 2006a), but a risk factor in a Japanese population (Imamura et al., 2006).

The reason for this difference in the association between the *KLOTHO* G-395A SNP and hypertension between men and women remains unclear. However, this finding was consistent with that of a previous study, which discovered significant associations between the G-395A SNP and essential hypertension in women, but not in men (Wang et al., 2010). One possibility is the gender-based difference in the maintenance of free radical homeostasis (Ali et al., 2006). The *KLOTHO* gene reduces oxidative stress by inhibiting the insulin/IGF-1 signaling pathway (Wang and Sun, 2009); however, oxidative stress may play a role in the genesis of hypertension (Harrison and Gongora, 2009).

In this study, the *KLOTHO* G-395 SNP was significantly associated with hypertension in smokers; however, this was inconsistent with the results obtained by Wang et al. (2010), who found that the corresponding association was significant in nonsmokers. This difference might be attributed in part to the different definitions of smokers. In this study, smokers were defined as subjects who smoked ≥ 100 cigarettes in their lifetime, according to the National Health Interview Survey (NHIS) (Ryan et al., 2012) definition of a smoker. Alternately, Wang et al. (2010), defined smokers as subjects who smoked ≥ 20 cigarettes/day for >2 years. In addition, the subjects included in our study had an extremely high rate of smoking (62.8%), because of the tradition of smoking self-made cigarettes (“Chinese-prepared tobacco”) in the Dujiangyan area.

The molecular mechanism with which the *KLOTHO* G-395A SNP modulated the blood pressure remains unclear. Animal studies indicated that the *KLOTHO* gene could protect against endothelial dysfunction (Nzietchueng et al., 2011). Yang et al. (2003) reported that the *KLOTHO* gene might play a role in the regulation of vascular tone through a homeostatic interplay between NO and the renin-angiotensin system. This might explain the association between the *KLOTHO* gene and hypertension. In addition, *KLOTHO* gene delivery is known to ameliorate vascular endothelial function and reduce blood pressure in rat models of atherosclerotic disease (Saito et al., 2000). Recently, Wang et al. (2010) reported that the -395A variant of the G-395A SNP might protect against the presence and development of essential hypertension by upregulating *KLOTHO* gene expression and increasing KLOTHO protein levels. This was believed to increase the NO production, alleviate arteriosclerosis, and suppress oxidative stress. However, further studies are required to investigate the complex regulatory mechanisms.

There are some limitations to this study. Firstly, 160 subjects were excluded from this study because of a lack of DNA sample availability. The exclusion of participants with missing data may undermine the association between the *KLOTHO* G-395A SNP and hypertension. Secondly, there was a gender imbalance in the study population, which is a common characteristic of an oldest-old population because women generally have a longer expected lifespan than men. Thirdly, approximately 15% of the hypertension patients in our study population used anti-hypertensive agents during the study, which may have influenced the blood pressure test results. Therefore,

the percentage of ISH patients might have been underestimated. Lastly, all participants were ≥ 90 years of age; in other words, all of the study participants were survivors of the whole population. This may induce selection bias in our study.

In conclusion, the *KLOTHO* G-395A SNP is associated with hypertension in Chinese nonagenarians and centenarians. The -395A allele of the *KLOTHO* gene may be a protective genetic factor for hypertension in the Chinese population. Further studies with a larger sample size are required to confirm this finding, and to identify the possible mechanism with which *KLOTHO* G-395A influences the blood pressure.

Conflicts of interest

The authors declare no conflict of interest.

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