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The differential effects of age on the association of *KLOTHO* gene polymorphisms with coronary artery disease

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Abstract

The Klotho knockout mouse is thought to be a good animal model for human aging. Recent studies have reported on the association of *KLOTHO* gene mutation with cardiovascular disease in humans. We observed the frequencies of single nucleotide polymorphisms, that is, G-395A in the promoter region, C1818T in exon 4, and a functional variant, KL-VS, of *KLOTHO* gene in Koreans, and we investigated their relationships with the presence of coronary artery disease (CAD) in patients who had undergone coronary angiograms. A total of 274 subjects who underwent coronary angiograms because of chest pain were enrolled, and their blood pressure, body mass index, fasting blood glucose level, and lipid profiles were measured. Genotypings were performed on samples of their blood with real-time polymerase chain reaction. Two single nucleotide polymorphisms, G-395A and C1818T, complied with Hardy-Weinberg equilibrium. For the KL-VS genotype, 1 homozygote subject for the adverse allele was detected among the entire population (GG for F352V and CC for C370S). When the subjects were classified into 4 groups according to the number of stenotic vessels, there were no differences among the mean values of the cardiovascular risk factors, except for age and the fasting blood glucose levels, which showed a significant difference between that of the normal and the diseased vessel groups. There were no differences in the prevalence of CAD according to the genotypes of the G-395A polymorphism; however, for the C1818T polymorphism, those subjects with the T allele showed a lower prevalence of CAD than those with the CC genotype. When the subjects were divided into 2 groups according to age, in the group younger than 60 years, T allele carriers of the C1818T polymorphism showed a lower prevalence of CAD than did the noncarriers. In the group older than 60 years, A allele carriers of the G-395A polymorphism showed a lower prevalence of CAD than did the noncarriers. On the haplotype analysis, the GG-CC haplotype showed an increased risk for CAD with an odds ratio of 2.594 (95% confidence interval, 1.385-4.858; $P = 0.003$). Differential effects of age were observed in the association of *KLOTHO* G-395A and C1818T polymorphisms with CAD in Koreans. The KL-VS variant seems to be rarely found in the Korean population. These results infer the possibility of the *KLOTHO* gene being a candidate gene of atherosclerosis in humans, and further research on this topic needs to be done.

1. Introduction

Atherosclerosis is a complex, polygenic disease, and its development is underpinned by the aberrant functioning of a plethora of factors from the various systems that are involved with the cardiovascular function [1]. It has been estimated that more than 400 genes can regulate the atherosclerosis-related processes, although most of these genes probably have only small effects. Extensive study has been done on the candidate genes that have been suggested to have possible associations with the atherosclerotic characteristics, but any direct causal relations have not been proven [2,3].

A novel mouse mutant, *klotho*, was incidentally discovered by Kuro-o et al [4] during the development of a hypertensive transgenic murine model. This mutant exhibits impressive phenotypes resembling premature aging of humans and so it was named after a Greek goddess who spins the thread of life. Mice that are homozygous for the transgene show various phenotypes that resemble premature

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aging syndrome, that is, a short life span, arteriosclerosis, osteoporosis, skin atrophy, pulmonary emphysema, ectopic calcification in various soft tissues, impaired sexual organ maturation, senile atrophy of the skin, and defective hearing. These mice showed no typical differences with wild-type or heterozygous mice until about postnatal age 3 to 4 months, after which time the systemic growth rate slowed down and the mice died during the postnatal 8 to 15 weeks. The precise causes for death were not explained clearly, and as the phenotypes were in good compliance with the aging phenomenon previously described by Martin [5], *klotho* mutant mice are now extensively studied as a good model for human aging.

Another interesting phenotype of *klotho* mutant mice is the expression of atherosclerosis. In *klotho* mice, Mönckeberg-type atherosclerosis, which is seen in aging humans, was observed from the aorta to the small arterioles [4,6], and the impairments of angiogenesis and vasculogenesis were additionally observed [7]. Also in *klotho* mice, low bone turnover that was caused by the impairment of the differentiation of osteoblasts and osteoclasts was seen, and this condition resembled the characteristics of bone loss and senile osteoporosis in the human aging process [4,8,9]. The simultaneous existence of atherosclerosis and osteoporosis resembles that in OPG knockout mice, which show aortic calcification and severe osteoporosis and are extensively studied as the possible link between those 2 diseases [10,11]. Kl/kl mice have increased serum plasminogen activator-inhibitor 1 levels, impaired endothelium-dependent vasodilation, and reduced synthesis of nitric oxide, which is a typical endothelium-derived vasodilator. An in vitro study has suggested that klotho protein could be involved in vascular tone and thrombogenesis as a humoral factor [12-14]. Further supporting this hypothesis are studies showing that adenovirus-mediated *klotho* gene delivery in Otsuka Long-Evans Tokushima fatty rats, an animal model of type 2 diabetes mellitus and diabetic nephropathy, ameliorated vascular endothelial dysfunction through the increment in nitric oxide production and the reduced blood pressure, and this all prevented medial hypertrophy and perivascular fibrosis [12,15,16].

More than 10 mutations or single nucleotide polymorphisms (SNPs) in the human *KLOTHO* gene have been reported, and some of these have been reported to be associated with bone mineral density (BMD) and the human life span [17-19]. Studies by Arking et al [17,20,21] reported the association of a functional variant of the human *KLOTHO* gene, KL-VS, with early-onset occult heart disease. In these studies, subjects with the adverse allele showed a significantly increased risk for occult coronary diseases compared with the wild-type allele carriers. Furthermore, a recent article by Arking et al [21] reported the association of this allele with high-density lipoprotein cholesterol (HDL-C) levels, systolic blood pressure, and the increased risk of stroke, which suggests the association of the *KLOTHO* gene with atherosclerosis in Caucasians. However, these types of analyses have never been performed in an Asian population.

In the study by Kawano et al [19], the association of SNPs of the *KLOTHO* gene, other than KL-VS, with BMD were analyzed in Caucasian and Japanese populations. In their study, G-395A of the promoter region and C1818T and C2298T of exon 4 were observed at a moderate frequency in the Japanese population; among them, the G-395A and C1818T SNPs showed significant associations with BMD. However, no reports have been published on the associations of those SNPs with coronary artery disease (CAD) in any ethnic groups.

Therefore, we performed genotype analyses for 2 SNPs of the *KLOTHO* gene, that is, G-395A in the promoter region and C1818T in exon 4, and also a functional variant of the *KLOTHO* gene, KL-VS, in Korean subjects who underwent coronary angiograms. We observed their associations with the cardiovascular risk factors and the presence of CAD to determine the possibility of the human *KLOTHO* gene being a candidate gene for the pathogenesis of atherosclerosis.

2. Methods

2.1. Study subjects

This study was performed in 274 patients whose chief complaint was chest pain and who underwent coronary artery angiography at Kangbuk Samsung Medical Center, Seoul, Korea, from May 2003 to August 2004. Of the total 1308 cases of coronary angiograms that were performed during the enrollment period, 274 subjects were enrolled in the study after exclusion of the subjects with problematic diseases or who were taking medication; repeat follow-up cases were excluded. The patients included 166 men and 108 women whose median age was 58.5 ± 10.9 years (range, 24-87 years). Those patients with medical illnesses such as acute infection, chronic renal failure (serum creatinine level ≥2.0 mg/dL), malignancies, and other severe medical illnesses were excluded from enrollment. A written informed

Table 1
General characteristics of the participants (N = 274)

	Mean ± SD
Age (y)	58.5 ± 10.9
Sex, male (%)	166 (60.6)
BMI (kg/m^2)	25.4 ± 2.9
Systolic BP (mm Hg)	134.3 ± 18.2
Diastolic BP (mm Hg)	84.3 ± 11.7
FBG (mg/dL)	113.5 ± 37.0
T-chol (mg/dL)	192.3 ± 43.8
TG (mg/dL)	159.2 ± 96.9
HDL-C (mg/dL)	50.5 ± 12.7
LDL-C (mg/dL)	111.2 ± 31.4
CAD (%)	
Normal	119 (43.4)
1-Vessel disease	91 (33.2)
2-Vessel disease	40 (14.6)
3-Vessel disease	24 (8.8)

BP indicates blood pressure; FBS, fasting blood glucose; T-chol, total cholesterol; TG, triglyceride.

Table 2
Differences of the variables between different groups according to the severity of CAD (N = 168)

	Number of involved vessels				P
	Normal (n = 119)	1 (n = 91)	2 (n = 40)	3 (n = 24)	
Age	55.3 ± 11.5	59.1 ± 9.9*,†	62.2 ± 9.1*	65.8 ± 8.1*,†	<.01
BMI (kg/m^2)	25.2 ± 2.9	25.7 ± 2.7	25.6 ± 3.0	24.6 ± 3.0	.319
Systolic BP (mm Hg)	132.0 ± 20.7	135.7 ± 16.3	134.8 ± 13.4	139.6 ± 18.5	.212
Diastolic BP (mm Hg)	84.0 ± 10.3	83.2 ± 14.6	85.8 ± 8.4	87.1 ± 10.0	.415
FBS (mg/dL)	105.5 ± 27.4	119.5 ± 45.1*	117.7 ± 37.6	123.8 ± 38.1*	.016**
T-chol (mg/dL)	193.9 ± 40.3	192.0 ± 47.5	186.2 ± 41.3	195.5 ± 51.4	.785
HDL-C (mg/dL)	52.6 ± 11.3	49.4 ± 14.5	47.7 ± 9.0	49.0 ± 15.7	.103
LDL-C (mg/dL)	110.9 ± 30.0	110.4 ± 32.2	110.1 ± 33.5	117.6 ± 32.4	.772
TG (mg/dL)	152.5 ± 100.9	171.0 ± 106.7	157.6 ± 76.3	150.0 ± 63.9	.553

Values are presented as mean ± SD. Abbreviations as in Table 1.

* $P < .05$ when analyzed between normal group in post hoc analyses.

** $P = .008$ (the significance was persistent even after adjustment for age by ANCOVA test).

† $P < .05$ when analyzed between normal group and 3-vessel disease group in post hoc analysis.

consent was obtained from each participant. The study's protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval from the institution's human research committee.

2.2. Anthropometric measurements and blood chemistry

Height, weight, and systolic and diastolic blood pressures were measured in duplicate and the results were then averaged. Weight and height were measured in kilograms and centimeters, respectively, down to 2 decimal points. The body mass index (BMI) was calculated by dividing the weight (kilograms) with the square of height (meters).

Blood sampling was done after 12 hours of fasting. The fasting blood glucose, total cholesterol, triglyceride, HDL-C, and low-density lipoprotein cholesterol (LDL-C) levels were measured from the samples; the hexokinase method was used to measure the blood glucose levels, and enzymatic calorimetric testing was used to measure the total cholesterol and triglyceride levels. The selective inhibition method was used to measure HDL-C level, and the homogeneous enzymatic calorimetric test was used to measure LDL-C level.

2.3. Coronary artery angiography

Coronary artery angiography was performed in all patients. The procedures were performed and interpreted by trained cardiologists who had been certified as cardiologists by the Korean Society of Circulation for at least 3 years. The investigators were blind to the genotypes at the time of the procedure. Significant stenosis was defined as a decrease of the internal diameter of more than 50%. Patients were grouped according to the number of significantly stenotic vessels into normal and 1-vessel, 2-vessel, and 3-vessel disease groups.

2.4. Genotyping of 2 SNPs of the KLOTHO gene by real-time polymerase chain reaction

The buffy coat was obtained from blood samples and then refrigerated at −70 °C, and genomic DNA was extracted by using Takara DNA Purification kits (Kyoto, Japan). The genotypings of G-395A in the promoter region, C1818T in exon 4, and the KL-VS variant of the *KLOTHO* gene were performed via an allelic discrimination assay with TaqMan probes (SCG, Seoul, Korea).

The KL-VS allele harbors 3 mutations in the coding region, of which is 1 is silent and 2 code for the missense mutations F352V (T→G) and C370S (G→C) [17]. Among the 6 SNPs in complete linkage disequilibrium (LD), F352V and C370S were genotyped, that is, known to alter klotho metabolism.

The detector used in this experiment was an ABI Prism 7200 sequence detection platform (Perkin Elmer Applied Biosystems, Foster City, CA). The quality control of the machine was performed with regular background calibration and pure dye calibration. The primers and probes used were as follows:

(1) G-395A
Forward primer, TAGGGCCCGGCAGGAT
Reverse primer, CCTGGAGCGGCTTCGTC

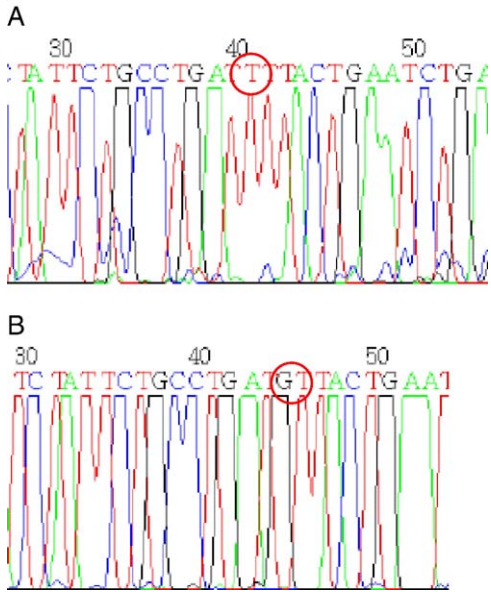

Fig. 1. DNA sequence analysis in (A) TT homozygote and (B) GG homozygote for F352V SNP of KL-VS variant of *KLOTHO* gene.

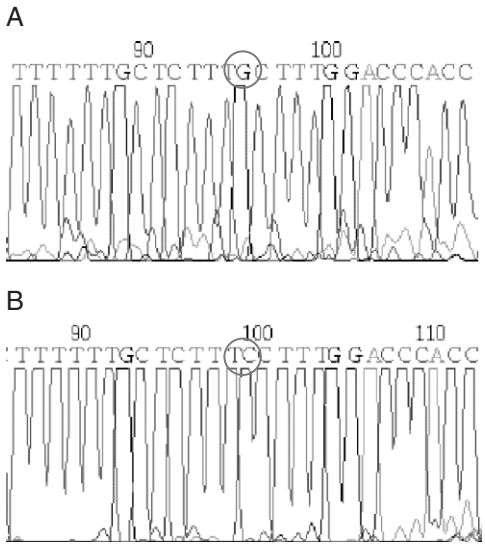

Fig. 2. DNA sequence analysis in (A) GG homozygote and (B) CC homozygote for C370S SNP of KL-VS variant of *KLOTHO* gene.

FAM, CAAGTCGGGGAAAG
VIC, AGTCGGGAAAAGT

(2) C1818T
Forward primer, CCAGCCCCAGATCGCTTTA
Reverse primer, GGCCCAGTCCAGGGAGAA
FAM, AAATGCATGTTACACATT
VIC, ATGCACGTTACACATT

(3) F352V
Forward primer, GTTTGCCAAACCCGTATTTATTG
Reverse primer, GTTGAAAACTCAAGGTGGGTCC
FAM, CATCTATTCTGCCTGATTTTACTGAATCTGAGAA
TET, CATCTATTCTGCCTGATGTTACTGAATCTGAGA

(4) C370S
Forward primer, AGTTCATCAAAGGAACTGCTGACTT
Reverse primer, CTTCATGTGAGGGTCCAAAAGTT
FAM, TTGCTCTTTGCTTTGGACCCACCT
TET, TTGCTCTTTCCTTTGGACCCACCTT

2.5. Statistical analysis

All results are expressed as means ± SD and significance was defined as a P value of <.05. The analysis was done by using SPSS version 10.0 (SPSS, Chicago, IL). Hardy-Weinberg equilibrium testing was performed by using the χ^2 test. Comparisons of the cardiovascular risk factors among the different groups that were categorized according to the severity of CAD were performed with analysis of variance tests, and multiple comparisons were done by a post hoc analysis. Student t test was used to compare the mean values between the different genotype groups, and analysis of covariance was done to adjust for confounding variables in the comparison of the mean values between the different genotype groups. χ^2 Testing was performed to calculate the odds ratio (OR) of the genotype for the occurrence of CAD.

Linkage disequilibrium analysis was performed with ARLEQUIN software version 2.0 (Geneva, Switzerland). For the haplotype analysis, the alleles in each polymorphism were paired 2 by 2, and 4 haplotype sets were generated. Logistic regression analysis was performed to calculate the OR of CAD according to the individual haplotypes [22].

The sample size of the study groups was calculated with the detected power of 90% in each group with a 95% confidence level. The formula used to calculate the sample size is as follows [23]:

$$D = \frac{\left(Z_{\alpha/2} + Z_{\beta}\right)^2 (\Delta + 1)^2}{(\Delta - 1)^2}$$

where D is the sample size, $Z_{\alpha/2}$ the 2-sided quantile for α, Z_{β} the quantile for β, and Δ the hazard ratio.

3. Results

3.1. Clinical characteristics of study subjects

The mean age of the study subjects was 59 years and the mean BMI was 25.4 kg/m^2, which was relatively obese. The mean fasting blood glucose level was 113.5 mg/dL; 113 (41.2%) subjects were in the range of normoglycemia

Table 3
Comparison of cardiovascular risk factors according to different groups of genotypes of *KLOTHO* gene

	Promoter G395A (n = 274)			Exon 4 C1818T (n = 273)		
	GG (n = 203)	GA + AA (n = 71)	*P*	CC (n = 188)	CT + TT (n = 85)	*P*
Age (y)	58.1 ± 11.2	59.6 ± 9.9	.310	58.8 ± 10.8	57.8 ± 11.1	.455
Sex, male (%)	125 (61.6)	41 (57.7)	.333	118 (62.8)	48 (56.5)	.197
BMI (kg/m^2)	25.3 ± 2.9	25.5 ± 2.7	.669	25.3 ± 2.8	25.5 ± 3.0	.638
Systolic BP (mm Hg)	134.0 ± 19.3	135.1 ± 14.9	.667	135.1 ± 18.8	132.5 ± 17.0	.274
Diastolic BP (mm Hg)	84.3 ± 11.1	84.3 ± 13.1	.957	84.8 ± 12.7	83.1 ± 9.0	.257
FBS (mg/dL)	113.6 ± 40.0	113.4 ± 27.0	.980	115.1 ± 37.5	110.1 ± 36.1	.302
T-chol (mg/dL)	192.2 ± 43.8	192.5 ± 44.3	.959	194.2 ± 45.0	187.9 ± 41.3	.278
TG (mg/dL)	161.5 ± 103.0	152.6 ± 77.6	.506	166.2 ± 93.3	144.2 ± 103.7	.084
HDL-C (mg/dL)	50.8 ± 14.0	49.7 ± 7.7	.401	50.1 ± 11.8	51.4 ± 14.5	.434
LDL-C (mg/dL)	110.2 ± 30.6	113.9 ± 33.6	.399	112.5 ± 32.3	108.0 ± 29.0	.272

Values are presented as mean ± SD. Abbreviations as in Table 1.

Table 4
Association of *KLOTHO* genotypes with CAD

	Promoter G395A (n = 274)		Exon 4 C1818T (n = 273)	
	GG (n = 203)	GA + AA (n = 71)	CC (n = 188)	CT + TT (n = 85)
Without coronary artery stenosis (%)	87 (42.9)	32 (45.1)	74 (39.4)	45 (52.9)
With coronary artery stenosis (%)	116 (57.1)	39 (54.9)	114 (60.6)	40 (47.1)
P	.746		.036	

All percentages are calculated within the same genotype groups. χ^2 Test was used to compare the frequencies of CAD according to different genotypes.

(<100 mg/dL), 108 (39.4%) subjects had fasting hyperglycemia (≥100 mg/dL and <126 mg/dL), and 53 (19.3%) subjects were in the range of provisional diabetes mellitus according to the newly recommended criteria of the American Diabetes Association [24].

According to the coronary angiogram results, 119 (43.4%) subjects had normal coronary arteries, 91 (33.2%) subjects had 1-vessel disease, 40 (14.6%) subjects had 2-vessel disease, and 24 (8.8%) subjects had 3-vessel disease (Table 1).

3.2. Comparisons of cardiovascular risk factors according to the number of stenotic coronary arteries

When the mean values of the cardiovascular risk factors were compared among the groups that were categorized by the number of stenotic coronary arteries (Table 2), the patients with 3-vessel disease were the oldest among the groups and mean fasting blood glucose level showed significant differences among the groups; these differences were significant even after adjustment for age ($P = 0.008$).

3.3. Genotyping of the KLOTHO SNPs and the comparisons of cardiovascular risk factors according to the different genotypes

For the G-395A polymorphism in the promoter region, 203 (74.1%) subjects had the GG genotype, 61 (22.3%) subjects had the GA genotype, and 10 (3.6%) subjects had the AA genotype. The allele frequencies were 0.852 for the G allele and 0.148 for the A allele; these were in compliance with Hardy-Weinberg equilibrium ($P = 0.16$).

For the C1818T polymorphism in exon 4, the genotype of 1 subject could not be determined; thus, a total of 273 samples were analyzed. Of these, 188 (68.6%) subjects had the CC genotype, 76 (27.7%) subjects had the CT genotype, and 9 (3.3%) subjects had the TT genotype. The allele frequencies were 0.830 for the C allele and 0.170 for the T allele, in compliance with Hardy-Weinberg equilibrium ($P = 0.93$).

The LD score (D') between the 2 polymorphisms was 0.245 ($P < .01$). Considering the small value of the score, the LD between the 2 polymorphisms could not be interpreted with this score.

Genotyping of F352V and C370S revealed complete linkage between these 2 SNPs. However, genotyping of all samples revealed wild-type homozygote alleles (ie, TT for F352V and GG for C370S) except for 1 sample that showed homozygous adverse alleles (ie, GG for F352V and CC for C370S). Sequencing of the polymerase chain reaction products confirmed the genotypes (Figs. 1 and 2). Analysis was impossible because nearly no adverse alleles existed for KL-VS.

When the cardiovascular risk factors were compared according to different genotypes, there were no differences between different genotypes in either of the SNPs (Table 3).

3.4. The association of KLOTHO SNPs with the presence of CAD

The association of *KLOTHO* SNPs with the presence of CAD was analyzed (Table 4). There were no differences in the prevalence of CAD between the different genotypes of the G-395A polymorphism. As for the C1818T polymorphism, the T allele carriers showed a significantly lower prevalence of CAD compared with the CC genotype group (47.1% vs 60.6%, respectively; $P = 0.036$; Table 4). The OR for the CC genotype for the subjects to have CAD was 1.733 (95% confidence interval [CI], 1.034-2.906).

There was no association between the number of stenotic vessels and the genotypes (data not shown).

Table 5
Association of *KLOTHO* genotypes with CAD according to different age groups

	Promoter G395A (n = 131)		Exon 4 C1818T (n = 130)	
Subjects aged <60 years	GG (n = 99)	GA + AA (n = 32)	CC (n = 92)	CT + TT (n = 39)
Without coronary artery stenosis (%)	57 (57.6)	14 (43.8)	44 (47.8)	27 (69.2)
With coronary artery stenosis (%)	42 (42.4)	18 (56.3)	48 (52.2)	12 (30.8)
P	.172		.025	
	Promoter G395A (143)		Exon 4 C1818T (n = 142)	
Subjects aged ≥60 years	GG (n = 104)	GA + AA (n = 39)	CC (n = 96)	CT + TT (n = 46)
Without coronary artery stenosis (%)	30 (28.8)	18 (46.2)	30 (31.3)	18 (39.1)
With coronary artery stenosis (%)	74 (71.2)	21 (53.8)	66 (68.8)	28 (60.9)
P	.041		.353	

All percentages are calculated within the same genotype groups. χ^2 Test was used to compare the frequencies of CAD according to different genotypes.

Table 6
Estimated haplotypes of *KLOTHO* gene and the association with CAD

Haplotype	G395A	C1818T	Frequency (%)	OR (95% CI)	*P*
H1	GG	T allele	57 (20.8)	(Referent)	–
H2	GG	CC	146 (53.3)	2.594 (1.385-4.858)	.003
H3	A allele	T allele	28 (10.2)	2.285 (0.906-5.760)	.080
H4	A allele	CC	42 (15.3)	1.478 (0.662-3.302)	.340

T allele and A allele denote the genotypes carrying T allele and A allele, respectively. Haplotype analyses were performed by using logistic regression model [22].

3.5. The association of KLOTHO genotypes with the presence of CAD as divided by the age groups

The associations of the *KLOTHO* polymorphisms with the presence of CAD were analyzed in the 2 groups that were subdivided according to age, that is, younger than and older than 60 years (Table 5). The cutoff for the age group of 60 years was determined as age of 60 according to the median for the age of all the subjects. For the subjects younger than 60 years, the C1818T polymorphism showed significant association with the presence of CAD, which was similar to the results for all the subjects; that is, the T allele carriers showed a lower prevalence of CAD than the subjects with the CC genotype (30.8% vs 52.5%, respectively; $P = 0.025$; Table 5). In contrast, for the subjects older than 60 years, the G-395A polymorphism showed significant association with the presence of CAD; that is, the A allele carriers showed a lower prevalence of CAD compared with the subjects having the GG genotypes (53.8% vs 71.2%, respectively, $P = 0.041$; Table 5).

On the haplotype analysis, subjects with the GG genotype of G-395A and the CC genotype of C1818T showed a significantly increased risk for CAD with an OR of 2.594 (95% CI, 1.385-4.858; $P = 0.003$) (Table 6).

4. Discussion

In this study, the allele frequencies of the G-395A polymorphism in the promoter region of the *KLOTHO* gene were 0.852 for the G allele and 0.148 for the A allele, and those of the C1818T polymorphism in exon 4 of the *KLOTHO* gene were 0.828 for the C allele and 0.172 for the T allele for Korean patients who underwent coronary angiogram because of chest pain. In a previous report [19], the allele frequency for the A allele in the G395A polymorphism was reported to be 0.196 in Caucasians and 0.143 in Japanese, suggesting there is a relatively lower frequency of adverse alleles in Asians. This result applies in a like fashion to the C1818T polymorphism, as the T allele frequencies were reported to be 0.411 in Caucasians and 0.247 in Japanese, which was similar to our results, suggesting the relatively lower frequency of adverse alleles in Asians. As for KL-VS, there was almost no adverse allele observed in the 274 Koreans genotyped in this study. Although there is a possibility that this could be due to the specific character of disease for the study population, KL-VS, which is prevalent in the general Caucasian population [17], seems to be rarely observed in the Asian population. Further analysis of different ethnic groups will be needed in the future.

The *klotho* gene encodes a novel cell surface protein of 1014 amino acids [17]. The extracellular domain consists of 2 internal repeats that exhibit 20% to 40% sequence identity to the β-glucosidases of bacteria and plants as well as to the mammalian lactase glycosylceramidase. The human *KLOTHO* gene is highly conserved (86% amino acid identity to the mouse klotho protein), and it is composed of 5 exons that range more than 50 kilobases on chromosome 13q12. Two isoforms exist, the membranous and secreted forms, and the secreted forms in humans are known to be the dominant forms. The secreted klotho protein is found predominantly over the membrane form in all the tissues that have been examined, for example, brain, placenta, kidney and small intestine; this is in contrast to mice, in which klotho protein was exclusively expressed in the kidney and brain [25]. Although there are not many reports on regulation of the expression of klotho protein, the expression of klotho was markedly decreased by lipopolysaccharide in vivo, suggesting that the expression of klotho is affected by acute inflammatory stress. In addition, in preadipocytes, triiodothyronine significantly increased the expression levels of the membrane form of the klotho protein [26,27]. Furthermore, *klotho* mRNA in the kidney was down-regulated under sustained circulatory stress in various animal models of vascular and metabolic diseases such as diabetes and chronic renal failure, suggesting the amelioration of the klotho protein expression by vascular stresses; this might suggest the role of this protein in regulating vascular health [28].

This was the first association study analyzing the relationship between the G-395A and C1818T SNPs of the *KLOTHO* gene and the presence of CAD in patients who underwent coronary angiograms. As for the 2 SNPs, previous studies were performed for the association with only BMD, and not with CAD [19]. These 2 SNPs are located relatively distantly apart and they do not seem to be in LD [25]; thus, they seem to act separately from each other. According to the study by Kawano et al [19], electrophoretic mobility shift analysis revealed that the G→A substitution in the promoter region of the *KLOTHO* gene affected the DNA-protein interaction in cultured human kidney cells, suggesting the possibility of the functional relevance of this SNP. In that study, G-395A and C1818T showed significant associations with the BMD

of postmenopausal women, and the significance was strengthened in the older group (>65 years old). These results are in line with our study result that the effects of the polymorphisms on CAD were enhanced in the older group compared with the younger group. Furthermore, haplotype analysis showed that subjects with the GG genotype of G-395A and the CC genotype of C1818T showed an increased risk for CAD with an OR of 2.594 (95% CI, 1.385-4.858), suggesting the CC genotype as a strong predictor for CAD in this population. A hypothesis could be suggested that, as subjects having CAD before the age of 60 years showed higher prevalence of CC genotype, this SNP could be associated with the premature atherosclerosis in humans. Although this is just a hypothesis to interpret the results of this study, these results could be a clue to solve the mystery of the klotho gene in human aging and atherosclerosis. Further research is strongly recommended to clarify and confirm the roles of these SNPs in the human body.

In this study population, almost no mutant allele was observed for KL-VS, which is known to be relatively prevalent in Caucasians [17,20,21]. This allele is characterized by 6 SNPs that occur within an 800-base-pair region spanning both exon 2 and the flanking sequence. Of the 3 mutations in exon 2, 1 is silent and 2 code for amino acid substitutions, F352V (T→G) and C370S (G→C). The transient transfection assays performed in that study revealed that the levels of secreted klotho protein in the cells harboring V352 and S370 showed significant differences compared with the wild types, suggesting the functional effect of this variant in the *KLOTHO* gene transcription [17]. As these studies were performed only on Caucasians or African Americans, we performed KL-VS genotyping in the Korean population, which could be considered as representative of the entire Asian population. However, the results failed to show the existence of the prevalent KL-VS variant in our population. Reviewing the literature, among the several SNPs genotyped in the Caucasian population in the study by Kawano et al [19], C1110S (G→C) seems to be C370S of the KL-VS variant genotyped by Arking et al [17]. In that study, no C1110S SNP was detected in the Japanese, which supports our hypothesis that the prevalence of KL-VS is strongly affected by the ethnic background, and this variant seems to be very rarely observed in the Asian population. Thus, the possibility that KL-VS is involved in the occurrence of CAD in Asian ethnic groups might be very small.

There are limitations in this study worth mentioning. The total number of studied subjects was relatively small to have enough power as an association study. Therefore, to show that our study results provided sufficient statistical power as a polymorphism study, we calculated the OR for CAD to be detected with our study samples, and a hazard ratio of 1.49 was determined with 90% power and a 95% confidence level for our sample size of 274 subjects [23]. Therefore, the results of our study could be interpreted with fairly adequate power to provide meaningful association. Second, because this study was performed in a population that included diseased subjects and this was the first such study performed on Koreans, we do not know which allele is the adverse allele in Koreans. In our previous genotyping data for *KLOTHO* SNPs in 251 apparently healthy Korean female subjects, the allelic frequency for the A allele in the G-395A SNP was 0.171 and the allelic frequency for the T allele in the C1818T SNP was 0.196, which were similar to our present study (data not shown). The strong points of this study were that the presence of CAD was confirmed by performing coronary angiograms in all subjects, which was different from previous studies in which the presence of CAD was diagnosed only with a thallium scan or an electrocardiogram. Furthermore, this study was the first study to genotype the KL-VS in an Asian population and to analyze the association of CAD with the *KLOTHO* polymorphisms G395A and C1818T in this population. More studies on humans have to be done to uncover the role of the *KLOTHO* gene in the pathogenesis of atherosclerosis, as more and more interesting reports are supporting its role as a link between other cardiovascular risk factors, such as insulin resistance and lipid metabolism, and the life span [29-31].

In conclusion, the KL-VS variant was not frequently observed in the Korean population; thus, it did not seem to be an effector for the life span or for the development of CAD in this population. Two *KLOTHO* polymorphisms, G395A in the promoter region and C1818T in exon 4, were observed at similar frequencies in the Korean population to those of other Asians. Furthermore, the presence of the CC genotype in the C1818T SNP increased the CAD risk, and aging enhanced the negative effects of these polymorphisms on CAD; this suggests the possibility that the *KLOTHO* gene might be involved in the age-related occurrence of CAD. Future investigations are needed to clarify the precise mechanism of involvement of the *KLOTHO* gene in atherosclerosis and on the life span of human beings, as was observed in the previous studies that used animal models.

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