

Klotho Gene Polymorphisms Associated With Bone Density of Aged Postmenopausal Women

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ABSTRACT

Because mice deficient in *klotho* gene expression exhibit multiple aging phenotypes including osteopenia, we explored the possibility that the *klotho* gene may contribute to age-related bone loss in humans by examining the association between *klotho* gene polymorphisms and bone density in two genetically distinct racial populations: the white and the Japanese. Screening of single-nucleotide polymorphisms (SNPs) in the human *klotho* gene identified 11 polymorphisms, and three of them were common in both populations. Associations of the common SNPs with bone density were investigated in populations of 1187 white women and of 215 Japanese postmenopausal women. In the white population, one in the promoter region (G-395A, $p = 0.001$) and one in exon 4 (C1818T, $p = 0.010$) and their haplotypes ($p < 0.0001$) were significantly associated with bone density in aged postmenopausal women (≥ 65 years), but not in premenopausal or younger postmenopausal women. These associations were also seen in Japanese postmenopausal women. An electrophoretic mobility shift analysis revealed that the G–A substitution in the promoter region affected DNA-protein interaction in cultured human kidney 293 cells. These results indicate that the *klotho* gene may be involved in the pathophysiology of bone loss with aging in humans. (J Bone Miner Res 2002;17:1744–1751)

Key words: osteoporosis, aging, pathophysiology, genetics, association

INTRODUCTION

OSTEOPOROSIS is a systemic bone disorder characterized by decreased bone density and disturbed skeletal architecture, which results in an increased risk for bone fractures with consecutively increased morbidity and mortality. Accumulating evidence has shown the involvement of genetic factors in the decrease of bone density.^(1–3) Twin and sibling studies have revealed that 50–90% of the variation in bone density is accounted for by genetic factors.^(4–9) In fact, some

loci, such as the vitamin D and estrogen receptor genes, as well as the collagen type I α 1 gene, have been reported as promising genetic determinants of bone density.^(10–15) However, this is controversial and the molecular basis of osteoporosis remains largely undefined.^(16–21) Considering that the effect of each candidate gene is expected to be modest, discrepancies between allelic association studies may have arisen because different populations carry different genetic backgrounds.

We recently established a mouse model for human aging termed *klotho*.⁽²²⁾ The mouse was serendipitously generated by insertional mutation in a transgenic mouse, which dis-

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rupted the *klotho* gene encoding a novel single-pass membrane protein (KL protein). Function of the KL protein remains to be determined; however, this may be involved in the suppression of aging because a defect in *klotho* gene expression leads to multiple aging phenotypes and age-related disorders. These include such maladies as a shortened lifespan, arteriosclerosis, decreased spontaneous activity, infertility, skin atrophy, premature thymic involution, pulmonary emphysema, lipodystrophy, ectopic calcification, and osteopenia. Osteopenia observed in the *klotho*-deficient mouse is accompanied by low turnover during bone metabolism, in which a decrease in bone formation that exceeds a decrease in bone resorption results in a net bone loss.⁽²³⁾ Because this state resembles bone loss by aging in humans, osteopenia observed in the *klotho*-deficient mouse can be regarded as one of the manifestations of generalized aging.

A human homologue of the *klotho* gene was isolated and its gene structure was determined.⁽²⁴⁾ The human *klotho* gene is composed of five exons and ranges over 50 kb on chromosome 13q12. To examine a possible contribution of the *klotho* gene to the pathophysiology of osteoporosis in humans, this study screened for single-nucleotide polymorphisms (SNPs) in and around the coding regions of the human *klotho* gene that could modify KL protein expression or function and examined the association of these SNPs with bone density. To avoid influence of the difference in genetic backgrounds, we analyzed two genetically distant populations: white and Japanese women.

MATERIALS AND METHODS

SNP screening

For the screening of SNPs in the *klotho* gene, DNA samples were extracted from peripheral blood obtained with written informed consent from 16 unrelated white women taking part in Gemini Genomics clinical genetics programs and 115 unrelated Japanese subjects (56 men and 59 women) who visited the orthopedic clinic of Tokyo University Hospital. All exons (exons 1–5) with their flanking sequences and ~2.0 kb of the promoter region were directly sequenced with DNA sequencer (ABI PRISM 310; Perkin Elmer, Foster City, CA, USA) using 17 sets of primers (the information of primers and polymerase chain reaction [PCR] conditions are available on request). The allelic frequency of each SNP in the Japanese population was calculated based on the results obtained from this direct sequencing. To determine the allelic frequency of each SNP in the white population, 288 unrelated white female samples were analyzed further in several ways as follows. For the G-395A, G1110C, C1818T, and C2298T SNPs, Taqman allelic discrimination assays were used (see the following paragraphs for details). The G-959C, -744delA, and IVS 4+22A->T SNPs were analyzed by allele-specific PCR, and the G1204A SNP was analyzed by PCR restriction fragment length polymorphism (RFLP) by ApoI endonuclease (details available on request).

Association study

For the study on the white population, DNA samples were obtained from 1187 unrelated white women recruited nationwide from the United Kingdom via media campaigns, as part of the St. Thomas' UK Adult Twin Registry, and written informed consent was obtained before investigation. No participant had medical complications known to affect bone metabolism, and no participant was receiving therapy for osteoporosis. Genotyping was performed for the three common SNPs (G-395A, C1818T, and C2298T) by Taqman allelic discrimination assay using primers and probes as follows (the polymorphic base in each probe is underlined):

G-395A—forward primer, TAGGGCCCGGCAGGAT; reverse primer, CCTGGAGCGGCTTCGTC; FAM-labeled probe, CCCCAAGTCGGGAAAAGTTGGTC; TET-labeled probe, CCCCAAGTCGGGGAAAGTTGGTC

C1818T—forward primer, GCCATCCAGCCCCAGATC; reverse primer, GGGCCCAGTCCAGGGA; FAM-labeled probe, TTTACTCCAGGAAATGCATGTTACACATTTT; TET-labeled probe, TTTACTCCAGGAAATGCACCGTTACACATTTT

C2298T—forward primer, CTGCCCCTTTCTCCCAAAA; reverse primer, AATCTCCAGAGCCGAAAATGG; FAM-labeled probe, CCAAAACTCTCTCAGCCACCTCTTTGT; TET-labeled probe, CCAAAACTCTCTCGCCACCTCTT.

Primer and probe concentrations were optimized according to the manufacturer's recommendations so that each reaction contained 50 nM of FAM-labeled probe, 200 nM of TET-labeled probe for assays G-395A and C1818T, and 350 nM of TET-labeled probe for C2298T, 300 nM of reverse primer, and 50, 300, or 900 nM of forward primer for G-395A, C1818T, and C2298T assay, respectively. Taqman reactions were thermocycled as follows: 50°C for 2 minutes, 95°C for 10 minutes; 40 cycles of 95°C for 15 s followed by 60°C for 1 minute. The completed reactions were analyzed on an ABI Prism 7200 sequence detection platform (Perkin Elmer). Bone mineral density (BMD), g/cm² of the whole body was measured by DXA (QDR 4500/w; Hologic, Inc. Waltham, MA, USA). This parameter was also recorded as a Z score that is a deviation from the weight-adjusted average BMD of each age based on data installed in the densitometer.

For the study on the Japanese population, DNA samples were obtained from the peripheral blood of 215 Japanese postmenopausal women living in a rural area of Akita prefecture on the mainland of Japan. All were unrelated volunteers and gave their written informed consent before the study. The exclusion criteria were the same as those of the white population described previously. Genotyping for the three common SNPs was also performed by Taqman allelic discrimination. BMD and its Z score of the distal one-third of the radius were measured by DXA using a bone mineral analyzer (DTX-200; Osteometer Co., Ltd., Hoersholm, Denmark).

Electrophoretic mobility shift assay

Two hundred ninety-three cells established from a human primary embryonal kidney were confirmed to express the

TABLE 1. SNPs DETECTED IN THE *KLOTHO* GENE OF THE WHITE AND JAPANESE POPULATIONS

	Location	Nucleotide change	Amino acid substitution	Allelic frequency
White population (<i>n</i> = 288)	Promoter	-959 (G → C)	—	0.003
	Promoter	-744 (del A)	—	0.212
	Promoter	-395 (G → A)	—	0.196
	Exon 2	1110 (G → C)	Cys → Ser	0.154
	Exon 2	1204 (G → A)	Lys → Lys	0.170
	Exon 4	1818 (C → T)	His → His	0.411
	Exon 4	2298 (C → T)	Ala → Ala	0.132
	Intron 4	IVS 4 + 22 (A → T)	—	0.121
	Japanese population (<i>n</i> = 115)	Promoter	-395 (G → A)	—
Exon 1		44 (A → C)	Gly → Pro	0.025
Exon 1		234 (C → G)	Ala → Gly	0.031
Exon 3		1541 (C → T)	Ser → Ser	0.043
Exon 4		1818 (C → T)	His → His	0.247
Exon 4		2298 (C → T)	Ala → Ala	0.270

Allelic frequency indicates the frequency of the minor allele in each SNP.

klotho transcript by reverse-transcription (RT)-PCR. Two hundred ninety cells were cultured in DMEM supplemented with 10% FBS and lysed to obtain nuclear extracts. Complementary single-stranded oligonucleotides were synthesized as follows (variant nucleotides underlined): 5'-TCG-ACAAGTCGGGG/AAAAGTTGGTG-3'. Complementary strands were annealed by combining 200 pmol of each oligonucleotide and 36 μ l of annealing buffer (10 mM of Tris-HCL, 1 mM of EDTA, and 0.1 M of NaCl, pH 8.0) in a 40- μ l reaction, incubating at 100°C for 5 minutes and allowing to cool to room temperature. The DNA-protein binding reaction was conducted in an 18- μ l volume containing 2.5 μ g of nuclear extract, 1 μ g of poly (dI-dC), 4 μ l of 5 \times binding buffer (Boehringer Mannheim, Mannheim, Germany), and 5.0 \times 10⁵ cpm of [³²P]-labeled oligonucleotide probe. For the competition experiment, various concentrations (X1-X100 of the labeled probe) of unlabeled probes with G- and A-bearing alleles were added to the solution. The reaction mixture was incubated at room temperature for 30 minutes and then was fractionated by 5% polyacrylamide gel. The DNA-protein complex was detected by exposing to X-ray film.

Statistical analysis

The χ^2 test was used for the Hardy-Weinberg equilibrium and the distribution of allelic frequencies. The difference in BMD between the major and minor alleles was determined by nonparametric analysis (Student-Newman-Keuls). The differences in BMD, body height, weight, body mass index (BMI) [BMI = (weight; kg)/(height; m)²] among genotypes, and haplotypic analysis were performed using nonparametric analysis (Kruskal-Wallis). This test indicates whether there are differences among the population means of the groups being compared, but it does not pinpoint which groups, if any, differ from the others. All statistical analyses were performed using the statistical package Stat View version J-5.0 (Abacus Concepts, Inc., Berkeley, CA,

USA). A value of *p* < 0.05 was considered statistically significant.

RESULTS

Identification of polymorphisms in the *klotho* gene in white and Japanese populations

In total, eight SNPs in the white population and six SNPs in the Japanese population were identified (Table 1). Among the 11 distinct SNPs identified in the two populations, three of them, one in the promoter region (G-395A) and two in exon 4 (C1818T and C2298T), were common in both populations. The SNPs in exon 4 were not accompanied by amino acid substitutions. Allelic frequencies of minor alleles in these SNPs were fairly frequent in both populations but were significantly different between populations.

Characteristics of the common polymorphisms in white and Japanese women

These three SNPs commonly identified in the two populations were used to study the association of the *klotho* gene with bone density in women. Unrelated white women (*n* = 1187, 18-72 years, 47.1 \pm 12.0 years, mean \pm SD) and unrelated Japanese postmenopausal women (*n* = 215, 66-92 years, 72.9 \pm 5.5 years) were analyzed for association. Because menopause is known to be a major factor for bone loss in women, we divided the white population into three subgroups according to their menopausal status: definite premenopausal women (*n* = 506, 18-58 years, 36.8 \pm 8.6 years), definite postmenopausal women (*n* = 364, 48-72 years, 57.9 \pm 6.7 years), and others whose menopausal status was unclear. Aging is also known to be another major factor affecting bone loss; therefore, we further divided the white postmenopausal women into three age groups: those \leq 54 years, 55-64 years, and \geq 65 years old (Table 2). The allelic frequency of minor alleles was not

TABLE 2. ASSOCIATION OF COMMON SNPs WITH MENOPAUSAL STATUS, AGE, AND BMI

	G-395A				C1818T				C2298T			
	BMI		Allelic frequency		BMI		Allelic frequency		BMI		Allelic frequency	
	G/G	G/A	A/A		C/C	C/T	T/T		C/C	C/T	T/T	
White population												
All (n = 1187)	24.75 ± 0.16	25.03 ± 0.23	25.03 ± 0.70	0.421	24.62 ± 0.21	25.03 ± 0.19	24.79 ± 0.30	0.124	24.90 ± 0.15	24.84 ± 0.26	22.58 ± 0.88	
Premenopausal (n = 506)	24.57 ± 0.25	24.40 ± 0.36	24.54 ± 1.35	0.448	24.14 ± 0.35	24.72 ± 0.30	24.50 ± 0.45	0.121	24.62 ± 0.23	24.22 ± 0.45	22.91 ± 1.35	
Postmenopausal (n = 364)	25.08 ± 0.29	25.75 ± 0.42	24.79 ± 0.99	0.416	25.65 ± 0.41	25.22 ± 0.32	24.78 ± 0.53	0.109	25.15 ± 0.26	25.91 ± 0.51	24.55 ± 0.30	
≤54 years (n = 112)	23.95 ± 0.57	25.16 ± 0.66	22.69 ± 1.18	0.426	24.77 ± 0.80	24.23 ± 0.59	23.64 ± 0.79	0.120	24.04 ± 0.47	25.30 ± 1.06	24.56 ± 0.52	
55-64 years (n = 197)	25.50 ± 0.36	26.11 ± 0.59	25.05 ± 1.66	0.417	25.99 ± 0.52	25.67 ± 0.44	25.13 ± 0.75	0.093	25.64 ± 0.35	25.93 ± 0.59	24.53 ± 0.32	
≥65 years (n = 55)	25.93 ± 0.72	25.65 ± 0.23	26.36 ± 1.76	0.382	25.84 ± 1.23	25.99 ± 0.78	25.36 ± 0.89	0.136	25.52 ± 0.66	26.77 ± 1.30	—	
Japanese population												
All: Postmenopausal (n = 215, > 65 years)	23.96 ± 0.34	22.73 ± 1.45	23.45 ± 0.53	0.248	23.46 ± 0.48	23.34 ± 0.46	21.02 ± 0.83	0.256	23.21 ± 0.28	22.89 ± 0.28	23.46 ± 2.24	

Allelic frequency indicates the frequency of the minor allele in each SNP. BMI data are mean ± SEM. There was no significant difference of BMI among genotypes of each SNP (all *p* > 0.05).

significantly different among subpopulations in each population and was similar to that obtained from the SNP screening study shown in Table 1. No significant difference in height, weight (data not shown), or BMI (Table 2) was seen among genotypes of these SNPs in any subpopulation (all *p* > 0.05). These results indicate that these SNPs are not associated with menopausal status, age, height, or weight in each population.

The genotypic frequencies for these SNPs in any subpopulations were not significantly different from those expected for populations in Hardy-Weinberg equilibrium (all *p* > 0.05, data not shown). Linkage disequilibrium among these SNPs was evaluated by calculating haplotype frequencies according to the method by Hill⁽²⁵⁾ and Thompson et al.⁽²⁶⁾ None of the disequilibrium values for marker pairs differed significantly from zero (the maximum-likelihood estimate of *D* = -0.047-0.058, all *p* > 0.05, data not shown), indicating there was no significant linkage disequilibrium among these SNPs.

Association of the common polymorphisms with bone density in white women

In all white women (n = 1187, 2374 alleles), there were no significant differences in the whole body BMD between major and minor alleles of these SNPs (Fig. 1A). We then investigated the association between BMD and the allele types in definite premenopausal women (n = 506, 1012 alleles) and definite postmenopausal women (n = 364, 728 alleles), respectively. No difference in BMD was seen between allele types of any SNPs in premenopausal women. However, there was a weak but significant association between BMD and C1818T SNP in postmenopausal women: the minor *T* allele was associated with lower BMD than the *C* allele (*p* = 0.029, Fig. 1A). These results were unchanged when we repeated the analysis using the Z score that was adjusted by age and weight. The *T* allele at the C1818T site was still associated with a lower Z score in postmenopausal women (*P*=0.004), although no association was found in the overall population or in the premenopausal subpopulation.

We performed further analysis by dividing the white postmenopausal women into three age groups (Fig. 1B). No significant association was seen between any of the SNPs and BMDs in the two younger subpopulations. However, in the oldest subpopulation (≥65 years), the association was stronger than that seen in the overall postmenopausal women and was detected not only with the C1818T SNP (*p* = 0.010) but also with the G-395A SNP (*p* = 0.001; Fig. 1B). Association between the Z score and allele types was also not seen in the two younger subpopulations but was observed in the oldest subpopulation: G-395A SNP (*p* = 0.001) and C1818T SNP (*p* = 0.018). Association analysis based on three genotypes was also performed (Fig. 1C). Again, both G-395A and C1818T SNPs showed a significant association with BMD in the oldest subpopulation (*p* = 0.003 and 0.014, respectively), and BMD was decreased dose dependently of the minor alleles.

Furthermore, G-395A and C1818T SNPs were examined jointly by haplotypic analysis (Table 3). Here again, the minor alleles were significantly associated with lower BMD

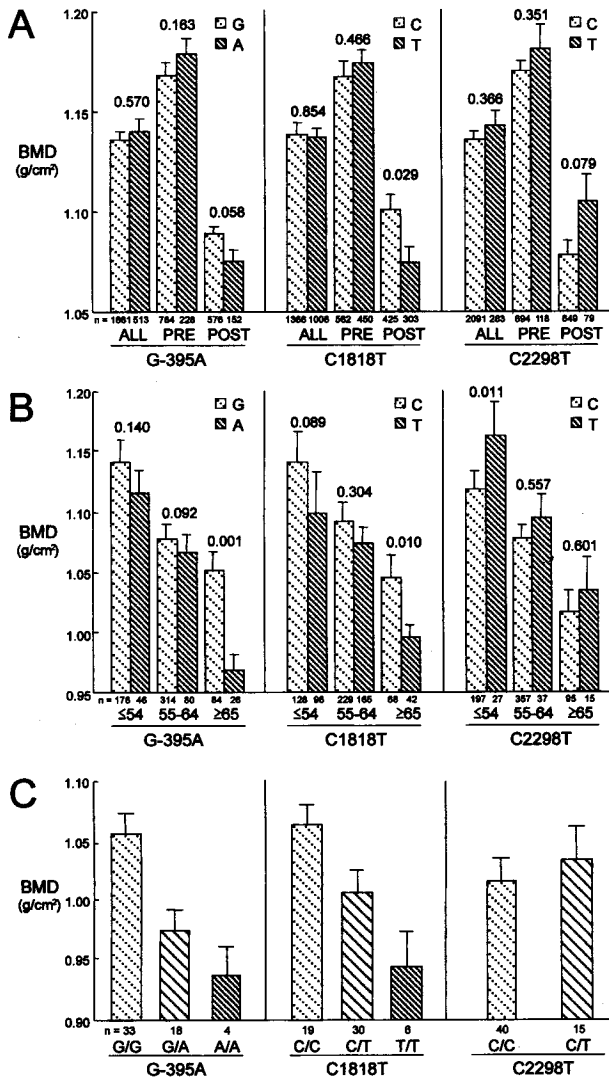


FIG. 1. Association of the three common SNPs with BMD in white women. BMD of the whole body was compared (A and B) between the major and minor alleles and (C) among genotypes. (A) Association of allele types with BMD in the three different subgroups classified according to their menopausal status: all women (ALL; $n = 1187$, 2374 alleles), definite premenopausal women (PRE; $n = 506$, 1012 alleles), and definite postmenopausal women (POST; $n = 364$, 728 alleles). (B) Association of allele types with BMD in the three different age groups of definite postmenopausal women ($n = 364$): those ≤ 54 years ($n = 112$, 224 alleles), 55–64 years ($n = 197$, 394 alleles), and ≥ 65 years ($n = 55$, 110 alleles). (C) Association of three genotypes with BMD in the oldest subpopulation (≥ 65 years old, $n = 55$). Data are expressed as means (bars) \pm SEMs (error bars) for the (A and B) number of alleles and (C) women shown under each bar. The p values of the difference in the mean BMD between major and minor alleles in panels A and B are shown as the numbers above the bars (Student–Newman–Keuls test) and those among genotypes in panel C are 0.003, 0.014, and 0.573 for G-395, C1818T, and C2298T, respectively (Kruskal–Wallis test).

in postmenopausal women ($p = 0.007$), especially in aged women (≥ 65 years, $p < 0.0001$), but not in all premenopausal or younger postmenopausal women.

TABLE 3. BMD OF EACH HAPLOTYPE OF G-395A AND C1818T SNPs IN THE WHITE POPULATION

Haplotype	Postmenopausal		
	All ($n = 1187$)	≤ 54 years ($n = 112$)	≥ 65 years ($n = 55$)
H1 (– –)	1.138 \pm 0.002 (301)	1.139 \pm 0.009(21)	1.052 \pm 0.010(15)
H2 (– +)	1.135 \pm 0.003 (423)	1.112 \pm 0.009(49)	1.009 \pm 0.014(18)
H3 (+ –)	1.138 \pm 0.006 (82)	1.101 \pm 0.014 (9)	0.985 \pm 0.020 (4)
H4 (+ +)	1.142 \pm 0.006 (381)	1.111 \pm 0.014(33)	0.944 \pm 0.015(18)
p Value	0.644	0.066	<0.0001

(–) Denotes women without the minor allele (G/G for C1818T) and (+) denotes those with the minor allele (G/A or A/A for G-395A, and C/T or T/T for C1818T). Data are means \pm SEM for the number of women in the parenthesis. p Values were determined by nonparametric analysis (Kruskal–Wallis).

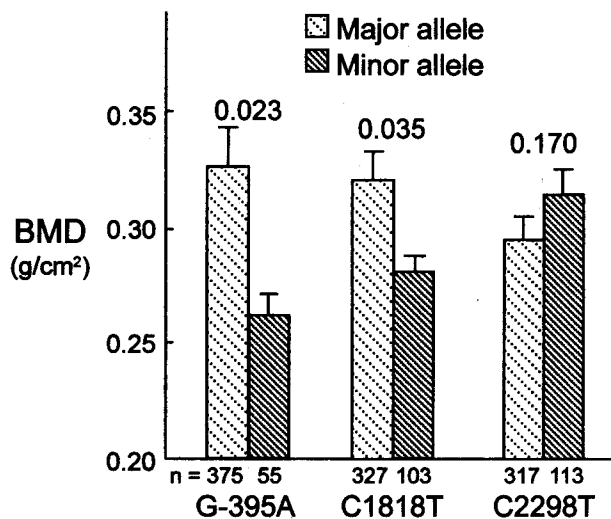


FIG. 2. Association of the three common SNPs with BMD in Japanese postmenopausal women. BMD of the distal radius was compared between the major and minor alleles in Japanese postmenopausal women ($n = 215, 430$ alleles, all >65 years old). Data are expressed as means (bars) \pm SEMs (error bars) for the number of alleles shown under each bar. The number above the bars is the p value of the difference in the mean BMD.

Association of the common polymorphisms with BMD in Japanese postmenopausal women

To examine if the association between the SNPs and BMD observed in white women would extend to other genetically distinct populations, we carried out similar analyses in Japanese postmenopausal women ($n = 215, 430$ alleles, all >65 years old). In this population we also observed significant association of allele types of G-395A and C1818T SNPs with bone density ($p = 0.023$ and 0.035 , respectively; Fig. 2). Again, these results were unchanged in the analysis of the Z score ($p = 0.013$ and 0.031 , respectively), and haplotypic analysis of these two SNPs also revealed significant association with BMD in this population ($p = 0.009$; data not shown).

Functional relevance of the G-395A polymorphism

We further explored the possible functional relevance of the SNPs that were associated with bone density of postmenopausal women. The C1818T SNP was not likely to affect the function of the KL protein directly because it was not accompanied by amino acid substitution; however, the G-395A SNP located at the promoter region possibly may be related to its function. To investigate the effect of the G->A substitution, we used electrophoretic mobility shift analysis to assess the DNA-binding activity (Fig. 3). Synthetic allele-specific oligonucleotides representing the G-395A site were incubated with nuclear protein extracts from human embryonal kidney 293 cells that were confirmed to express *klotho* by RT-PCR. Differential binding patterns were detected between the G- and A-bearing alleles. Amount of DNA-protein complex formed by the

G-bearing allele was greater than that by the A-bearing allele (lanes 1 and 2). Cold competition with various concentrations of unlabeled probes dose dependently decreased the formation of the complex (lanes 3–12), and the 100-fold excess of the competitor abrogated it (lanes 3 and 4). In each concentration of cold competitors, the competition was stronger by the G-bearing allele than that by the A-bearing allele (lanes 5–12). These results indicate that the binding of one or more proteins (presumably transcription factors) in the complex is impaired by the G->A substitution of the promoter region, and this may change the expression of the *klotho* gene.

DISCUSSION

Based on the finding that the *klotho*-deficient mouse exhibits multiple aging phenotypes,^(22,23) this study provides the first evidence that the *klotho* gene may be involved in pathophysiology of a common age-related disorder of humans, osteoporosis. The *klotho* gene polymorphisms were correlated with bone density in postmenopausal women in two genetically distinct racial groups. There are three major factors that determine bone density in women: the peak bone mass in adolescence, a rapid bone loss after menopause as a result of estrogen withdrawal, and a gradual age-related bone loss thereafter.^(1–3) This study therefore classified the white women into subgroups by menopausal status and age. The *klotho* polymorphisms were not associated with either of these factors; however, they showed much stronger association with bone density of aged postmenopausal women than that of premenopausal women or younger postmenopausal women. This indicates that the *klotho* gene may be involved in the pathophysiology of bone loss by aging rather than in peak bone mass or menopausal bone loss.

Because osteopenia observed in the *klotho*-deficient mouse was seen more predominantly in the cortical bone rather than in the cancellous bone,⁽²³⁾ in this study we measured BMDs of the whole body and the distal radius, which are reported to be better indicators of cortical bone density than that of the spine or the hip.⁽²⁷⁾ Although a significant correlation of these two BMDs is described in the manufacturer's data of DTX-200, the associations of G-398A and C1818T SNPs with bone density of aged women (>65 years old) were stronger in the white population than in the Japanese population. This may be because of not only the difference in genetic background between the two races, but also the difference in the sites where BMD was measured: the whole body for the white population and the distal radius for the Japanese population. Previous reports strongly suggest that the pleiotropic *klotho* gene functions are mediated by unknown humoral factors or by the KL protein itself functioning as a circulating "antiaging" hormone.^(22–24,28) Thus, it is possible that the bone metabolism of the whole body might be affected more strongly by alterations of the *klotho* gene than specific bone site.

To study the functional relevance of the SNPs, we performed electrophoretic mobility shift assay using cultured human embryonal kidney 293 cells. This is because the

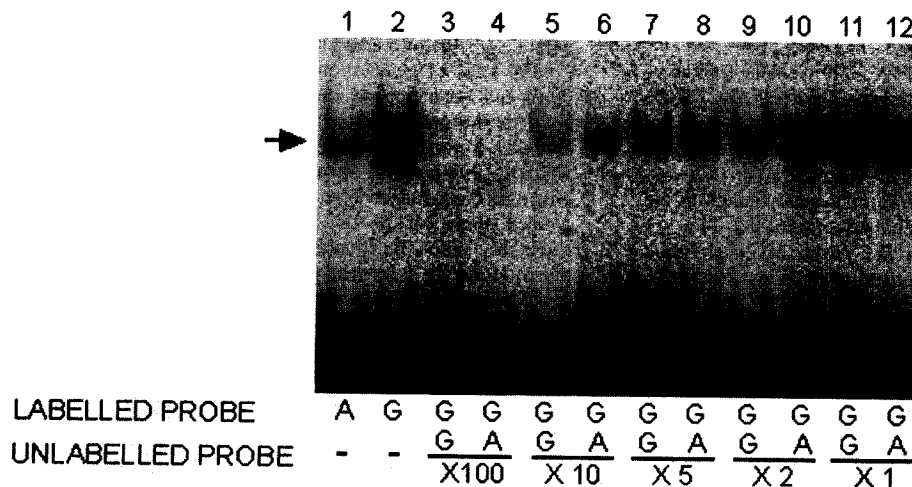


FIG. 3. Differential binding patterns between G- and A-bearing alleles of the *klotho* promoter. Synthetic allele-specific oligonucleotides representing the polymorphic G-395A site were incubated with nuclear extracts from cultured human embryonal kidney 293 cells. Lanes 1 and 2 show the DNA-protein complex formed by labeled probes with A- and G-bearing alleles. Lanes 3–12 show cold competition with various concentrations of unlabeled probes with G- and A-bearing alleles (X1–X100 of the labeled probe) against the complex formation by the labeled probe with G-bearing alleles.

klotho gene is known to be expressed most predominantly in the kidney but not in bone or bone marrow, in mice and humans.^(22–24) In fact, we confirmed the expression of the *klotho* in 293 cells. Consequently, it was indicated that some transcription factors, coactivators, or co-repressors bound to the sequence including the polymorphic site in the promoter region (G-395A) and the substitution affected its binding affinity. Sequence analysis of the 5' flanking region revealed that there was no typical TATA box, but there were five potential binding sites for Sp1 that are known to be found often in TATA-less promoter.⁽²⁴⁾ DNA sequences around the G-395A site are highly conserved with those of murine *klotho* gene (> 70%), and this site is located close to Sp1. It is interesting to note here that the polymorphism in the collagen I α 1 gene associated with low bone density is also located at the Sp1 binding site.^(14,15) However, our functional study using 415 bp of the human *klotho* promoter construct containing the G-395A site ligated to the luciferase reporter gene failed to show a significant difference of the reporter activity by the G/A substitution in transfected 293 cells (data not shown). This discrepancy might be because there are other important elements than the G-395A site in the promoter region that regulate the *klotho* gene transcription. Another possibility might be that the expression of the transcription factors/cofactors in the 293 cells was sufficient for the binding of the *klotho* promoter but might be insufficient for the activation of exogenously transfected promoter construct.

C1818T, the SNP in exon 4 associated with bone density, was a variation that caused no amino acid substitution. Although several reports have suggested the possibility that a silent mutation in an exon may yield an alternative transcript with abnormal function or affect the expression level of the product,^(29,30) our preliminary analysis has so far failed to detect splicing variants of the *klotho* transcript by RT-PCR using human kidney samples obtained from 18 renal disease patients (data not shown). Another possibility is that the association of C1818T may be linked physically to an SNP that could influence the function of the *klotho* gene. Because C2298T that is located downstream of C1818T did not exhibit any association with BMD, the

functional variant might possibly be located upstream of C1818T.

Among identified SNPs, three of them, one in the white population (G1110C) and two in the Japanese population (A44C and C234G), resulted in amino acid substitutions that might affect the structure of the protein. Recently, the G1110C was identified by another group as a functional variant that contributes to the longevity of humans⁽³¹⁾; however, our association study failed to show significant associations between G1110C and bone density in any subpopulation (all $p > 0.05$, data not shown). In addition, the allelic frequency of the minor C allele was not significantly different among subpopulations classified by ages in the white postmenopausal women. This discrepancy might possibly be caused by the difference of races. Neither A44C nor C234G was applicable for the association study because of the shortage of the number of patients with the minor allele in the Japanese population.

Aging is a common and potent risk factor in all age-related disorders in humans, and for the first time this study indicated the involvement of an aging-related gene *klotho* in the pathophysiology of a major age-related disorder, osteoporosis. The SNPs identified in this report will be useful for testing the association between *klotho* and other age-related diseases. We propose that further studies on the function of the *klotho* gene will provide new insight into the understanding of molecular mechanisms of age-related disorders.

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