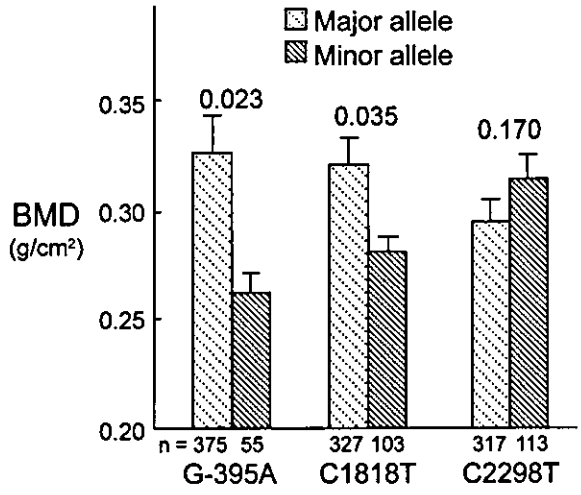

FIG. 2. Association of the three common SNPs with BMD in Japanese postmenopausal women. BMD of the distal radius was compared between the major and minor alleles in Japanese postmenopausal women (n = 215, 430 alleles, all >65 years old). Data are expressed as means (bars) ± SEMs (error bars) for the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

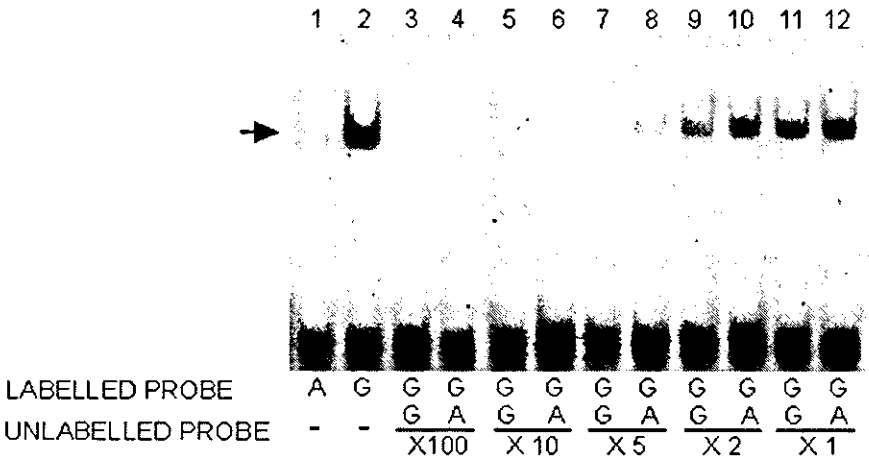

FIG. 3. Differential binding patterns between G- and A-bearing alleles of the *klotho* promoter. Synthetic allele-specific oligonucleotides representing the polymorphic G-395A site were incubated with nuclear extracts from cultured human embryonal kidney 293 cells. Lanes 1 and 2 show the DNA-protein 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 Iα1 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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ORIGINAL ARTICLE

Association of tumor necrosis factor receptor 1 gene polymorphism with bone mineral density

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Background: Estrogen deficiency in postmenopausal women causes an increased production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α. These cytokines are associated with an increase of bone turnover and an acceleration of bone loss. Tumor necrosis factor-α is known to promote osteoclastogenesis via TNFR1, one of the tumor necrosis factor receptors (TNFR). Therefore, the purpose of the present report was to investigate the association of *TNFR1* gene polymorphism with bone mineral density (BMD) in postmenopausal Japanese women.

Methods: The question of whether a polymorphism of the *TNFR1* gene would correlate with osteoporosis in 320 unrelated healthy postmenopausal women in Japan, was investigated. A single nucleotide polymorphism (SNP) located at Pro12 (CCA to CCG) in exon 1 of *TNFR1* was utilized.

Results: The subjects were categorized into three genotypes: AA, AG, and GG. The frequency of each genotype was 72.2%, 23.8%, and 4.0%, respectively. The association of this polymorphism with BMD of the lumbar spine and total body, and several bone metabolic markers was then examined. Concerning the *TNFR1* gene, the AA group had significantly low total body BMD, compared with the AG + GG group (Z score; 0.285 *vs* 0.568; $P = 0.03$), although BMD of the lumbar spine was not statistically different.

Conclusion: These results suggest an association between this SNP of the *TNFR1* gene and BMD, and an involvement of TNFR1 in postmenopausal osteoporosis among Japanese.

Keywords: bone mineral density, genetics, osteoporosis, polymorphism, tumor necrosis factor receptor 1.

Introduction

Osteoporosis is characterized by pathologically low bone mass and an increased risk of fracture.[1,2] Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors for fractures, and it has been suggested that low bone mass may be partly hereditary.[3,4] The report of Morrison *et al.* was the first to demonstrate an association of common allelic variants of the *vitamin D receptor* gene (*VDR*) with bone mineral density (BMD).[5] Following this study, several groups reported on the *VDR* gene polymorphism[6] and BMD, but its genetic implications are still controversial.[7,8]

Estrogen deficiency in postmenopausal women causes an increased production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis fac-

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tor (TNF)-α.[9] An increased production of proinflammatory cytokines is associated with an increase of bone turnover and an acceleration of bone loss, which leads to an increased susceptibility to fractures.[10] Thus, we investigated in this report the association with a *tumor necrosis factor receptor* (*TNFR*) gene polymorphism and BMD in postmenopausal Japanese women.

Tumor necrosis factor-α has a tumor necrosing activity.[11] It is secreted from macrophages and monocytes in several pathological conditions, such as systemic inflammation, malignant tumor, and autoimmune diseases.[12] Moreover, TNF-α regulates lipoprotein lipase (LPL) activity, and elevates the serum level of triglyceride.[13] The overproduction of TNF-α, not only from macrophages and monocytes, but also from adipocytes, induces so-called 'insulin resistance'.[14] Tumor necrosis factor receptors are members of transmembrane receptors, and are mediators of various TNF-α actions.[15,16] There are two subtypes of TNFR: TNFR1 and TNFR2.[17,18] Tumor necrosis factor receptor 1 is expressed constitutively in various cell lineages, including osteoblast-progenitor cells and osteoclasts, and is reported to suppress osteoblast formation and promote osteoclast formation.[19–22] In contrast, the roles of TNFR2 in the bone metabolism are still unclear. A *TNFR2* gene polymorphism is reported to be related to glucose metabolism, especially 'insulin resistance'.[23,24] With regards to the involvement of *TNFR2* gene polymorphism in the bone metabolism, it is reported that a polymorphism in the 3′ untranslated region (UTR) is associated with low BMD.[25] However, to our knowledge the association of *TNFR1* gene polymorphism with BMD has not been reported as yet. Therefore, we have studied the association of a single nucleotide polymorphism (SNP) of *TNFR1* gene[26] with BMD.

Methods

Subjects

Genotype analysis was performed using the blood samples obtained from 320 unrelated postmenopausal women (mean age ± SD: 64.9 ± 9.6 years) living in Nagano prefecture, Japan. Subjects who had endocrinological disorders (e.g. hyperthyroidism, hyperparathyroidism), diabetes mellitus, liver disease, renal disease, use of medications that were known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin), or unusual gynecologic history were excluded. Subjects who were affected by collagen diseases were also excluded. All volunteers were unrelated and gave informed consent before the study.

Genotyping

The DNA was extracted from white blood cells of peripheral blood.[27] The DNA fragment containing one base substitution (CCA to CCG) at Pro12 in exon 1 of *TNFR1* was amplified by polymerase chain reaction (PCR) using a Gene Amp PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA). The reaction was carried out in a final volume of 25 μL containing 100 ng of genomic DNA, 10 pmol of each primer, 200 mmol/L dNTP, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% gelatin and 0.1 U Taq DNA polymerase (Takara, Kyoto, Japan). The sense and antisense primers to detect TNFR1 gene polymorphism were 5′-GAGCCCAAATGGGGGAGTGAGAGG-3′ (TNFR1S) and 5′-ACCAGGCCCGGGCAGGAGAG-3′ (TNFR1AS),[26] respectively. Template DNA was amplified by 35 cycles by the following steps: denaturation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The PCR products were digested with MspA1I (Takara), which can recognize A to G substitution and electrophoresed in 2% agarose gel.

Clinical data

Bone turnover markers, pyridinoline (Pyr), deoxypyridinoline (Dpyr) in urine[28] and intact-osteocalcin (IOC) in serum,[29] in addition to parathyroid hormone (PTH), calcitonin, 1, 25(OH)2D3, calcium (Ca), phosphate (P) and alkaline phosphatase (AL-P), total cholesterol (TC), triglyceride (TG) were measured. Moreover, the BMD of the lumbar spine (L2–4) and total body was measured by dual-energy X-ray absorptiometry (DXA) using fast scan mode (DPX-L; Lunar, Madison, WI, USA). The Z score (average 0, SD 1) was calculated by the installed software of Lunar DPX-L based on the data from 20 000 Japanese women.[30]

Statistical analysis

Comparison of BMD in Z scores and biochemical markers between the groups of each allelic genotype were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied the Student's *t*-test, using the STATVIEW-J4.5 software (SAS Institute, Cary, NC, USA).

Results and discussion

After amplification of DNA fragments containing the CCA to CCG substitution at Pro12 in exon 1 of *TNFR1*, the PCR products were digested with MspA1I, which can recognize A to G substitution. The polymorphic MspA1I site was detected as 108-bp and 75-bp fragments. The subjects were categorized into three genotypes: AA, AG, and GG (Fig. 1).

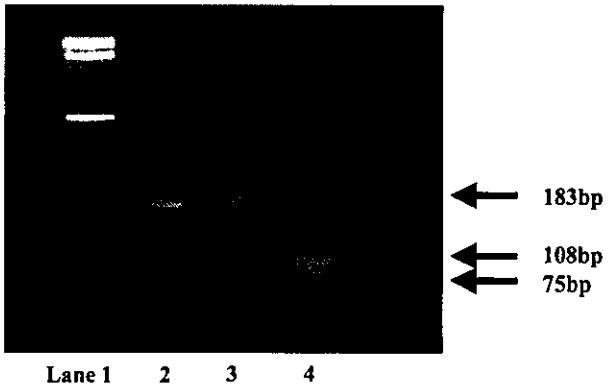

Figure 1 Electrophoresis of polymerase chain reaction (PCR) products revealing polymorphism in tumor necrosis factor receptor (TNFR) 1, after digestion of DNA with MspA1I. In recombinant DNA samples, a single 183-bp product was derived from AA homozygotes (lane 2); 183-, 108-, and 75-bp products were derived from AG heterozygotes (lane 3); and 108- and 75-bp products were derived from GG homozygotes (lane 4). Lane 1, molecular-weight marker.)

Frequencies of alleles in the *TNFR1* gene were 231 AA homozygotes, 76 AG heterozygotes and 13 GG homozygotes. The risk ratio of the Hardy–Weinberg formula was 0.0421 and slightly significant, possibly due to recruitment of subjects from a relatively small isolated area in Japan. We analyzed the correlation of the genotypes with BMD of the lumbar spine and total body between two groups of 231 AA homozygotes and 89 AG heterozygotes + GG homozygotes. As a result, the AA group had significantly lower total body BMD, compared with the AG + GG group. (Z score; 0.285 *vs* 0.568; $P = 0.03$) (Table 1). There was a high level of deoxypyridinoline (Dpyr) in the urine in the AA group (Dpyr; 7.60 *vs* 6.93; $P = 0.059$).

The cytokines, such as IL-1, IL-6, and TNF-α, are thought to be important promoters for bone loss in postmenopausal women. Fontova *et al.* reported the association of femoral BMD with *IL-1 receptor antagonist* (*IL-1ra*) and *TNF-α* gene polymorphism.[31] Genotype combination of A2 allele (A2+) of the *IL-1ra* gene and

Table 1 Comparison of Z scores of lumbar spine (L2–L4) and total body bone mineral density, background and biochemical data of the subjects between the groups of genotypes

Variable	Genotype		*P*
	AG + GG ($n = 89$)	AA ($n = 231$)	
Age (years)	64.7 ± 8.5	65.0 ± 10.0	NS
Body height (cm)	149.4 ± 6.1	150.8 ± 6.4	NS
Bodyweight (kg)	50.8 ± 8.1	51.1 ± 8.2	NS
Years after menopause	15.4 ± 8.4	15.6 ± 10.4	NS
BMI (kg/m^2)	22.5 ± 3.3	22.7 ± 3.1	NS
% fat	31.8 ± 8.4	32.4 ± 7.6	NS
Lumbar spine BMD (Z score)	0.203 ± 1.2	0.041 ± 1.45	NS
Total body BMD (Z score)	0.568 ± 0.98	0.285 ± 0.95	0.03
Serum			
Ca (mg/dL)	9.13 ± 0.41	9.15 ± 0.38	NS
P (mg/dL)	3.55 ± 0.44	3.48 ± 0.45	NS
AL-P (IU/l)	163.6 ± 49.4	175.7 ± 54.7	NS
I-OC (mg/dL)	7.35 ± 3.3	7.77 ± 4.0	NS
Intact PTH (pg/mL)	38.6 ± 14.2	37.3 ± 14.2	NS
Calcitonin (pg/mL)	22.6 ± 9.5	23.1 ± 9.9	NS
1, 25 (OH)2D3 (pg/mL)	34.9 ± 11.3	33.4 ± 12.7	NS
TC (mg/dL)	200.7 ± 33.1	196.7 ± 36.1	NS
TG (mg/dL)	160.4 ± 92.9	144.4 ± 78.6	NS
Urinary			
Pyr (pmol/μmol Cr)	32.2 ± 8.87	34.7 ± 11.4	0.09
Dpyr (pmol/μmol Cr)	6.93 ± 1.98	7.60 ± 2.82	0.06

NS, not significant; BMI, body mass index; BMD, bone mineral density; AL-P, alkaline phosphatase; I-OC, intact osteocalcine; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; Pyr, pyridinoline; Dpyr, deoxypyridinoline.
Values are given as mean ± SD.

GG allele of the *TNF-α* gene was associated with greater hip BMD at femoral neck and Ward triangle levels ($P = 0.02$). We focused on *TNFR* gene polymorphisms in the TNF-α signal in the present study. Here, we have noticed the influence of a polymorphism of the *TNFR1* gene on bone metabolism. The AA group of *TNFR1* polymorphism had significantly lower total body BMD, compared with the AG + GG group, although there was no significant difference in BMD of the lumbar spine between these two groups. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass. At present, two hypotheses could be proposed: (i) this silent polymorphism may be linked with other mutations in exons, which contributes to the change of the TNFR1 protein function or may be linked with a mutation in regulatory elements affecting the levels of expression through variable transcriptional regulation; or (ii) the polymorphism in the *TNFR1* gene may be linked with a mutation of another undefined gene adjacent to the *TNFR1* gene, which cause low BMD directly or indirectly. Our results suggest that TNFR1 may be involved in the bone metabolism among postmenopausal Japanese women, and that the SNP in the *TNFR1* gene is useful as a genetic marker for osteoporosis. In addition, the contribution of other genetic factors should be analyzed further by utilizing other candidate genes that are known to play important roles in the bone metabolism.

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Association of a Haplotype (196Phe/532Ser) in the Interleukin-1-Receptor-Associated Kinase (*IRAK1*) Gene With Low Radial Bone Mineral Density in Two Independent Populations

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ABSTRACT

Osteoporosis, a multifactorial common disease, is believed to result from the interplay of multiple environmental and genetic determinants, including factors that regulate bone mineral density. Interleukin-1 (IL-1) is one of the most potent bone-resorbing factors, and interleukin-1-associated kinase 1 (IRAK1) is an essential effector of the IL-1 receptor signaling cascade. In genetic studies of two independent populations of postmenopausal women (cohort A: 220 individuals and cohort T: 126 individuals) from separated geographical regions of Japan, we found that radial bone mineral density levels had similar associations with IRAK1 genotypes in both populations. Two amino acid–substituting variations in the gene, encoding Phe196Ser and Ser532Leu, were in complete linkage disequilibrium ($D' = 1.0000, r^2 = 1.0000, \chi^2 = 192.000, p = 1.2 \times 10^{-43}$), and we found two exclusive haplotypes (196F/532S, frequency 0.74; 196S/532L, frequency 0.26) of the *IRAK1* gene among our test subjects. In both populations, a significant association with decreased radial bone mineral density was identified with haplotype 196F/532S (in cohort A: $r = 0.21, p = 0.0017$; in cohort T: $r = 0.23, p = 0.011$). Radial bone mineral density was lowest among 196F/532S homozygotes, highest among 196S/532L homozygotes, and intermediate among heterozygotes. Accelerated bone loss also correlated with the 196F/532S haplotype in a 5-year follow-up. These results suggest that variation of *IRAK1* may be an important determinant of postmenopausal osteoporosis, in part through the mechanism of accelerated postmenopausal bone loss. (J Bone Miner Res 2003;18:419–423)

Key words: **single nucleotide polymorphism, interleukin-1-receptor-associated kinase, bone mineral density, association study, quantitative trait**

INTRODUCTION

CYTOKINE PATHWAYS INCLUDING interleukin-1 (IL1) and tumor necrosis factor α (TNFα) are considered to be among the most potent of all bone-resorbing mechanisms.[1–4] These signaling cascades participate in osteoclast differentiation and can affect bone mineral density (BMD). IL-1 receptor-associated kinase (IRAK1), one of the key regulators in the IL1 pathway, is likely to play an important role in bone metabolism by regulating c-*Jun* N-terminal kinase (JNK) and NF-κB–activating pathways, through stimulation of both IL-1 and TNFα.

Two IRAK1 isoforms exist because of alternative splicing: IRAK1a encodes a 713-amino acid polypeptide and IRAK1b encodes a 683-amino acid polypeptide that lacks residues 514–543.[5] In vitro cell culture experiments have revealed that the latter isoform is not only functionally active but is also highly stable and resistant to degradation; this isoform therefore would prolong signal transduction after stimulation of IL-1 ligand.[5] We speculate that amino acid variations that affect IRAK1 kinase activity or protein stability might bring about variations in bone mineral metabolism.

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Osteoporosis is characterized by low BMD and by deterioration of the microarchitecture of bone tissue with a consequent increase in fragility and susceptibility to fracture.[6] BMD, an important predictor of fracture, seems to be determined by genetic as well as environmental factors.[7–11] Because people carrying genetic risk are considered more susceptible to deleterious lifestyle factors, genetic elements need to be clarified for adequate diagnosis, prevention, and early treatment of osteoporosis. If the relevant genes were identified, the pathogenesis of osteoporosis presumably could be explained by variations in those genes or in loci adjacent to them. Although several genes have already been investigated as potential risk factors for osteoporosis,[12–17] an extended panel of genes needs to be examined to elucidate in detail the genetic background of this disease, considering the polygenic nature of BMD distribution and the multiplicity of endocrine and cytokine factors known to regulate bone mass and bone turnover.

For this study, we chose IRAK1 as the most probable candidate marker for osteoporosis that acts in the most upstream step of the IL-1 receptor signaling pathways, possibly regulating osteoclastogenesis from osteoblastic or stromal cells. We investigated a possible association between known amino acid variations in the gene product and radial BMD in samples from two independent Japanese populations of postmenopausal women.

MATERIALS AND METHODS

Subjects

DNA samples for our association study were obtained from peripheral blood of 220 postmenopausal Japanese women living in Akita, Japan. In this cohort (cohort A), mean age and body mass index (BMI) were 71.9 ± 4.7 years and 23.9 ± 3.8 kg/m^2, respectively. All were nonrelated volunteers and all provided informed consent before the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none was receiving estrogen replacement therapy. A second cohort (cohort T), consisting of 126 postmenopausal women living in Tokyo, was sampled independently and studied in the same manner. Values for age and BMI in cohort T were 57.0 ± 5 years and 22.9 ± 3.5 kg/m^2, respectively.

Measurement of radial BMD

The BMD of radial bone (expressed in g/cm^2) of each participant was measured by DXA using a DTX-200 Osteometer (Meditech Inc., Hawthorne, CA, USA). This parameter was normalized for differences in age, height, and weight using multiple regression analysis.[18,19] Mean values and SD of the measured BMD and adjusted BMD of cohort A were 0.321 ± 0.071 and 0.308 ± 0.057 g/cm^2, respectively. Values for cohort T were 0.403 ± 0.072 and 0.394 ± 0.057 g/cm^2, respectively. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening in a health check-up program in Japan.[20] Five-year bone loss (g/cm^2) was calculated in subjects from cohort T by subtracting the adjusted BMD levels recorded 5 years before the present analyses from the current values.

Genotyping for molecular variants in the IRAK1 *gene*

We examined two amino acid–substituting single nucleotide polymorphisms (SNPs) (rs1059702 and rs1059703) archived in the dbSNP at http://www.ncbi.nlm.nih.gov/SNP. The first of these, IRAK1 +587T>C, corresponded to Phe196Ser (F196S) and the other (IRAK1 +1595C>T) to Ser532Leu (S532L). Both SNPs were genotyped by a single-base extension assay using the ABI PRISM SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA).[21] For detection of SNP +587T>C, genomic DNAs were amplified with primers 5′-GAAGGAATTCAGCCTTTGATGTAG-3′ and 5′-ATGAGACCCTCCAGCTACGC-3′; for SNP +1595C>T, primers were 5′-AGGCCATTCTCAGTCCTTGC-3′ and 5′-AGTCGGGACAGACACTCTGC-3′. Column-purified 1492-bp (IRAK1 +587T>C flanking) and 1203-bp (IRAK1 +1595C>T flanking) polymerase chain reaction (PCR) products used for the single-base extension assay were mixed with fluorescently labeled ddNTPs and 1 μM of appropriate primer (5′-AGAGGGGCCAGCAAAACGGA-3′ for IRAK1 +587T>C or 5′-GGATGCAGCTGGCGGCCTCC-3′ for IRAK1 +1595C>T). Genotypes were analyzed by electrophoresis on an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Statistical analysis of linkage disequilibrium and tests for association with BMD

To estimate haplotype frequencies, genotypes of two SNPs were determined among 96 subjects selected from Akita population. Haplotype frequencies among the representative 192 alleles investigated were calculated by Arlequin software (http://lgb.unige.ch/arlequin/software/). Linkage disequilibrium was investigated for all two-way comparisons of the polymorphisms according to Thompson's method (D, D′, and r^2).[22,23] The coefficient of disequilibrium, D, is the difference between the observed haplotype frequency and the frequency expected under statistical independence: D = pAB × pab − pAb × paB. The normalized disequilibrium coefficient is obtained by dividing D by its maximum possible value (D′ = D/ D max); D max = min (pA × pB, pa × pb) if D < 0, and D max = min (pA × pb, pa × pB) if D > 0. r^2 = D^2/(A × B × a × b). Significance levels were determined by χ^2 statistics for the corresponding 2 × 2 table.

Adjusted BMD (g/cm^2) values between genotypic (haplotypic) categories were compared using ANOVA and linear regression analysis as a post-hoc test. Statistical significance was determined by ANOVA, f test. Differences were considered significant when p values were less than 5% ($p < 0.05$).

RESULTS

Complete linkage disequilibrium between F196S and S532L

Our test populations were genotyped for two SNPs encoding amino acid substitutions in the *IRAK1* gene (rs1059702 and rs1059703 in the NCBI dbSNP database). These two sites, located at either end of an important

catalytic kinase domain of IRAK1 at nt + 587 and nt + 1595, respectively, could conceivably affect the enzymatic activity of the gene product. The SNP at IRAK1 + 587T>C brings about substitution at amino acid residue 196 from phenylalanine to serine (F196S); the other, at IRAK1 + 1595C>T, causes substitution of leucine for serine at residue 532 (S532L). We first genotyped 220 Japanese postmenopausal women from Akita (cohort A) for the two sites; an obvious consistency led us to analyze linkage disequilibrium for possible two-way comparisons of the variations using Thompson's method (D, D', and r^2). The results confirmed complete linkage disequilibrium of the SNP F196S at position + 587 with variation S532L at position + 1595 (D' = 1.0000, r^2 = 1.0000, c^2 = 192.000, $p = 1.2 \times 10^{-43}$). On this basis we constructed haplotypes, calculated their frequencies using the Arlequin algorithm, and identified only two distinct haplotypes among the test subjects. The major haplotype, 196F/532S or +587T/+1595C, constituted 74.0%, and the minor haplotype (196S/532L or +587C/+1595T) represented 26% of the haplotypes present in the Japanese populations examined. No haplotypic assortments of 196F/532L or 196S/532S were found.

Association of 196F/532S with low BMD in the A population

Genotypes and haplotypes for each of the two variant sites were determined and correlated with BMD among cohort A of 220 postmenopausal women living in Akita, on the western side of northern mainland Japan. A significant correlation with BMD was identified with both +587T>C SNP and +1595C>T SNPs, thus with the haplotypes of IRAK1. Haplotype 196F/532S was associated with decreased BMD in an ANOVA and a linear regression test as a post-hoc test. When BMD values were compared among the three haplotypic categories (196F/532S homozygotes, heterozygotes, and 196S/532L homozygotes), BMD was lowest among 196F/532S homozygotes (0.312 ± 0.056 g/cm^2), highest among 196S/532L homozygotes (0.353 ± 0.059 g/cm^2), and intermediate among heterozygotes (0.330 ± 0.055 g/cm^2; r = 0.21, p = 0.0017), indicating a codominant effect of 196F532S haplotype for lowered BMD (Fig. 1A). The codominant effect of the haplotype was also supported by multiple comparison test of Tukey-Kramer ($p < 0.05$). Coefficient of determination value (r^2) was 0.44, indicating about 4% of population variance in BMD was explained by these two SNPs in this cohort. No deviation of genotype frequencies from Hardy-Weinberg equilibrium was observed in any of the polymorphisms among our subjects (cohort A: p = 0.86, cohort T: p = 0.93).

Association of 196F/532S with low BMD in the T population

To examine reproducibility of the noted association of IRAK1 variation with BMD, we examined a second cohort (cohort T) of Japanese postmenopausal women, independently collected in Tokyo, on the eastern side of central mainland Japan. ANOVA and a linear regression test in these subjects again revealed a significant correlation between the IRAK1 haplotypes and the adjusted BMD, indicating a codominant effect; that is, it was lowest among

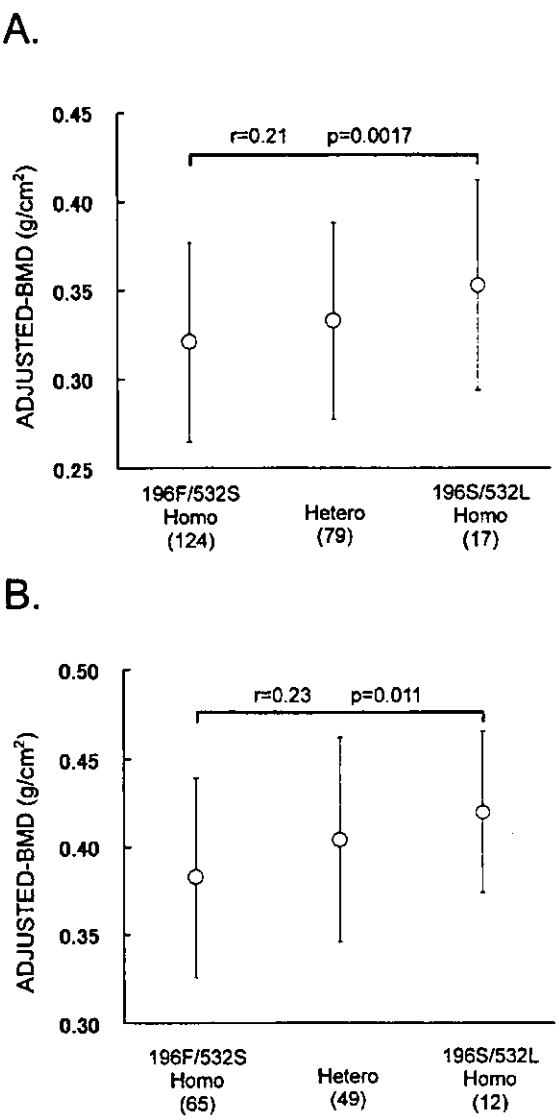

FIG. 1. Effect of *IRAK1* alleles on quantitative adjusted BMD. Open circles indicate mean values; error bars indicate SD. (A) Akita population (cohort A). (B) Tokyo population (cohort T). r, correlation coefficient; p, p values, calculated by ANOVA f test.

196F/532S homozygotes (0.383 ± 0.057 g/cm^2), highest among 196S/532L homozygotes (0.420 ± 0.046 g/cm^2), and intermediate among heterozygotes (0.404 ± 0.058 g/cm^2; r = 0.23, p = 0.011; Fig. 1B). Coefficient of determination value (r^2) was 0.53, indicating about 5% of population variance in BMD was explained in this cohort.

Significant difference in bone loss between 196F/532S and non-196F/532S

To test whether bone loss of postmenopausal women is affected by the variation of *IRAK1* gene, Student's t-test was examined. Five-year bone loss of the subjects in cohort T who had been followed up longitudinally for over 5 years

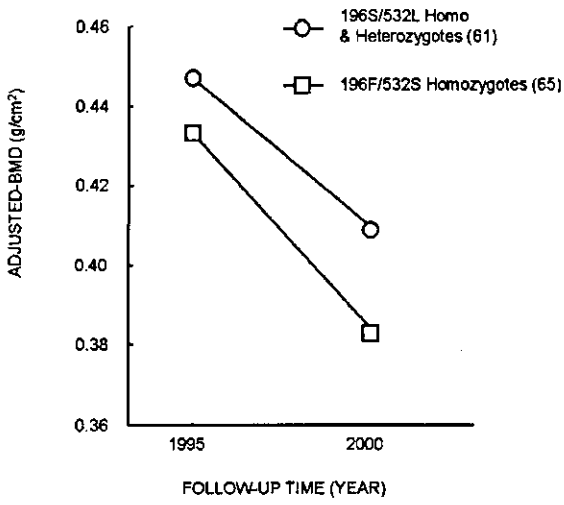

FIG. 2. Five-year longitudinal bone loss between subjects harboring major haplotype 196F/532S and other subjects in cohort T. Mean values of adjusted BMD are plotted on the diagram from data at 5 years ago (1995) and the present data (2000). Open circles indicate values among 196S/532L homozygous or heterozygous individuals. Open squares are for individuals with 196F/532S homozygotes. Lines are synonymous to regression lines drawn for each haplotypically classified subject group. Significant difference was indicated for inclination of two lines by Student's *t*-test ($p = 0.01$).

was examined. Five-year bone loss (g/cm^2) was calculated in each individual of cohort T by subtracting the adjusted BMD value obtained in previous BMD measurement carried out 5 years ago from the BMD value measured in the present analysis. Significant difference in 5-year bone loss was identified between women with the major haplotype 196F/532S homozygotes (0.0506 ± 0.0304 g/cm^2, $n = 65$) and other women (0.0382 ± 0.0252 g/cm^2, $n = 61$; $p = 0.014$; Fig. 2). This result suggests that genetic variation of IRAK1 contribute to development of osteoporosis in part through the mechanism of accelerated postmenopausal bone loss. We hypothesized that haplotype 196F/532S is an important risk factor for decreased BMD in postmenopausal women.

DISCUSSION

In the work reported here, we studied two SNPs encoding amino acid substitutions in the *IRAK1* gene among two independent populations of Japanese women, analyzing haplotypes and correlating them with forearm cancellous BMD. We demonstrated complete linkage disequilibrium between the two SNPs and determined that two (and only two) haplotypes were present in our test populations. Significant association of BMD with these variants was detected; adjusted BMD was lowest in 196F/532S homozygotes, intermediate among heterozygotes, and highest among 196S/532L homozygotes in both populations. The data implied that variation in the coding region of the *IRAK1* gene might have affected bone metabolism in these women, eventually introducing variation in BMD. Lowered BMD in postmenopausal women could be a result of accelerated bone loss and/or lesser acquisition of bone mass before maturation. A trend of correlation between rate of bone loss and variation of *IRAK1* was indicated by analysis of bone loss over 5 years in one of the cohorts. The trend may suggest that the main contribution of the *IRAK1* variation is to increase bone turnover and bone loss.

There are several interpretations for the observed associations, given the complete linkage disequilibrium between the two SNP sites. First, one site or the other may play the predominant mechanistic role that explains the associations. Second, both amino acid changes together are required to impart the functional change that underlies the observed association (codominant effect of the two polymorphisms). Third, these markers may themselves be in linkage disequilibrium with other unmeasured and functional variants at or near IRAK1 that are the true mechanistic basis for the associations. Functional studies will be required to distinguish between these possibilities. However, the S532L variation may be more important on theoretical grounds because it is located just downstream from the C-terminal end of the essential kinase domain (IRAK1 + 1595) and also because it lies within the sequence that is variably spliced between the two IRAK1 isoforms; a 30-amino acid domain is retained in IRAK1a but spliced out in IRAK1b.[5] Analysis of the shorter isoform in cultured cells showed that IRAK1b is functionally active but highly stable and resistant to degradation after stimulated by receptor binding of IL-1 ligand, implying that signal transduction is prolonged.[5] Three serine residues and one tyrosine residue are present within the spliced-out 30 amino acid sequence, and they represent potential phosphorylation sites required for hyperphosphorylation/destabilization of the enzyme.[5] The lack of a serine residue may therefore affect efficiency of degradation. Detailed characterization of the function of IRAK1 protein will be required for testing this hypothesis, as well as examinations of both variants as to their effects on signaling pathways. Such investigations may help to clarify mechanisms of postmenopausal bone loss as well as general bone loss associated with interleukin-exerted pathways.

Along with others, we have focused on molecules acting on inflammatory cytokine pathways such as IL-1, TNFα, and their effectors, which are known to be involved in bone loss.[24–29] Among many other effectors on this pathway, IRAK1 seems to be especially important for bone metabolism. However, many other effectors and inhibitors act on this signaling cascade, and clarification of their contributions awaits for further investigation.

In summary, we have identified a novel candidate osteoporosis-susceptibility gene *IRAK1*, whose two amino acid variations were significantly associated with BMD among postmenopausal Japanese women, where rate of bone loss, in part, seemed to be affected. Structural inspection indicated a possible contribution of S532L variation in *IRAK1*, which alters an amino acid immediately downstream of the kinase domain. The possible involvement of genetic variations in *IRAK1* may explain, at least in part, the pathogenesis of postmenopausal osteoporosis, and may contribute to establishment of designs for suitable treatments and preventive strategies for this disease.

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ORIGINAL ARTICLE

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Association of the –381T/C promoter variation of the brain natriuretic peptide gene with low bone-mineral density and rapid postmenopausal bone loss

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Abstract Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone-mineral density (BMD). Recent quantitative trait locus analysis in human suggested a possible involvement of chromosomal region 1p36.2–p36.3 for determination of BMD. The brain natriuretic peptide (*BNP*, also named *NPPB*) gene lies within this candidate region for BMD determination. Overexpression of the *BNP* resulted in skeletal overgrowth in transgenic mice. Association analysis between nucleotide variations of the *BNP* gene and radial BMD in 378 Japanese postmenopausal women revealed a significant association of the –381T/C variation of the *BNP* gene with radial BMD (r = 0.17, P = 0.01). Homozygous T-allele carriers had the lowest BMD values (0.395 ± 0.056 g/cm^2), homozygous C-allele carriers had the highest (0.429 ± 0.051 g/cm^2), and heterozygous individuals had intermediate radial BMD values (0.405 ± 0.048 g/cm^2), indicating a dosage effect. Accelerated bone loss also correlated with the –381 T allele in a 5-year follow-up study (r = 0.21, P = 0.017). These results suggest that variation of BNP may be an important determinant of postmenopausal osteoporosis, in part through the mechanism of accelerated postmenopausal bone loss.

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Key words Single-nucleotide polymorphism · Brain natriuretic peptide (BNP) · Bone-mineral density · Association study · Quantitative trait · Osteoporosis

Introduction

Osteoporosis is characterized by low bone-mineral density (BMD) and by deterioration of the micro-architecture of bone tissue with a consequent increase in fragility and susceptibility to fracture. BMD, an important predictor of fracture, appears to be determined by genetic as well as environmental factors (Giguere and Rousseau 2000; Stewart and Ralston 2000).

Natriuretic peptides comprise a family of three structurally related molecules: atrial natriuretic peptide, brain natriuretic peptide (BNP; also known as B-type natriuretic peptide), and C-type natriuretic peptide (CNP) (LaPointe et al. 1996; Maack 1996). Accumulating evidence indicates that natriuretic peptides are important regulators of bone and cartilage differentiation and maintenance, whose biological actions are mediated through two types of guanylyl cyclase (GC)-coupled receptor subtypes (GC-A and GC-B) (Yamamoto et al. 1996; Suda et al. 1998; Chusho et al. 2001; Yasoda et al. 1998; Hasegawa et al. 1994). For instance, transgenic mice overexpressing BNP presented with skeletal overgrowth (Suda et al. 1998). The targeted disruption of a related gene, *CNP*, in mouse resulted in congenital abnormalities in skeletal development (Chusho et al. 2001).

A quantitative trait locus (QTL) analysis on BMD in human suggested a possible involvement of chromosomal region 1p36.2–p36.3, spanning approximately 7Mb, for determination of BMD (Devoto et al. 2001). The responsible gene within the region has not been defined to date (Spotila et al. 2000; Albagha et al. 2002).

Because of the chromosomal location within the QTL candidate region, as well as skeletal abnormalities displayed in BNP-transgenic mice, we investigated the association between genetic variations in the *BNP* gene and radial

Table 1. Summary of analyzed polymorphisms on *BNP* gene

No.	Name	Location	JSNP ID[a]	NCBI dbSNP[b]	Allele frequency	% Heterozygosity
1	-2158A/G	Promoter	IMS-JST106520	—	0.88:0.12	19
2	-1563A/G	Promoter	—	rs1800773	Monomorphic	0
3	-1299G/T	Promoter	IMS-JST106522	—	0.86:0.14	21
4	-381T/C	Promoter	IMS-JST083611	—	0.84:0.16	24
5	R25L	Exon 1 (+)	—	rs5227	Monomorphic	0
6	R47H	Exon 2 (+)	—	rs5229	Monomorphic	0
7	M93L	Exon 2 (+)	—	rs5230	Monomorphic	0

SNP, Single-nucleotide polymorphism
[a]JSNP ID, Number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html)
[b]dbSNP ID, Number from the dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)

BMD levels and rate of bone loss in a 5-year longitudinal follow-up study.

Subjects and methods

Subjects

DNA samples were obtained from the peripheral blood of 378 postmenopausal Japanese women. Mean age and body mass index (BMI) with standard deviation (SD) was 58.4 ± 8.6 (range 32–69) years and 23.7 ± 3.61 (range 14.7–38.5) kg/m^2, respectively. The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry using a DTX-200 (Osteometer Meditech, Hawthorne, CA, USA). To calculate adjusted BMD, we normalized the measured BMD for differences in age and BMI by multiple regression analysis (Kleinbaum et al. 1998; Tsukamoto et al. 2000), using the Instat 3 software package (GraphPad Software, San Diego, CA, USA). The adjustment equation for the study samples was as follows: [adjusted BMD (g/cm^2)] = [measured BMD (g/cm^2)] − 0.006375 × {58.39 − [age (years)]} + 0.008961 × {23.65 − [BMI (kg/cm^2)]}. BMD in the distal radius was measured according to the Guidelines for Osteoporosis Screening in a health check-up program in Japan (Orimo et al. 2001). All subjects were nonrelated volunteers who gave their informed consent prior to the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none was receiving estrogen replacement therapy.

Genotyping for molecular variants in the *BNP* gene

We examined in our test population seven polymorphisms (SNPs) archived in the National Center for Biotechnology Information database for SNPs (NCBI dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) and the Japanese SNP (JSNP) database (http://snp.ims.u-tokyo.ac.jp/index.htmlamino); three SNPs from JSNP were confirmed to be polymorphic in our test population, whereas four other SNPs archived in the NCBI dbSNP turned out to be monomorphic in our test population, as described in Results (Table 1).

Genotypes of SNPs were determined using the SNP dependent PCR (Sd-PCR) method, a refined allele-specific PCR to discriminate polymorphic sequences, as described previously (Iwasaki et al. 2002). In brief, the Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. The procedure incorporates double-nucleotide mismatches at the 3′ end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other designed to allow distinct allelic discrimination through almost exclusive amplification of one allele over the other.

Two allele-specific primers (AS primers) and one reverse primer were prepared per SNP. AS primers (long and short) have a five-base difference between them, and each has a polymorphic nucleotide of the SNP sequence at the 3′ ends and an additional artificial mismatch introduced near the 3′ end. These primer sets allowed distinct discrimination of alleles. Each genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10 mM deoxyribonucleoside triphosphates, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-labeled deoxycytidine triphosphate (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out on a thermal cycler (Gene-amp system 9600, Perkin-Elmer) with initial denaturation at 94°C for 4 min, followed by five cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, and 72°C for 20 s, terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, we carried out direct resequencing using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

BMD data of each subject were normalized with age and BMI, using Instat 3 software package (GraphPad Software) via multiple regression analysis (Iwasaki et al. 2002). Quantitative association between genotypes and adjusted BMD values (g/cm^2) was analyzed via one-way analysis of variance (ANOVA) with regression analysis as a post hoc test. Three genotypic categories of each SNP were converted into incremental values, 0, 1, and 2, corresponding to the number of chromosomes possessing a minor allele nucleotide. Statistical significance was determined by ANOVA, *F*-test. To ascertain the Hardy-Weinberg equilibrium among genotypes of the subjects, a chi-square test was used. Prediction of binding sites for transcription factors was performed using MatInspector V2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) based on the TRANSFAC4.0 database (http://transfac.gbf.de/TRANSFAC/index.html).

Results

The *BNP* gene was examined for association with BMD as one of the likely candidates for osteoporosis susceptibility genes. We first examined the polymorphic nature of seven archived SNPs in 32 chromosomes of the subjects from our test population; three SNPs were archived in the JSNP database, and four SNPs were archived in the NCBI-dbSNP database, as shown in Table 1. Three SNPs from the JSNP database were moderately polymorphic, whereas the other four SNPs from the NCBI-dbSNP database turned out to be monomorphic in the test population.

Among the three polymorphic SNPs as defined earlier, promoter SNP –381T/C, localized at 283 bp upstream of the transcription initiation site (referenced contig; NT_004488.10 from GenBank), revealed a significant correlation with variation in radial BMD (r = 0.13, P = 0.01). Statistical analyses of two other SNPs did not reach statistical significance (–2158A/G; r = 0.06, P = 0.23, –1299G/T; r = 0.03, P = 0.58). As to the –381T/C SNP, homozygous T-allele carriers had the lowest adjusted BMD (0.395 ± 0.056 g/cm^2), heterozygous individuals had an intermediate adjusted BMD (0.405 ± 0.048 g/cm^2), and homozygous C-allele carriers had the highest adjusted BMD (0.429 ± 0.051 g/cm^2), implying an allelic dose effect of this variation on influences to BMD (Fig. 1).

Accelerated bone loss in T-allele carriers in a 5-year follow-up study. To test whether bone loss of postmenopausal women is affected by the variation of the *BNP* gene, we examined the 5-year bone loss of the 126 subjects who had been followed up longitudinally for over 5 years. Five-year bone loss (g/cm^2) was calculated for each individual by subtracting the adjusted BMD value obtained in the BMD measurement carried out 5 years previously from the BMD value measured in the recent analysis (Fig. 2). A significant difference in 5-year bone loss was identified between women with the T-allele homozygotes for the –381T/C SNP

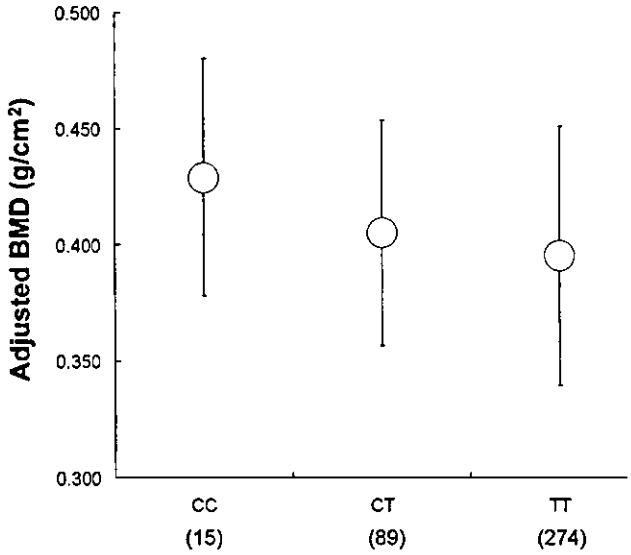

Fig. 1. Association of –381T/C variations with adjusted-radial bone mineral density (BMD). Adjusted BMD of three genotypically classified subgroups among 378 subjects were plotted. *Open circles* indicate mean values and *error bars* indicate standard deviations. The correlation between the number of minor alleles possessed and the adjusted BMD was tested by linear regression analysis (P = 0.01)

(0.048 ± 0.030 g/cm^2, n = 90) and other women (0.036 ± 0.023 g/cm^2, n = 36) (P = 0.017). The results suggest that genetic variation of BNP contributes to the development of osteoporosis, in part through the mechanism of accelerated postmenopausal bone loss. We hypothesized that T-allele homozygotes for the BNP –381 SNP is an important risk factor for decreased BMD in postmenopausal women.

Discussion

In the work reported here, we showed an association of the –381T/C SNP variation in the promoter region of the *BNP* gene with radial BMD in a population of postmenopausal Japanese women. Adjusted BMD was lowest in T/T homozygotes, intermediate among heterozygotes, and highest among C/C homozygotes in the test population. The data implied that variation in the promoter region of the *BNP* gene might have affected bone metabolism in these women, eventually introducing variation in BMD. Lowered BMD in postmenopausal women could be a result of accelerated bone loss and/or lesser acquisition of bone mass before maturation. A correlation between rate of bone loss and variation of the *BNP* gene was indicated by analysis of bone loss over 5 years in a longitudinal follow-up study. The correlation may suggest that the main contribution of the BNP variation is to increase bone turnover and bone loss.

The –381 variation may be important on theoretical grounds because it is located just upstream of the gene transcription initiation site. Predictive analysis of binding motifs for transcription factors using the MatInspector pro-

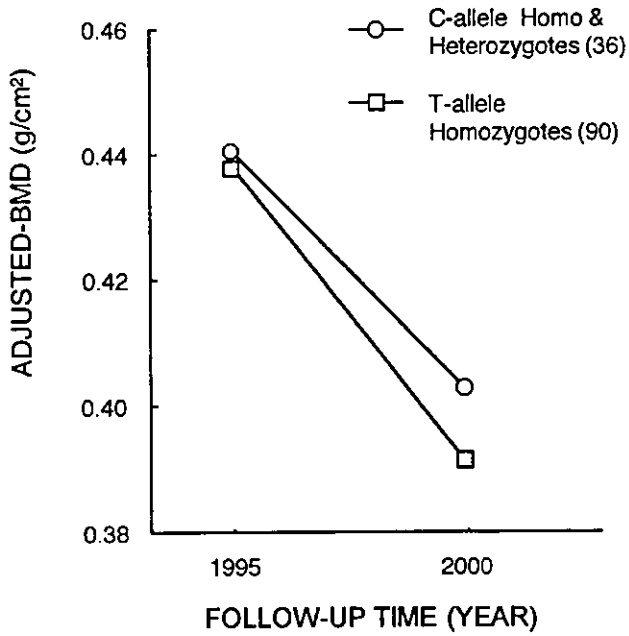

Fig. 2. Five-year longitudinal bone loss between subjects harboring the T allele of the -381T/C single-nucleotide polymorphism and other subjects in 126 postmenopausal women. Mean values of adjusted BMD are plotted on the diagram from data at 5 years previously (1995) to those at a recent measurement (2000). *Open circles* indicate values among C homozygous and heterozygous individuals. *Open squares* are for individuals with T homozygotes. *Lines* are synonymous to regression lines drawn for each genotypically classified subject group. A significant difference was indicated for the inclination of two lines ($P = 0.017$)

gram revealed that the -381T/C variation is located within the consensus binding sequence of δ-crystallin/E2-box factor 1 (δ-EF1; GGACACCTGGA) and upstream stimulatory factor (ACACCTGG), both of which contain an E-box binding element (CAnnTG) inside them (Sekido et al. 1994; Dillner and Sanders 2002; Viollet et al. 1996). Because δ-EF1 is an important transcription factor that distributes in many organs including connective tissues of the skeletal system (Davies et al. 2002; Funahashi et al. 1993; Terraz et al. 2001; Sooy and Demay 2002), it regulates not only tissue-specific expression of the crystalline gene in the lens, but also many genes including collagens and osteocalcin genes important for bone and cartilage development and maintenance (Funahashi et al. 1993; Terraz et al. 2001; Sooy and Demay 2002). Altered promoter function could account for the different clinical features of bone mass in individuals, which should be examined in the future by means of a binding assay for each transcription factor, or by a promoter activity test using reporter constructs, for example.

In summary, we showed a significant association of the -381T/C variation in the promoter region of the *BNP* gene with radial BMD of postmenopausal Japanese women. Structural inspection suggests a possible contribution of a transcription factor delta-EF1 binding to the SNP site. The possibility cannot be ruled out, however, that this SNP marker may itself be in linkage disequilibrium with other unmeasured and functional variants at or near *BNP* that are the true mechanistic basis for the associations. Functional studies will be required to rule out these possibilities. Nevertheless, our data is in accord with the previous data from human QTL linkage analysis in search of an osteoporosis susceptibility gene at 1p36.2–p36.3.

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Association of a Trp16Ser variation in the gonadotropin releasing hormone signal peptide with bone mineral density, revealed by SNP-dependent PCR typing

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Abstract

Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone mineral density (BMD). Among those factors, adequate estrogen is essential for achievement of peak bone mass as well as for postmenopausal maintenance of skeletal homeostasis. Gonadotropin-releasing hormone (GnRH) from the hypothalamus is the primary determinant in the hypothalamic–pituitary–gonadal feedback system. In genetic studies of 384 postmenopausal Japanese women, we found a significant association between BMD and an amino acid variation (Trp16Ser) located within the signal peptide of GnRH ($r = 0.143$, $P = 0.005$). These results were achieved by genotyping all subjects using a newly developed SNP-dependent PCR method. This automated, high-throughput, and inexpensive procedure is suitable for typing large numbers of samples. BMD was lowest among 16Ser/Ser homozygotes, highest among 16Trp/Trp homozygotes, and intermediate among heterozygotes. A case-control study involving 125 osteoporosis patients and 92 healthy controls revealed a significant association between the presence of a 16Ser GnRH allele and affected status ($\chi^2 = 4.74$, $P = 0.041$). The results suggested that variation of the GnRH signal peptide may be an important risk factor for postmenopausal osteoporosis.

Keywords: Single nucleotide polymorphism; GnRH; Bone mineral density; Association study; Quantitative trait

Introduction

Osteoporosis is a common, multifactorial disease characterized by reduced bone mass and microarchitectural deterioration of bone tissue, typically in association with postmenopausal estrogen deficiency. Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone mineral density (BMD) or determine bone geometry [1,2].

Adequate estrogen is essential for achievement of peak bone mass [3] as well as for postmenopausal maintenance of skeletal homeostasis; estrogen deficiency in the postmenopausal period or after ovariectomy results in bone mineral loss and increased risk of osteoporosis [4,5] and estrogen replacement therapy is a common choice for treatment of osteoporosis [6,7]. Homeostasis of the sex hormones is controlled through a feedback system involving the hypothalamic–pituitary–gonadal axis [8,9]. Recently an amino acid variation (Trp16Ser, W16S) was identified in the 16th

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residue of the signal peptide of gonadotropin-releasing hormone (GnRH), the primary determinant in the hierarchical regulatory system. Kallmann syndrome [10] and anorexia nervosa [11] are clinical examples of hypogonadism due to dysfunction of GnRH. We considered that the genetic variation in this hormone could make it one of the most likely candidates for involvement in susceptibility to osteoporosis.

A number of efforts have been expended for identifying genetic factors involved in the onset of osteoporosis [3,5]. An accurate, automated, high-throughput, and inexpensive method is desirable if one is to carry out large-scale genotyping of human DNAs for single-nucleotide polymorphisms (SNPs) as a potentially effective way of determining disease susceptibility or drug sensitivity among individuals. Numerous methods have been introduced, each based on different technologies, including various types of hybridization, enzymatic reactions, electrophoretic separation, sequence-specific incorporation of nucleotides, and oligonucleotide ligation. Allelic discrimination based on PCR priming has the advantages of simplicity and within-assay control for exclusion of false-negative results. We took advantage of the basic principles of mutagenically separated PCR, a method developed originally by Rust et al. for detection of disease-causing mutations [12], and refined those principles to meet the requirements of large-scale genomewide SNP screening. In this way we defined an automated, high-throughput, and inexpensive SNP typing procedure suitable for hundreds of samples at once, which we named SNP-dependent PCR (Sd-PCR).

In the work reported here we examined the potential involvement of the GnRH gene in pathogenesis of osteoporosis by investigating a possible association between the Trp16Ser amino acid variation and BMD in 384 postmenopausal women. Subsequently we undertook a case-control comparison using the Sd-PCR method.

Materials and methods

Subjects

DNA samples for our association study were obtained from peripheral blood of 384 postmenopausal Japanese women. Mean ages and body mass indices (BMI) with standard deviations (SD) were 58.4 ± 8.6 (range 32–69) years and 23.7 ± 3.61 (range 14.7–38.5) kg/m^2 [3]. The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry (DEXA) using DTX-200 (Osteometer Meditech Inc., Hawthorne, CA). To calculate adjusted BMD, the measured BMD was normalized for differences in age, height, and weight, using the Instat 3 software package (GraphPad Software, San Diego, CA) and logistic regression analysis [13]. The adjustment equation for the study samples was {adjusted BMD (g/cm^2)} = {measured BMD (g/cm^2)} − 0.006375 × [58.39 − {age (years)}] + 0.008961 × [23.65 − {BMI (kg/cm^2)}] [10,12]. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening in a health checkup program in Japan [14]. All were nonrelated volunteers and gave their informed consent prior to the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none was receiving estrogen replacement therapy.

For case-control correlation, two groups of adult Japanese female subjects were ascertained: (1) osteoporosis patients, consisting of 125 women who exhibited low lumbar BMD (Z score ≤ -1), (2) healthy controls, consisting of 92 women with sufficient lumbar BMD (Z score ≥ 1). The BMD of lumbar vertebral bodies (expressed in g/cm^2) was measured in each participant by DEXA using DPX-L. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20,000 Japanese women [15]. The patients and the control subjects were ascertained from the Research Institute and Practice for Involutional Diseases, and all gave their informed consent prior to the study. This project was approved by the IRB ethical committee of the Institute.

SNP-dependent PCR (Sd-PCR) procedure

Using the Sd-PCR method that we had defined for large-scale SNP genotyping, we placed the polymorphic nucleotide of the SNP sequence at the 3′ end of each polymorphic (forward) primer, and an additional artificial mismatch was introduced near the 3′ end, after being selected to be the best choice by a computer algorithm, a program that attempts to balance the strength of the mutations on the two allele-specific primers. This procedure allowed distinct discrimination of alleles, due to almost exclusive amplification of one allele over the other. Two allele-specific primers (AS primers), each specific for respective allelic sequences, were prepared, with a five-base difference between them, i. e., the TTTTT sequence is placed at the 5′ end of one AS primer to make one allele longer than the other. Either a GG or a CC sequence is also placed at the 5′ portion of each AS primer in order to stop the short product from unfavorable annealing to long product and filling in the five-base extension.

For the C allele (16Ser) of the GnRH gene, a specific forward primer was constructed having at its 5′ end a five-T stretch and double G: 5′-TTTTTGGGCTGGCCTTATTC-TACTGAATTG-3′. The forward primer specific to the G allele (16Trp), having only double C at the 5′ end, was designed as 5′-CCGCTGGCCTTATTCTACTGACCTC-3′; polymorphic, discriminating nucleotides are marked here by single and double underlines. The nonpolymorphic reverse primer (R) having a four-base stretch of GTTT at its 5′ end was 5′-GTTTTTTTCGGCATCTCTCTTTCC-3′. Two AS primers were mixed with a single nonpolymorphic