cations or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary disease, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal disease or collagen disease. Individuals under medical treatments with oestrogen, bisphosphonates, calcitonin, vitamin D, or other drugs at the time of examination were excluded from participation in this study.

2.2. Measurement and criteria of BMD

The BMD of radial bone (expressed in $g\,cm^{-2}$) of each participant was measured by dual energy X-ray absorptiometry (DPX-L, Lunar Co., Madison, WI, USA). This parameter was recorded as adjusted BMD, in order to correct for differences in age, height and weight. The formulae were as follows: body mass index (BMI) = (body weight) (kg)/(body height)^2 (m), adjusted BMD (ADJBMD) = BMD − 0.0052432908 × (73.1716102 − age) + 0.0088382998 × (23.2271299 − BMI) (Kleinbaum, Kupper and Muller 1988). We have previously established the regression constants for the adjusted BMD formula in a large panel of individuals from the Japanese population. These have been used in numerous previous studies under the guideline of Japanese Society of Bone and Mineral Metabolism (Sano *et al.* 1995, Miyao *et al.* 1998, 2000, Hosoi *et al.* 1999, Ogawa *et al.* 1999, Tsukamoto *et al.* 1999, Ota *et al.* 2001). The individuals in the present study consist of the same genetic background, i.e. all participants belonged to the Japanese population. Although some researchers measure BMD in the lumbar spine, we measure it in the radius according to the Guideline for Osteoporosis Screening in a health check-up programme conducted by the Ministry of Health and Welfare of Japan (Orimo, Sugioka, Fukunaga *et al.* 1996). This method is recommended for measuring BMD in elderly females, in whom osteoporosis is often accompanied by osteoarthritis of the spine.

2.3. Genotyping for molecular variants in the TNFα *gene*

Polymorphisms in the promoter region of *TNFα* were genotyped by single-strand conformational polymorphism (SSCP) analysis performed on PCR-amplified segments (Tsukamoto, Haruta, Shiba *et al.* 1998, Ota *et al.* 2001). Variant results were confirmed by direct sequencing as described previously (Hopkins, Wu, Stephenson *et al.* 1999). The DNA segment containing the SNP site at −1031 was amplified according to procedures described previously (Ota *et al.* 2001); using primers TNFα-CF (5′-CAATGGGTAGGAGAATGTCC-3′)/TNFα-CR (5′-AACTCTGGGGACCCTGATTT-3′). DNA segment containing the SNP sites at −863 and −857 were amplified using primers TNFα-CTF (5′-AAGATATGGCCA-CACACTGG-3′)/TNFα-CTR (5′-TGAAATCACCCCCGGGAATT-3′). SSCP analysis was carried out according to procedures described elsewhere (Hopkins *et al.* 1999). Briefly, each PCR product was mixed with loading buffer, heated rapidly, cooled on ice, and applied to a 5% polyacrylamide gel containing 5% glycerol in 0.5X TBE buffer. Electrophoresis was performed under two different conditions: 150 V for 16 h at room temperature, and 240 V for 16 h at 4°C. Each variant PCR product was sequenced using the ABI 377 system with 3.2 pmol of the appropriate primer and 0.1 pmol/μl of PCR product. Cycle sequencing conditions were 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, for 25 cycles.

2.4. Statistical analysis

Adjusted BMD values between individuals having different *TNFα* genotypes were compared using a non-parametrical (Student–Newman–Keuls) analysis, according

to previously published similar works (Sano *et al.* 1995, Miyao *et al.* 1998, 2000, Hosoi *et al.* 1999, Ogawa *et al.* 1999, Tsukamoto *et al.* 1999, Ota *et al.* 2001). Differences in means were considered statistically significant for p values <0.05. Before testing differences in BMD, values were adjusted to ADJBMD (adjusted BMD) as described above.

3. Results

We examined three molecular variants of the *TNFα* gene that were described recently in Japanese alleles by Higuchi *et al.*, for association with BMD in 390 postmenopausal Japanese women living in northern Japan. Minor-allele frequencies for these three SNPs (−1031C, −863A and −857T) in this population were 0.16, 0.13 and 0.20, respectively. As the −863 C/A alleles were in complete disequilibrium with the T/C variation at −1031 in this population, we regarded them as haplotypes. Association of SNP variations at both −308 and −238 was not included in this study, because the frequencies of minor alleles of both SNPs in this Japanese population were less than 0.017; allelic association could not be contemplated as over 98% of population was homozygous for the major allele. Mean BMD values in our panel of women, classified by their *TNFα* genotypes, are presented in table 1.

Among the three SNPs examined, we observed a significant correlation between the presence of a T allele at nt −1031 and decreased BMD, by analysis of variance. BMD values were significantly higher in the T-negative genotype (C/C homozygotes; mean ± SD = $0.342 \pm 0.052\,g\,cm^{-2}$) compared to T-positive genotypes (T/T homozygotes, $0.309 \pm 0.062\,g\,cm^{-2}$; $p = 0.0253$ and T/C heterozygotes, $0.304 \pm 0.062\,g\,cm^{-2}$; $p = 0.0164$) (table 1). No significant differences in distributions of age, weight or height were observed among these genotypic groups (table 2).

We defined affected status for osteoporosis as a decrease of BMD below 70% of the mean in young adult women (cut-off value, 0.333 ± 2.6 SD), and unaffected status as a BMD above 80% of the mean in young adult women (cut-off value, 0.381 ± 1.8 SD), according to general criteria recommended by the Japanese Society for Bone and Mineral Research (Orimo *et al.* 1996). Of the 390 individuals

Table 1. Distribution of genotypes for TNFα polymorphisms and age-adjusted BMD.

Position	Nucleotide substitution (*X*/*x*)	Allele frequency		ADJBMD ($g\,cm^{-2}$)		
		X	*x*	*XX*	*Xx*	*xx*
−1031	T/C	0.836	0.164	0.309 ± 0.062 ($n = 281$)	0.304 ± 0.062 ($n = 90$)	0.342 ± 0.052 ($n = 19$)
−857	C/T	0.800	0.200	0.307 ± 0.062 ($n = 251$)	0.313 ± 0.058 ($n = 122$)	0.336 ± 0.082 ($n = 17$)

Values of ADJBMD ($g\,cm^{-2}$) shown are means ± SD.

Table 2. Clinical data for the 390 post-menopausal women studied for TNFα genotypes at position −1031.

	TT ($n = 281$)	TC ($n = 90$)	CC ($n = 19$)
Age (years)	73.054 ± 5.842	73.634 ± 5.735	72.511 ± 5.115
Weight (kg)	49.5 ± 8.6	49.4 ± 8.0	49.9 ± 8.9
Height (cm)	145.1 ± 6.0	144.5 ± 5.8	143.8 ± 7.9

in the present study, 168 and 72 were classified as unaffected and affected, respectively. Allelic association examined by comparing the frequency of each of TNFα alleles at −1031, −863 and −857 among affected and unaffected groups in our test population by chi-square test failed to reveal significant association between alleles of each polymorphism and affected state ($p > 0.05$). This suggests that no linkage disequilibrium exists between specific TNFα alleles and a causative mutation, although the numbers of affected individuals tested in the statistical analysis as mentioned above were small.

4. Discussion

A relationship between the immune system and bone resorption was recognized almost 40 years ago, with the first description of 'osteoclast activating factor' (OAF) and other molecules produced by cells of the immune system (Luben *et al.* 1974). It has gradually become clear since then that several molecular entities produced by cells of the immune system can affect differentiation and maturation of osteoblasts or osteoclasts *in vitro*. The most widely studied cytokines in that respect are interleukin-1 (IL-1), interleukin-6 (IL-6) and TNFα. TNFα, a multi-functional cytokine, is involved in tumour-induced bone resorption (Johnson, Boyce, Mundy *et al.* 1989, Mundy 1991) and non tumour-induced osteopenia (Bertolini, Nedwin, Bringman *et al.* 1986) and, like IL-1, it is secreted from mononuclear cells of postmenopausal women (Cohen-Solal, Graulet, Denne *et al.* 1993). TNFα is essential for regulating the immune response, haematopoiesis and bone resorption; in bone it stimulates formation of osteoclasts by inducing proliferation of osteoclast precursor cells or by activating differentiated osteoclasts (Pfeilschifter, Chenu, Bird *et al.* 1989), especially when triggered by oestrogen deficiency (Ralston, Russell and Gowen 1990).

We reported earlier that the *TNFα* locus was linked to osteoporosis by sib-pair linkage analysis and suggested that *TNFα* may be an important susceptibility gene for determination of BMD (Ota *et al.* 2000). The data presented in the present study suggest that variation or mutation in *TNFα* locus or unravelled adjacent locus in linkage disequilibrium may affect bone metabolism and eventually cause variation in BMD. However, since our study population is in a single ethnic group, further confirmation in different populations would merit to draw definitive conclusion regarding association between *TNFα* and BMD. TNFα synthesis is regulated at the transcriptional level (Beutler 1992). The well-characterized *TNFα* promoter contains a number of important regulatory elements that affect *TNFα* transcription in response to various stimuli (Economou 1989, Jongeneel 1995, Kroeger, Carville and Abraham 1997). Several polymorphisms have been reported in this region (Wilson, di Giovine, Blakenbore *et al.* 1992, Wilson, Symons, McDowell *et al.* 1997), and evidence exists that the region encompassing a polymorphic site at −308 has a role in the transcription of *TNFα* (Braun, Michel, Ernst *et al.* 1996, Wilson, Symons, McDowell *et al.* 1997). Genetic linkages between many autoimmune diseases and *TNFα* polymorphism are well characterized (Jacob, Froken, Lewis *et al.* 1990, Prociot, Briant, Jongeneel *et al.* 1993). For example, certain SNPs within the *TNFα* promoter are associated with a particular clinical subtype of systemic lupus erythematosus or with cerebral malaria, septic shock, rheumatoid arthritis, or preterm premature rupture (McGuire, Hill, Allsopp *et al.* 1994, D'Alfonso, Colombo, Della Bella *et al.* 1996, Kaijzel, van Krugten, Brinkman *et al.* 1998, Mira, Cariou, Grall *et al.* 1999, Roberts, Monozon-Bordonaba, Van Deerlin *et al.* 1999). However, allele frequencies of those polymorphisms are extremely low in the Japanese popula-

tion (Higuchi, Seki, Kamizono *et al.* 1998). Therefore in the present study we chose to examine variations known to be present in Japanese to elucidate the mechanism of osteoporosis, which is very common in Japan.

How sequence variation of the *TNFα* locus is related to elements responsible for controlling transcriptional activity in the promoter is not clear at present; some polymorphic sequences may bind to unknown genetic elements or alter the secondary structure of DNA that affects accession of *cis*-acting transcription factors to the promoter/enhancer regions of the *TNFα* gene. However, there are documented instances where nucleotide polymorphisms within the regulatory regions of cytokine genes have been associated with an altered rate of expression of the genes in question. For instance, a strong association exists between a certain minor allele of the *TNFα* promoter and a worse outcome from malaria or from mucocutaneous leishmaniasis (McGuire *et al.* 1994). That particular polymorphism is a stronger inducer of reporter-gene expression than the common allele.

In the present study, we detected significant association between a molecular variant in the regulatory region of the *TNFα* gene, a T/C substitution at position −1031, with BMD. These observations may provide some insight into a possible mechanism of genetic predisposition to osteoporosis. The data presented here imply that the −1031 variation in the *TNFα* promoter may affect bone metabolism and eventually cause variation in BMD. Further genetic studies in different populations, and functional studies of this *TNFα* variant, should clarify those issues in the near future.

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Zusammenfassung. *Hintergrund:* Der Tumor-Nekrose-Faktor Alpha (TNFα) wird als potentieller Osteoporose auslösender Faktor betrachtet, da er auf die Osteoklasten einen stimulierenden Effekt hat und in die Pathogenese des durch Östrogenmangel bedingten Knochenverlustes einbezogen ist. Kürzlich beschrieben wir einen genetischen Zusammenhang zwischen TNFα Locus und humaner Osteoporose mittels Geschwisterpaaranalyse. Jedoch bleibt der molekulare Mechanismus, durch welchen dieser Locus die Knochenmineraldichte (BMD) beeinflusst, unbekannt.
Ziel: Wir untersuchten, ob der beobachtete Zusammenhang auf einer Sequenzvariation beruht, welche die Expression des TNFα Gens, oder die Funktion des TNFα Proteins beeinflussen könnte.
Material und Methoden: Wir untersuchten drei Einzelnukleotid Polymorphismen (SNPs) des TNFα Gens in einer Gruppe von 390 japanischen Frauen in der Postmenopause aus Nordjapan. Die Minor-Allel Frequenzen für die drei SNPs (−103C, −863A und −857T) betrugen in dieser Population 0.16, 0.13 und 0.20.
Ergebnisse: Bei den drei untersuchten SNPs wurde mittels Varianzanalyse eine signifikante Korrelation zwischen dem Auftreten eines T Allels auf nt -1031 und verringerter BMD nachgewiesen. Unter den drei Genotypen auf nt -1031, waren die mittleren BMD-Werte im T-negativen Genotyp (C/C homozygot; Mittelwert $\pm$ SD = 0.342 ± 0.052 g cm^{-2}) signifikant höher als im T-positiven Genotyp (T/T homozygot, 0.309 ± 0.062 g cm^{-2}; p = 0.0253 und T/C heterozygot, 0.305 ± 0.062 g cm^{-2}; p = 0.0164).
Schlussfolgerungen: Aufgrund von unterschiedlichen genetischen Studien vermuten wir, dass TNFα eine Rolle in der Pathogenese der Osteoporose spielt.

Résumé. *Arrière plan*: Le facteur de nécrose tumorale alpha (TNFα) est maintenant connu comme facteur potentiel d'ostéoporose parce qu'il a un effet stimulant sur les lignées de cellules ostéoclastes et a été impliqué dans la pathogénie de la perte osseuse associée à la déficience en oestrogène. On a récemment décrit un linkage génétique entre le locus TNFα et l'ostéoporose humaine par analyse de paires de germains, cependant le mécanisme moléculaire par lequel ce locus régule la densité minérale osseuse (DMO) reste obscur.
But: Etudier si le linkage observé reflète une variation de séquence qui pourrait affecter l'expression du gène TNFα ou altérer la fonction de la protéine TNFα .
Sujets et méthodes: On a examiné trois polymorphismes d'un unique nucléotide du gène TNFα dans un groupe de 390 japonaise ménopausées, vivant dans le nord du Japon. Les fréquences de l'allèle mineur pour les trois SNPs (−1031C, −863A et −857T) dans cette population sont respectivement 0,16 0,13 et 0,20.
Résultats: On a seulement observé par analyse de variance, une corrélation significative entre la présence d'un allèle T à nt −1031 et une DMO diminuée dans les trois SNPs examinés. Dans les trois groupes génotypiques à nt −1031, les valeurs moyennes de DMO sont significativement plus élevées dans le génotype T-négatif (C/C homozygotes; moyenne $\pm$ ET = $0{,}342 \pm 0{,}052$ g cm^{-2}), comparés avec les génotypes T-positifs (T/T homozygotes, $0{,}309 \pm 0{,}062$ g cm^{-2}; p = 0,0253 et T/C hétérozygotes, $0{,}305 \pm 0{,}062$ g cm^{-2}; p = 0,0164).
Conclusions: Etant donné l'évidence provenant de plusieurs études génétiques, on suggère que *TNFα* puisse jouer un rôle dans la pathogénie de l'ostéoporose.

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ORIGINAL ARTICLE

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Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis

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Abstract Osteoprotegerin (OPG), a secreted glycoprotein and a member of the tumor necrosis factor receptor superfamily, is considered to play an important role in the regulation of bone resorption by modifying osteoclast differentiation. Overexpression of OPG in mice has been reported to result in osteopetrosis, whereas targeted disruption of OPG in mice has been associated with osteoporosis. Accordingly, *OPG* could be a strong candidate gene for susceptibility to human osteoporosis. Here, we analyzed whether *OPG* is involved in the etiology of osteoporosis using both linkage and association analyses. We recruited 164 sib pairs in Gunma prefecture, which is located in the central part of Honshu (mainland Japan), for a linkage study, and 394 postmenopausal women in Akita prefecture, which is in the northern part of Honshu, for an association study. We identified two microsatellite polymorphisms in the linkage study, and six single-nucleotide polymorphisms (SNPs) in the *OPG* region for the association study. Although, no evidence of significant linkage between *OPG* and osteoporosis was found, a possible association of one SNP, located in the promoter region of the gene, was identified. A haplotype analysis with the six SNPs revealed that four major haplotypes account for 71% of the alleles in the Japanese population.

Key words Osteoprotegerin · Osteoporosis · Single-nucleotide polymorphism · BMD · Sib pair analysis

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Introduction

Osteoporosis is a systemic skeletal disease characterized by excessive bone resorption, typically in association with postmenopausal estrogen deficiency, which leads to low bone mass and microarchitectural deterioration with a consequent increase in bone fragility and susceptibility to fracture. Bone mineral density (BMD) is a complex trait that is influenced by multiple genes and environmental factors. Genetic factors have been estimated by twin studies to account for up to 80% of the variance in BMD (Giguere and Rousseau 2000). A number of candidate genes have been analyzed for involvement in the etiology of osteoporosis. These include, for example, the vitamin D receptor (Morrison et al. 1994), type I collagen (Grant et al. 1996), estrogen receptor (Kobayashi et al. 1996), interleukin 6 (Ota et al. 1999), and calcitonin receptor genes (Taboulet et al. 1998). Genome-wide screening of 330 DNA markers with 149 members of seven large pedigrees has been performed and several possible loci identified (Devoto et al. 1998). However, the contribution of these genes to the etiology of osteoporosis is still controversial, possibly because of racial difference, type I error, or misgenotyping (Morrison et al. 1997).

Osteoprotegerin (OPG) is a secreted glycoprotein, which was independently identified by three laboratories (Simonet et al. 1997; Tsuda et al. 1997; Tan et al. 1997) and which is considered to be a member of the tumor necrosis factor receptor superfamily. Transgenic mice that overexpress OPG exhibit a generalized increase in bone density (Simonet et al. 1997). Two separate studies using OPG-deficient mice, which showed severe early onset of osteoporosis with increased osteoclast numbers, indicated that the function of OPG is to block osteoclast formation and bone resorption (Mizuno et al. 1998; Bucay et al. 1998). Consistent with this, OPG administration protected against the decrease in bone mass that occurs in ovariectomized rats, an animal model of postmenopausal osteoporosis (Simonet et al. 1997). From these findings, *OPG* appears to be one of the most attractive candidate genes responsible

for postmenopausal-type osteoporosis susceptibility. To investigate possible effects of genetic variations at the *OPG* loci, we performed a linkage study by a sib pair analysis, and an association study with postmenopausal women by identifying single-nucleotide polymorphisms (SNPs). A possible association with one of the studied SNPs located in the promoter region of the gene was detected: individuals with TT genotype in the osteoporosis group had significantly decreased bone mineral density (BMD) when compared with those with TC or CC genotypes.

Subjects and methods

Subjects

For the sib pair analysis, DNA samples were obtained from peripheral blood of 283 Japanese women from 131 families, comprising 164 sib pairs. To determine the frequency distribution of CA repeats in Gunma Prefecture 77 unrelated Japanese women were recruited and their DNA analyzed. All lived in the area, which is in the central part of Honshu (mainland Japan). Their ages ranged from 50 to 86 years old (mean 66.2 ± 7.1 years).

For the association study, DNA samples were extracted from peripheral blood of 394 postmenopausal Japanese women ranging in age from 66 to 92 (mean 73.2 ± 5.8 years) living in Akita prefecture, which is in the northern part of Honshu. No participant in either study group had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary disease, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal disease, or collagen disease, and none was receiving estrogen replacement therapy. To analyze the haplotype pattern of *OPG* in the Japanese population, 48 DNA samples from volunteers of both sexes ranging in age from 20 to 22, recruited in our medical school, were used. The ethics committee of the Asahikawa Medical College approved the protocol of this study. The nature, purpose, and potential risks of the study were carefully explained to all patients before they agreed to participate. All were volunteers and gave informed consent prior to the study.

Measurement of bone mineral density (BMD)

Instruments for BMD measurements were different between Gunma and Akita prefectures because we used the installed machine in each health check-up center. The BMD of the radial bone (expressed in g/cm^2) of each participant was measured by means of dual energy X-ray absorptiometry by using a DTX-200 (Osteometer MediTech, Hawthorne, CA, USA) instrument in Gunma, and with a DPX-L (Lunar Madison, WI, USA) instrument in Akita. In the sib pair linkage analysis, osteoporosis and osteopenia were defined as a decrease in BMD below 70% and 80%, respectively, of the mean in young adult women (cut-off values 0.333 = −2.6SD and 0.381 = −1.8SD), according to the general criteria recommended by the Japanese Society for Bone and Mineral Research (Orimo et al. 1996). On the other hand, in the association study with the Akita population, the analysis was performed on the quantitative phenotype, BMD. Consequently, factors (age, height, and weight) that would affect BMD values had to be adjusted. Thus, we used the following formula: body mass index (BMI) = body weight (kg)/body height^2 (m); adjusted BMD (adjBMD) = BMD − 0.0052 × (73.2 − age) + 0.0088 × (23.2 − BMI) (Ota et al. 2001).

Genotyping of microsatellite polymorphisms

A human genomic clone containing the *OPG* gene was identified by a P1-derived chromosome (PAC) Human Genomic polymerase chain reaction (PCR) Screening Kit (Incyte Genomics, Palo Alto, CA, USA), by using primer sequences derived from the 3′ portion of the gene. A fragment containing the CA repeat was identified by Southern blotting of PAC DNA digested by *Hae*III or *Sau*3AI with a $(GT)_{20}$ oligonucleotide probe, and then subcloned and sequenced. Two informative repeat sequences, named OPG1 and OPG2 and shown in Fig. 1, were identified. PCR primers were designed to flank the repeat sequences for the polymorphism analysis (Fig. 1). The PCR primers used were OPG1F (forward), 5′-GCACACACGCTCTGTTTC TC-3′; OPG1R (reverse), 5′-GGAGGGTGGTAACTTG GGAT-3′; OPG2F (forward), 5′-AGTCTGGGCAACA GAGCAAG-3′; and OPG2R (reverse), 5′-CTAGCCTGA TGAATTGTCATC-3′. Fluorescent-labeled primers were used for genotyping. PCR amplification was carried out as described below for genotyping except that the annealing temperature was 55°C. Electrophoresis was performed with an ABI 377 DNA sequencer; the data were extracted by using GeneScan Analysis software and analyzed by the Genotyper program (Applied Biosystems, Foster City, CA, USA).

a. OPG1

GCACACACGCTCTGTTTCTCTCTCTCTCTGTCTGTCTCTCT
CTCTGTGCATGTGAGTGCTTTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGGAATCAATATAGTAATAAGATATTTA
AAATTGTTAAATCCCAAGTTACCACCCTCC

b. OPG2

AGTCTGGGCAACAGAGCAAGATTTCATCACACACACACA
CACACACACACACACACACATTAGAAATGTGTACTTGGC
TTTGTTACCTATGGTATTAGTGCATCTATTGCATGGAAC
TTCCAAGCTACTCTGGTTGTGTTAAGCTCTTCATTGGGT
ACAGGTCACTAGTATTAAGTTCAGGTTATTCGGATGCAT
TCCACGGTAGTGATGACAATTCATCAGGCTAG

Fig. 1a,b. Nucleotide sequences of the polymorphic repeats and their flanking region in the osteoprotegerin (*OPG*) gene locus. Sequences used for forward and reverse primers are *underlined*. CA (or GT) repeats are shown in *bold*. a *OPG1*. b *OPG2*

Search for single-nucleotide polymorphisms (SNPs)

A total of 23 primer sets were designed to amplify 12kb of the *OPG* gene containing 1100 bases of the promoter region, all five exons and introns, and 840 base pairs of the 3′ flanking region. SNPs were detected by sequencing DNA samples from ten independent volunteers (20 alleles) on an ABI 310 sequencer (Applied Biosystems).

Genotyping of detected SNPs

Primers for each SNP were designed for the amplification refractory modification system (ARMS) technique (Newton et al. 1989). In all, six SNPs were analyzed: one in the 5′ untranslated region (5′UTR) (SNP1), one in exon 1 (SNP2), two in intron 2 (SNP3 and SNP4), and two in intron 3 (SNP5 and SNP6). ARMS primers were designed to amplify a region of about 250bp. Sequences of the primers used are as follows: OPG5UTRA (forward), 5′-GGCTGC GGAGACGCACCCGCA-3′; OPG5UTRC (forward), 5′-GGCTGCGGAGACGCACCCGCC-3′; OPG5UTRAS (reverse), 5′-AGCATGGCATAACTTGAAAGC-3′; OPGE1K (forward), 5′-CGGGGACCACAATGAAC TAG-3′; OPGE1N (forward), 5′-CGGGGACCACAATG AACTAC-3′; OPGE1AS (reverse), 5′-GCTGTCTTCCA TAAAGTCAGC-3′; OPGi21C (forward), 5′-ATGCTAG AGTTTTGTGCATC-3′; OPGi21T (forward), 5′-ATG CTAGAGTTTTGTGCATT-3′; OPGi21AS (reverse), 5′-TTTCCTTTCTGAGTTAGCAGG-3′; OPGi22C (forward), 5′-ACTAAATTGCTTGGTATTTGCC-3′; OPGi22T (forward), 5′-ACTAAATTGCTTGGTATTTG CT-3′; OPGi22AS (reverse), 5′-TACAAAATCGTACAA AGACGT-3′; OPGi31G (forward), 5′-TCTCCCCAAAC AGTTTTGCG-3′; OPGi31A (forward), 5′-TCTCCCCAA ACAGTTTTGCA-3′; OPGi31AS (reverse), 5′-GTGCA CAATAAATGAAAAAAAGT-3′; OPGi32T (forward), 5′-CAGTTCCAGCATTGTTTAAT-3′; OPGi32C (forward), 5′-CAGTTCCAGCATTGTTTAAC-3′; and OPGi32AS (reverse), 5′-CTACTACCTATATTCATCT GA-3′. To confirm the reaction, a part of the β-globin gene was amplified as well, as a positive control. The PCR primers used were BGLOS (forward), 5′-ACACAACTGTG TTCACTAG-3′ and BGLOAS (reverse), 5′-CATGAGC CTTCACCTTAGGG-3′, which amplified a 360-bp region. After amplification, 3% agarose gel or 12% acrylamide gel electrophoresis were performed for genotyping. For some of the SNPs, the PCR products were sequenced to confirm the results obtained by the ARMS method. The PCR primers for sequencing were as follows: for SNP1 and SNP2, OPGSNP1F (forward), 5′-GCTCTCCCAGGGGACAGA CA-3′ and OPGSNP1R (reverse), 5′-AGACCAGGTGGC AGCAGCCT-3′; for SNP3 and SNP4, OPGSNP2F (forward), 5′-TAGCGTCTTTAGTTGTGGACT-3′ and OPGSNP2R (reverse), 5′-CCGGAACATATGTTGTCG TG-3′; and for SNP5 and SNP6, OPGSNP3F (forward), 5′-GTGTTAAGCTCTTCATTGGGTA-3′ and OPGSNP3R (reverse), 5′-AAATGGGAGTAATGGGTGTTTG-3′. PCR was performed in a volume of 12.5μl containing 20ng genomic DNA, 10mM Tris HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 200μM deoxyribonucleotide triphosphates (dNTPs), 10pmol of each primer, and 0.25 units of *Taq* polymerase. PCR amplification was performed with 30 cycles of 94°C for 30s, 49°–62°C for 30s, and 72°C for 30s, depending on the region analyzed, with a final extension step of 5min at 72°C in a Gene Amp PCR9600 System (Applied Biosystems). The amplified mixture was electophoresed in 1.5% agarose gel to isolate the fragment containing the PCR product. After that, the PCR product was extracted by using a Geneclean III Kit (Bio 101, Vista, CA, USA). The sequencing reaction and electrophoresis were performed with a BigDye Terminator kit (Applied Biosystems) following the manufacturer's protocol.

Statistical analysis

In an analysis using osteoporosis and osteopenia as the affected status, we analyzed three classes of sib pairs: (1) both sib unaffected (clinically concordant unaffected sib pairs); (2) one sib affected and the other not (clinically discordant sib pairs); and (3) both sibs affected (clinically concordant affected sib pairs). A nonparametric linkage analysis was performed by using the SIBPAL program (version 2.7) of the SAGE package (Case Western Reserve University, Cleveland, OH, USA). A significant increase in allele sharing (>0.5) for concordant pairs and/or a significant decrease in allele sharing (<0.5) for discordant pairs was considered evidence for linkage. Here, the term "allele sharing" means the proportion of shared alleles in a sib; thus, the value ranges from 0 to 1 and the expected value is 0.5.

For the association analysis, we compared BMD and adjBMD as a quantitative phenotype between genotype groups (TT vs. TC+CC and TT+TC vs. CC in SNP1; GG vs. GC+CC and GG+GC vs. CC in SNP2) by using both *t*-test (parametric) and the Mann-Whitney U test (nonparametric) from the statistical analysis system package (SAS Institute, Cary, NC, USA). A P value of < 0.05 was considered statistically significant.

Haplotype analysis

Haplotype frequency was estimated by the maximum-likelihood method by using a simplified version of the computer program GENEF (J-M Lalouel, University of Utah, unpublished). Procedures to generate the haplotype, are described in detail in Jeunemaitre et al. (1997). Briefly, two SNPs were chosen to generate a haplotype, followed by sequential inclusion of one SNP at a time. All haplotypes below a frequency of 1/4N, where N is the sample size, were automatically eliminated.

The strength of linkage disequilibrium (LD) was calculated by using Arlequin software for population genetic data analysis (http://anthropologie.unige.ch/arlequin). Pairwise LD was estimated as $D = x_{ij}-p_ip_j$, where x_{ij} is the frequency of haplotype A_iB_i, and p_i and p_2 are the frequencies of alleles A_i and B_i at loci A and B, respectively. A

standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where q_1 and q_2 are the frequencies of the other alleles at loci A and B, respectively (Hill and Robertson 1968). Lewontin's coefficient D′ is given by D/D_{max}, where $D_{max} = \min[p_1p_2, q_1q_2]$ when $D < 0$ and $D_{max} = \min[q_1p_2, p_1q_2]$ when $D > 0$ (Lewontin 1984). An appropriate LD measure for association studies, d, is given by $d = D/p_1q_2$, where p_1 is the variant frequency and p_2 is the marker allele frequency (Kruglyak 1999).

Results

Sib pair linkage analysis

The frequency distribution of two microsatellite polymorphisms, OPG1 and OPG2, in the Gunma area was determined by using a randomly selected group of 77 unrelated Japanese women (Table 1). The polymorphic PCR products of OPG1 and OPG2 contained 16–23 and 15–21 repeats, respectively. The frequency of heterozygotes was calculated as 80.1% for OPG1 and 53.7% for OPG2. The total number of successfully genotyped sib pairs for the linkage analysis was 164 for OPG1 and 153 for OPG2. The results of the analysis for osteoporosis and osteopenia are shown in Table 2. No significant linkage of OPG1 or OPG2 to either osteoporosis or osteopenia was observed.

Table 1. Frequency distribution of CA repeat alleles in the *OPG* gene locus among 77 Japanese women

	OPG1		OPG2	
Alleles	Repeat no.	Freq.	Repeat no.	Freq.
A1	16	0.0001	15	0.0001
A2	17	0.2208	16	0.6474
A3	18	0.2662	17	0.1282
A4	19	0.2078	18	0.0641
A5	20	0.1169	19	0.1538
A6	21	0.1429	20	0.0000
A7	23	0.0455	21	0.0064

OPG, osteoprotegerin

Association analysis

A total of six SNPs (SNP1 to SNP6, see Subjects and methods) were detected in the *OPG* gene locus. The observed number of genotypes in each SNP site did not differ significantly from what would be expected from the Hardy-Weinberg equilibrium. Two SNPs (SNP1 and SNP2) out of six, located in the 5′-UTR and in exon 1, appeared to be more interesting in terms of OPG function. SNP1, either T or C at 223 bp upstream from the translation initiation site, could have some influence on promoter activity. SNP2, either G or C at the 9th base of the signal peptide coding sequence, changed the third amino acid from lysine to asparagine. Because of their possible role in OPG function and the limited amount of available DNA, we decided to genotype these two SNPs for the 394 samples from postmenopausal women of Akita Prefecture. Age, height, weight, BMI, BMD, and adjusted BMD in each genotype group are shown in Table 3 as mean ± SD. The analysis was performed with BMD and adjBMD as a quantitative phenotype between genotype groups (see Subjects and methods). In SNP1, individuals with the TT genotype showed significantly low BMD and adjBMD when compared with those of the TC or CC genotypes (Table 4) ($P = 0.028$ in BMD and $P = 0.021$ in adjBMD, by the Mann-Whitney U-test). Because our samples showed a nearly normal distribution, a *t*-test was also used, and it also indicated a significant difference ($P = 0.023$). This result may indicate that the allele with C in SNP1 has a protective effect with respect to osteoporosis. On the other hand, in SNP2, no significant result was obtained ($P = 0.561$ for BMD and $P = 0.369$ for adjBMD by the Mann-Whitney U-test; $P = 0.242$ by the *t*-test when GG was compared with GC+CC).

Table 2. Sib pair linkage

Locus	Status	Pairs	Mean	SD	SE	*t*-values	*P*-values
Osteoporosis							
OPG1	0	82	0.517	0.262	0.029	0.591	0.28
	1	49	0.470	0.259	0.037	0.807	0.21
	2	33	0.499	0.277	0.048	−0.020	0.51
OPG2	0	77	0.492	0.205	0.023	−0.324	0.63
	1	45	0.448	0.221	0.033	1.567	0.06
	2	31	0.447	0.185	0.033	−1.593	0.94
Osteopenia							
OPG1	0	44	0.516	0.252	0.038	0.425	0.34
	1	59	0.491	0.271	0.035	0.244	0.40
	2	61	0.495	0.268	0.034	−0.140	0.56
OPG2	0	41	0.483	0.216	0.034	−0.501	0.69
	1	57	0.492	0.200	0.026	0.313	0.38
	2	55	0.438	0.205	0.028	−2.231	0.99

Status: 0, concordant unaffected pairs; 1, discordant pairs; 2, concordant affected pairs. Criteria for diagnosis were bone mineral density (BMD) <70% (osteoporosis) and <80% (osteopenia) of the mean among young adult females. Mean is the average value of allele sharing. SD and SE are standard deviation and standard error, respectively, of the value of allele sharing

Table 3. Polymorphic status and clinical characteristics

	SNP1			SNP2		
	Genotype TT	TC	CC	Genotype GG	GC	CC
n	171	168	55	203	159	32
Age (years)	73.2 ± 6.1	73.1 ± 5.7	73.1 ± 5.9	73.0 ± 6.1	73.1 ± 5.7	74.2 ± 5.2
Height (cm)	144.7 ± 6.1	144.6 ± 5.9	145.4 ± 6.4	144.6 ± 6.2	144.4 ± 5.8	145.5 ± 6.0
Weight (kg)	49.1 ± 8.0	49.0 ± 8.2	50.3 ± 8.7	49.4 ± 8.0	48.9 ± 8.6	50.0 ± 7.7
BMI	23.4 ± 3.4	23.0 ± 3.2	23.4 ± 3.4	23.2 ± 3.4	23.0 ± 3.3	23.3 ± 3.5
BMD (g/cm^2)	0.296 ± 0.075	0.309 ± 0.075	0.314 ± 0.070	0.303 ± 0.074	0.307 ± 0.077	0.297 ± 0.059
adjBMD (g/cm^2)	0.297 ± 0.060	0.311 ± 0.058	0.312 ± 0.060	0.303 ± 0.058	0.308 ± 0.061	0.302 ± 0.060

BMI, body mass index; adjBMD, adjusted bone mineral density; SNP, single-nucleotide polymorphism

Table 4. Comparison of adjBMD between SNP1 genotype groups

	SNP1 genotype	
	TT	TC+CC
n	171	223
Age (years)	73.2 ± 6.1	73.1 ± 5.7
Height (cm)	144.7 ± 6.1	144.8 ± 6.0
Weight (kg)	49.1 ± 8.0	49.3 ± 8.3
BMI	23.4 ± 3.4	23.4 ± 3.3
BMD (g/cm^2)	0.296 ± 0.075*	0.310 ± 0.074
adjBMD (g/cm^2)	0.297 ± 0.060**	0.311 ± 0.059

Values are mean ± SD
* $P = 0.028$, ** $P = 0.021$ (Mann-Whitney U test, TT versus TC+CC)

Haplotype analysis

In order to find the OPG susceptibility haplotype for osteoporosis or other diseases in the future, a haplotype analysis was performed with all six SNPs, based on 37–48 Japanese DNA samples. Unfortunately, the amount of DNA collected from the 394 postmenopausal women of Akita Prefecture was not enough for genotyping these four additional SNPs. Thus, a haplotype association study could not be performed in the present study. Genotype and allele frequencies of these SNPs are shown in Table 5. As shown in Table 6, a strong linkage disequilibrium of variable degree was observed among these SNPs. Haplotype construction with these SNPs revealed that four major haplotypes accounted for 71% of the population (Table 7).

Discussion

In the current study, we investigated the role of OPG in the pathogenesis of osteoporosis by both linkage and association analyses. In the sib pair linkage analysis, two disease criteria (osteoporosis and osteopenia) were used to classify sib pairs. As shown in Table 2, even including osteopenia, it was possible to recruit only 61 affected sib pairs for OPG1 and 55 for OPG2. If λ_s is 3.0, a power to detect an effect of about 80% can be attained with 100 affected pairs (Risch 1990). However, the role of OPG in the pathogenesis of osteoporosis or osteopenia is not estimated to be very strong (Giguere and Rousseau 2000). Thus, the negative result of the present linkage analysis may be because the sample size was too small for detection, even if OPG has some role in the pathogenesis of osteoporosis.

In the association study, we analyzed the quantitative phenotype itself, BMD and adjBMD, which is considered a more powerful technique than a comparison between a disease group and a control group classified according to a quantitative variable (Duggirala et al. 1997). When a group of TT genotype in SNP1 was compared with a group of TC or CC genotype, significantly lower BMD and adjBMD values were identified by both parametric and nonparametric tests (Table 4), with marginal *P* values. SNP1, a T to C change 233 bp upstream from the translation initiation site, was located in the promoter region. Thus, this polymorphism could derive its functional significance by altering the level of promoter activity. A promoter assay with a reporter gene fusion construct could clarify this point. SNP2 changed the third amino acid (lysine to asparagine) of the signal peptide, which is necessary for OPG to be secreted from the cell. Lysine is a basic amino acid, while asparagine is an uncharged polar amino acid. In angiotensinogen, another secreted protein, a basic amino acid in the signal peptide was shown to drastically affect secretory kinetics (Nakajima et al. 1999a). Therefore, although we could not detect a significant disease association with SNP2, the point mutation could also influence OPG's secretory kinetics.

An association study with a haplotype analysis is a powerful tool for determining a genetic contribution to a common disease. Our analysis of the *OPG* gene revealed that four major haplotypes account for 71% of the population as reflected by the considerable linkage disequilibrium among the six SNPs (Table 6). With this information, another association analysis could be performed in the future with a larger sample size to have a reasonable power to detect an effect. Moreover, our finding of the haplotype profile of the gene is useful information for the study of the role of OPG in other disease conditions. In fact, since OPG was identified as a novel secreted protein involved in the regulation of bone density in 1997 (Simonet et al. 1997), several findings suggesting the relevance of OPG to other conditions have been reported. First, OPG-deficient mice had arterial calcification in the large arteries by 2 weeks of age (Bucay et al. 1998), indicating a possible role of OPG in diseases showing

Table 5. Genotype and allele frequencies of all six SNPs

Polymorphism	Nucleotide position	Genotype			No.	Allele frequency	
SNP1	−223[a]	TT	TC	CC		T	C
		18	22	8	48	0.6	0.4
SNP2	+9[a]	GG	GC	CC		G	C
		27	17	4	48	0.74	0.26
SNP3	IVS2-749G>T[b]	TT	TG	GG		T	G
		35	10	1	46	0.87	0.13
SNP4	IVS2-5C>T[b]	CC	CT	TT		C	T
		29	8	0	37	0.89	0.11
SNP5	IVS3-1059G>A[b]	GG	GA	AA		G	A
		35	9	2	46	0.86	0.14
SNP6	IVS3-915T>C[b]	TT	TC	CC		T	C
		16	21	8	45	0.59	0.41

[a]Nucleotide position is identified from the translation initiation site
[b]SNPs designated according to the mutation nomenclature in den Dunnen and Antonarakis (2000)

Table 6. Strength of linkage disequilibrium

Polymorphism		SNP1	SNP2	SNP3	SNP4	SNP5
SNP2	D′	0.90	...	...	...	...
	r^2	0.42				
SNP3		−0.65	−0.64	...	...	...
		0.06	0.03			
SNP4		0.53	−1	−1	...	...
		0.05	0.05	0.03		
SNP5		−0.78	−1	−1	−0.21	...
		0.09	0.08	0.04	0	
SNP6		0.81	1	1	−0.03	−1
		0.30	0.24	0.13	0	0.33

Table 7. Haplotype analysis of the *OPG* gene

SNP1 T→C	SNP2 G→C	SNP3 T→G	SNP4 C→T	SNP5 G→A	SNP6 T→C	Frequency
C	C	T	C	G	T	0.249
T	G	T	C	G	C	0.174
T	G	T	C	A	C	0.160
T	G	G	C	G	T	0.123
T	G	T	C	G	T	0.084
C	G	T	T	G	T	0.069
C	G	T	C	G	T	0.039
T	G	T	T	G	C	0.032
C	G	G	C	G	T	0.023
C	G	T	T	A	C	0.016
C	G	T	C	G	C	0.015
T	C	G	C	G	T	0.015
					Total	1.000

arterial calcification. In addition, an association of serum OPG levels with diabetes and cardiovascular mortality suggests the possibility that OPG may be a cause of vascular calcification (Browner et al. 2001). Second, expression of OPG is not restricted to bone; it is expressed in a variety of tissues and cell systems, such as heart, lung, kidney, placenta, liver, thyroid gland, spinal cord, and brain. In addition, it is expressed in various immune and hematological tissues and mesenchymal organs (Hofbauer 1999). Third, a possible role of OPG in the immune system was shown in experiments with OPG-deficient mice by Yun et al. (2001), who reported that OPG regulates B cell maturation and development.

Ethnic differences in the genetic background of diseases are sometimes observed. For example, although Grant et al. (1996) reported the association of osteoporosis with a polymorphic SP1-binding site in the collagen type Iα1 gene, we could not find the polymorphism in a study of Japanese individuals (Nakajima et al. 1999b). Thus, similar OPG studies should be performed with samples from other ethnic groups because OPG might also be related to osteoporosis in populations other than Japanese.

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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 Iα1 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-

The authors have no conflict of interest.

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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, CCCCCAAGTCGGGGAAAGTTGGTC

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

C2298T—forward primer, CCTGCCCTTTCTCCCAAAA; reverse primer, AATCTCCAGAGCCGAAAATGG; FAM-labeled probe, CCAAAACTCTCTCAGCCACCTCTTTGT; TET-labeled probe, CCAAAACTCTCTCGGCCACCTCTT.

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 mM 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/cm^2) 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 (n = 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 (n = 115)	Promoter	−395 (G → A)	—	0.143
	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′-TCGACAAGTCGGGG/AAAAGTTGGTG-3′. Complementary strands were annealed by combining 200 pmol of each oligonucleotide and 36 μl of annealing buffer (10 mM of Tris-HCL, 1 mM of EDTA, and 0.1 M of NaCl, pH 8.0) in a 40-μl 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-μl volume containing 2.5 μg of nuclear extract, 1 μg of poly (dI-dC), 4 μl of 5× binding buffer (Boehringer Mannheim, Mannheim, Germany), and 5.0×10^5 cpm of [^{32}P]-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 popultion 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 ± 12.0 years, mean ± SD) and unrelated Japanese postmenopausal women (n = 215, 66–92 years, 72.9 ± 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 ± 8.6 years), definite postmenopausal women (n = 364, 48–72 years, 57.9 ± 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 ≤54 years, 55–64 years, and ≥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				BMI				BMI		
	Allelic frequency	G/G	G/A	A/A	Allelic frequency	C/C	C/T	T/T	Allelic frequency	C/C	C/T	T/T
White population												
All (n = 1187)	0.215	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)	0.225	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)	0.213	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)	0.204	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)	0.210	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)	0.236	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)	0.128	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

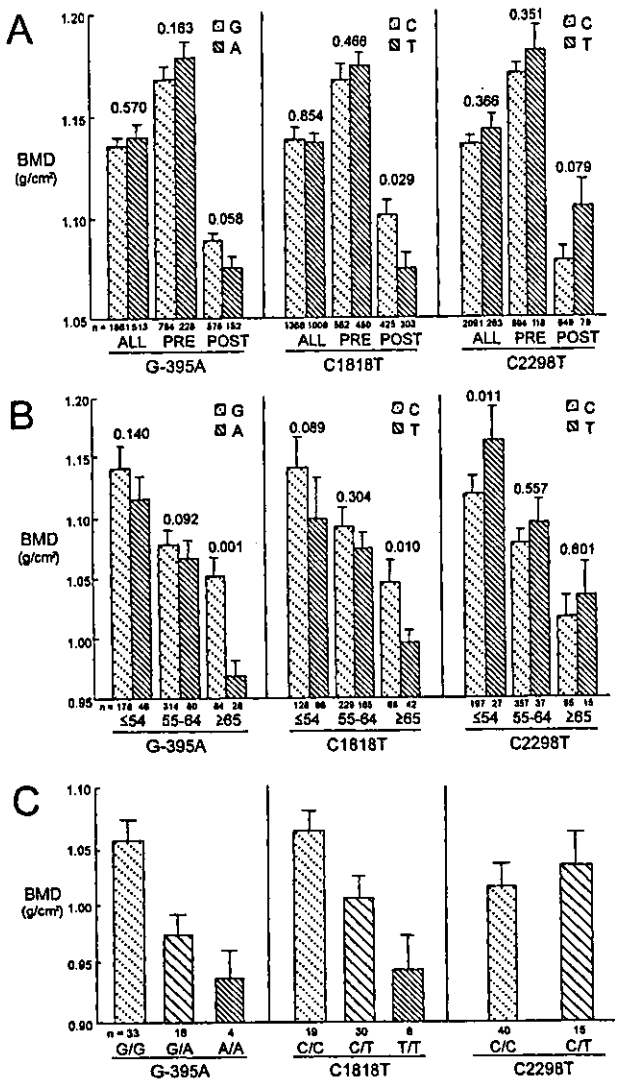

FIG. 1. Association of the three common SNPs with BMD in white women. BMD of the whole body was compared (A and B) between the major and minor alleles and (C) among genotypes. (A) Association of allele types with BMD in the three different subgroups classified according to their menopausal status: all 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) ± 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

		Premenopausal	*Postmenopausal*			
					55–64 years	
Haplotype	*All (n = 1187)*	*(n = 506)*	*All (n = 364)*	*≤54 years (n = 112)*	*(n = 197)*	*≥65 years (n = 55)*
H1 (− −)	1.138 ± 0.002 (301)	1.169 ± 0.003 (120)	1.113 ± 0.004 (87)	1.139 ± 0.009(21)	1.116 ± 0.006(51)	1.052 ± 0.010(15)
H2 (− +)	1.135 ± 0.003 (423)	1.170 ± 0.004 (181)	1.098 ± 0.006 (140)	1.112 ± 0.009(49)	1.115 ± 0.009(73)	1.009 ± 0.014(18)
H3 (+ −)	1.138 ± 0.006 (82)	1.173 ± 0.007 (30)	1.081 ± 0.010 (30)	1.101 ± 0.014 (9)	1.097 ± 0.014(17)	0.985 ± 0.020 (4)
H4 (+ +)	1.142 ± 0.006 (381)	1.186 ± 0.006 (175)	1.069 ± 0.012 (107)	1.111 ± 0.014(33)	1.080 ± 0.019(56)	0.944 ± 0.015(18)
p Value	0.644	0.140	0.007	0.066	0.054	<0.0001

(−) Denotes women without the minor allele (G/G for G-395A and C/C 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 ± SEM for the number of women in the parenthesis. p Values were determined by nonparametric analysis (Kruskal-Wallis).

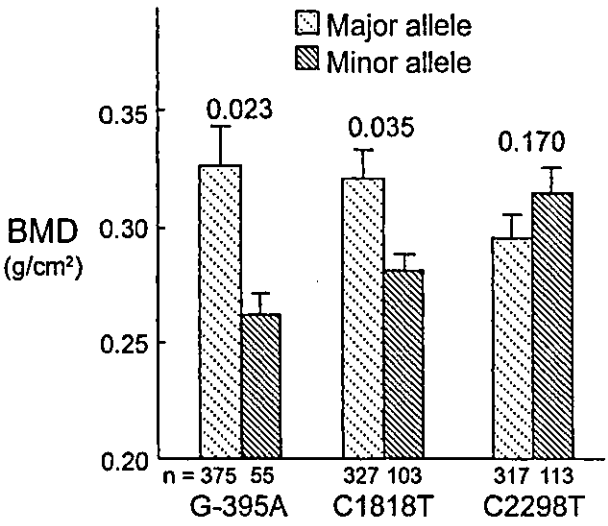

FIG. 2. Association of the three common SNPs with BMD in Japanese postmenopausal women. BMD of the distal radius was compared between the major and minor alleles in Japanese postmenopausal women (n = 215, 430 alleles, all >65 years old). Data are expressed as means (bars) ± SEMs (error bars) for the 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 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