Association of a −1997G → T Polymorphism of the Collagen Iα1 Gene with Bone Mineral Density in Postmenopausal Japanese Women

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Abstract Genetic variants that affect collagen Iα1 metabolism may be important in the development of osteoporosis or osteoporotic fractures. A −1997G → T polymorphism in the promoter of the collagen Iα1 gene (COL1A1) was shown to be associated with bone mineral density (BMD) for the lumbar spine in postmenopausal Spanish women. The relation of this polymorphism to BMD in Japanese women or men has now been examined in a population-based study. The subjects (1,110 women, 1,126 men) were 40 to 79 years of age and were randomly recruited for a population-based prospective cohort study of aging and age-related diseases. BMD for the lumbar spine, right femoral neck, right trochanter, and right Ward's triangle was measured using dual-energy x-ray absorptiometry. Genotypes for the −1997G → T polymorphism of COL1A1 were determined with a fluorescence-based allele-specific DNA primer assay system. When all women were analyzed together, BMD for the lumbar spine and trochanter was significantly lower in subjects with the *COL1A1*G/*G* genotype than in those in the combined group of *COL1A1*G/*T* and *COL1A1*T/*T* genotypes. When postmenopausal women were analyzed separately, BMD for the femoral neck and trochanter was also significantly lower in those with the *COL1A1*G/*G* genotype than in those with the *COL1A1*G/*T* genotype or those in the combined group of *COL1A1*G/*T* and *COL1A1*T/*T* genotypes. BMD was not associated with −1997G → T genotype in premenopausal women or in men. Multivariate regression analysis revealed that −1997G → T genotype affected BMD at various sites with a variance of 0.46–0.62% for all women and 0.61–1.01% for postmenopausal women. The −1997G → T genotype was not related to the serum concentration of osteocalcin, the serum activity of bone-specific alkaline phosphatase, or the urinary excretion of deoxypyridinoline or cross-linked N-telopeptides of type I collagen in men or in premenopausal or postmenopausal women. These results suggest that COL1A1 is a susceptibility locus for reduced BMD in postmenopausal Japanese women.

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Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures (Kanis et al. 1994). Although several environmental factors, including diet, smoking, and physical exercise, influence BMD, a genetic contribution to this parameter has been recognized (Peacock et al. 2002). Genetic linkage analyses (Morrison et al. 1994; Johnson et al. 1997; Devoto et al. 1998; Koller et al. 1998, 2000; Niu et al. 1999) and candidate gene association studies (Morrison et al. 1994; Kobayashi et al. 1996; Uitterlinden et al. 1998; Yamada et al. 2001; Ishida et al. 2003) have implicated several loci and candidate genes in the regulation of bone mass and the prevalence of osteoporosis or osteoporotic fractures. However, the genes that contribute to genetic susceptibility to osteoporosis remain to be identified definitively.

Type I collagen is the most abundant protein of bone matrix. Mutations in the coding regions of the genes for the two type I collagen chains (COL1A1 and COL1A2) result in a severe autosomal dominant pediatric condition known as osteogenesis imperfecta (Sykes 1990). A G → T single nucleotide polymorphism (SNP) at the first base of a consensus binding site for the transcription factor Sp1 in the first intron of COL1A1 was associated not only with BMD in white women (Grant et al. 1996) but also with osteoporotic fractures in postmenopausal women (Langdahl et al. 1998; Uitterlinden et al. 1998). The *COL1A1*T* allele of this polymorphism affects collagen gene regulation in such a manner that it increases the production of the α1(I) collagen chain relative to that of the α2(I) chain and leads to reduced bone strength by a mechanism that is partly independent of bone mass (Mann et al. 2001). These observations thus implicate genetic variants that affect collagen Iα1 metabolism as important determinants of the development of osteoporosis and osteoporotic fractures. Other studies, however, have shown only a weak association of the Sp1 binding site polymorphism with BMD or osteoporotic fractures in premenopausal French women (Garnero et al. 1998) or a lack of association in postmenopausal women in Sweden (Liden et al. 1998), in American women (Hustmyer et al. 1999), or in postmenopausal Danish women (Heegaard et al. 2000).

A −1997G → T SNP in the promoter of COL1A1 was also associated with BMD for the lumbar spine in postmenopausal Spanish women, and this SNP and the G → T SNP of the Sp1 binding site of COL1A1 were shown to be in linkage disequilibrium (Garcia-Giralt et al. 2002). Given the ethnic divergence of gene polymorphisms, it is important to examine polymorphisms potentially related to BMD in each ethnic group. We have now examined whether the −1997G → T SNP of COL1A1 is associated with BMD in Japanese women or men in a population-based study.

Materials and Methods

Study Population. The National Institute for Longevity Sciences Longitudinal Study of Aging (NILS-LSA) is a population-based prospective cohort study

of aging and age-related diseases (Shimokata et al. 2000). The subjects of the NILS-LSA are stratified by both age and sex and are randomly selected from resident registrations in the city of Obu and the town of Higashiura in central Japan (Yamada et al. 2003a, 2003b). Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, renal diseases, rheumatoid arthritis, and thyroid, parathyroid, and other endocrinologic diseases, were excluded from the study. Women who had taken drugs such as estrogen, progesterone, glucocorticoids, and bisphosphonates were also excluded.

We examined the relation of BMD at various sites to the $-1997G \rightarrow T$ SNP of COL1A1 in 2,236 participants (1,110 women, 1,126 men). All analyses were performed separately for men and for women. In addition, to uncover potential differences between women according to menopausal status, we conducted all analyses separately for premenopausal and postmenopausal women. Menopausal status was evaluated by a detailed questionnaire, and menopause was defined as complete cessation of menstruation. Furthermore, the relation of biochemical markers of bone turnover to $-1997G \rightarrow T$ genotype of COL1A1 was examined for men or premenopausal or postmenopausal women separately. The study protocol was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS, and written informed consent was obtained from each subject.

Measurement of BMD. BMD for the lumbar spine (L2–L4), right femoral neck, right trochanter, and right Ward's triangle was measured using dual-energy x-ray absorptiometry (DXA) (QDR 4500; Hologic, Bedford, Mass.). The coefficients of variation (CVs) of the DXA instrument were 0.9% (L2–L4), 1.3% (femoral neck), 1.0% (trochanter), and 2.5% (Ward's triangle); these values were determined by measurement of BMD three times at each site in 10 healthy subjects (mean age ± SE, 38.7 ± 2.4 years).

Determination of Genotypes. Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) (Yamada et al. 2002). The polymorphic region of COL1A1 was amplified using the polymerase chain reaction with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-TGGGTCAGTTCCAAGAGXCC-3′) or Texas red (5′-TGGGTCAGTTCCAAGAGXAC-3′) and with an antisense primer labeled at the 5′ end with biotin (5′-TCTAAATGTCTGTTCCCTCCAA-3′). The reaction mixture (25 μL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 3.5 mmol/L $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol was initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 2 min.

The amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature.

The plate was then placed on a magnetic stand, and the supernatants from each well were transferred to the wells of a 96-well plate containing 0.01 mol/L NaOH and measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 nm and 538 nm, respectively, for fluorescein isothiocyanate and of 584 nm and 612 nm, respectively, for Texas red.

Measurement of Biochemical Markers of Bone Turnover. Venous blood and urine samples were collected in the early morning after the subjects had fasted overnight. Blood samples were centrifuged at 1,600 × *g* for 15 min at 4°C, and the serum fraction was separated and stored at −80°C until analysis. The serum concentration of intact osteocalcin was measured with an immunoradiometric assay kit (Mitsubishi Chemical, Tokyo, Japan). The activity of bone-specific alkaline phosphatase in serum was measured with an enzyme immunoassay kit (Metra Biosystems, Mountain View, Calif.). Urine samples were collected in plain tubes and stored at −80°C. Urinary deoxypyridinoline was measured with an enzyme immunoassay kit (Metra Biosystems); the values were corrected for urinary creatinine and expressed as picomoles per micromole of creatinine. The urinary concentration of cross-linked N-telopeptides of type I collagen (NTx) was measured with an enzyme-linked immunosorbent assay kit (Mochida Pharmaceutical, Tokyo, Japan); the values were expressed as picomoles of bone collagen equivalents per micromole of creatinine. Urinary creatinine was enzymatically measured with a creatinine test kit (Wako Chemical, Osaka, Japan).

Statistical Analysis. Quantitative data were compared among the three groups using one-way analysis of variance and the Tukey-Kramer post hoc test and between two groups using the unpaired Student's *t* test. BMD values were analyzed with adjustment for age and body mass index (BMI) using the least-squares method in a general linear model. The effect of −1997G → T genotype on BMD at various sites was evaluated using multivariate regression analysis; R^2 and *P* values were calculated from the analysis including age, BMI, and COL1A1 genotype (0 = *COL1A1*G/*G*, 1 = *COL1A1*G/*T* = *COL1A1*T/*T*) . Allele frequencies were estimated using the gene-counting method, and the chi-square test was used to identify significant departure from Hardy-Weinberg equilibrium. A *P* value less than 0.05 was considered statistically significant.

Results

The distribution of −1997G → T genotypes was in Hardy-Weinberg equilibrium, and age and BMI did not differ among genotypes for all women (Table 1). BMD for the lumbar spine and trochanter was significantly lower in women with the **G/*G* genotype than in those in the combined group of **G/*T* and

Table 1. BMD and Other Characteristics of All Women (n = 1,110) According to the −1997G → T Genotype of COL1A1

Characteristic	**G/*G*	**G/*T*	**T/*T*	**G/* T + *T/*T*
Number (%)	407 (36.7)	526 (47.4)	177 (15.9)	703 (63.3)
Age (years)	60.0 ± 0.5	58.9 ± 0.5	58.4 ± 0.8	58.8 ± 0.4
BMI (kg/m^2)	22.9 ± 0.2	22.8 ± 0.1	23.0 ± 0.2	22.9 ± 0.1
BMD values (g/cm^2)				
L2–L4	0.855 ± 0.006	0.870 ± 0.006	0.878 ± 0.010	0.872 ± 0.005[a]
Femoral neck	0.672 ± 0.004	0.681 ± 0.004	0.680 ± 0.007	0.681 ± 0.003
Trochanter	0.564 ± 0.004	0.575 ± 0.004	0.574 ± 0.006	0.575 ± 0.003[b]
Ward's triangle	0.500 ± 0.006	0.512 ± 0.005	0.508 ± 0.009	0.511 ± 0.004

Data are means ± SE. BMD values are adjusted for age and BMI.
a. P = 0.039 vs. **G/*G*.
b. P = 0.033 versus **G/*G*.

**T/*T* genotypes; the difference in BMD between the **G/*G* genotype and the combined group of **G/*T* and **T/*T* genotypes (expressed as a percentage of the corresponding larger value) was 1.9% for both the lumbar spine and the trochanter.

We also analyzed BMD and other characteristics for premenopausal and postmenopausal women independently. The distributions of −1997G → T genotypes were in Hardy-Weinberg equilibrium, and age and BMI did not differ among genotypes for premenopausal or postmenopausal women (Table 2). For postmenopausal women there was no difference in years after menopause among genotypes. For premenopausal women BMD was not associated with −1997G → T genotype. In contrast, BMD for the femoral neck or trochanter was significantly lower in postmenopausal women with the **G/*G* genotype than in those with the **G/*T* genotype or those in the combined group of **G/*T* and **T/*T* genotypes; the differences in BMD between the **G/*G* genotype and the combined group of **G/*T* and **T/*T* genotypes were 2.5% for the femoral neck and 2.2% for the trochanter.

The distribution of −1997G → T genotypes was in Hardy-Weinberg equilibrium, but BMD did not differ among these genotypes in men (Table 3).

The effect of −1997G→T genotype on BMD was evaluated using multivariate regression analysis (Table 4). The analysis revealed that the −1997G → T genotype affected BMD at various sites with a variance of 0.46–0.62% for all women and of 0.61–1.01% for postmenopausal women.

The relation of biochemical markers of bone turnover to −1997G → T genotype of COL1A1 was also examined. No association of −1997G → T genotype with the serum concentration of intact osteocalcin, serum activity of bone-specific alkaline phosphatase, or urinary excretion of deoxypyridinoline or NTx was apparent for men or premenopausal or postmenopausal women (Table 5).

Table 2. BMD and Other Characteristics of Women (n = 1,093) According to Menopausal Status and the −1997G → T Genotype of COL1A1

	Premenopausal Women (n = 278)				*Postmenopausal Women* (n = 815)			
Characteristic	**G/*G*	**G/*T*	**T/*T*	**G/*T + *T/*T*	**G/*G*	**G/*T*	**T/*T*	**G/*T + *T/*T*
Number (%)	94 (33.8)	140 (50.4)	44 (15.8)	184 (66.2)	306 (37.5)	377 (46.3)	132 (16.2)	509 (62.5)
Age (years)	45.9 ± 0.4	46.3 ± 0.4	45.8 ± 0.6	46.2 ± 0.3	64.6 ± 0.5	63.8 ± 0.4	62.8 ± 0.7	63.5 ± 0.4
Years after menopause					15.4 ± 0.5	15.1 ± 0.5	13.7 ± 0.8	14.7 ± 0.4
BMI (kg/m^2)	22.8 ± 0.3	22.9 ± 0.3	22.4 ± 0.5	22.8 ± 0.2	23.0 ± 0.2	22.8 ± 0.2	23.2 ± 0.3	22.9 ± 0.1
BMD values (g/cm^2)								
L2–L4	1.018 ± 0.012	1.026 ± 0.010	1.044 ± 0.018	1.030 ± 0.009	0.798 ± 0.007	0.813 ± 0.007	0.821 ± 0.011	0.815 ± 0.006
Femoral neck	0.782 ± 0.009	0.767 ± 0.008	0.771 ± 0.014	0.768 ± 0.007	0.634 ± 0.005	0.650 ± 0.004[a]	0.647 ± 0.007	0.650 ± 0.004[b]
Trochanter	0.656 ± 0.009	0.661 ± 0.007	0.656 ± 0.013	0.660 ± 0.006	0.532 ± 0.005	0.544 ± 0.004	0.545 ± 0.007	0.544 ± 0.004[c]
Ward's triangle	0.663 ± 0.012	0.657 ± 0.010	0.658 ± 0.018	0.657 ± 0.009	0.444 ± 0.007	0.459 ± 0.006	0.455 ± 0.010	0.458 ± 0.005

Data are means ± SE. BMD values are adjusted for age and BMI.

a. P = 0.034 vs. **G/*G*.

b. P = 0.011 vs. **G/*G*.

c. P = 0.033 vs. **G/*G*.

Table 3. BMD and Other Characteristics of Men (n = 1,126) According to the −1997G → T Genotype of COL1A1

Characteristic	**G/*G*	**G/*T*	**T/*T**	*G/*T + *T/*T*
Number (%)	457 (40.6)	511 (45.4)	158 (14.0)	669 (59.4)
Age (years)	58.5 ± 0.5	59.7 ± 0.5	59.2 ± 0.9	59.6 ± 0.4
BMI (kg/m^2)	22.9 ± 0.1	23.0 ± 0.1	22.9 ± 0.2	22.9 ± 0.1
BMD values (g/cm^2)				
L2–L4	0.990 ± 0.007	0.975 ± 0.007	0.983 ± 0.012	0.977 ± 0.006
Femoral neck	0.754 ± 0.005	0.754 ± 0.004	0.744 ± 0.008	0.751 ± 0.004
Trochanter	0.672 ± 0.005	0.665 ± 0.004	0.667 ± 0.008	0.665 ± 0.004
Ward's triangle	0.557 ± 0.006	0.552 ± 0.005	0.540 ± 0.010	0.549 ± 0.005

Data are means ± SE. BMD values are adjusted for age and BMI.

Discussion

The −1997G → T SNP of the COL1A1 promoter has previously been associated with BMD for the lumbar spine and, to a lesser extent, with BMD for the femoral neck in postmenopausal Spanish women, and with the **T/*T* genotype, which represents a risk factor for reduced BMD (Garcia-Giralt et al. 2002). We have now shown that the −1997G → T SNP is associated with BMD for the femoral neck and trochanter in postmenopausal Japanese women and with the **G/*G* genotype, which represents a risk factor for reduced BMD. The −1997G → T genotype affected BMD at various sites with a variance of 0.61–1.01% for postmenopausal women, although this SNP was not associated with biochemical markers of bone turnover.

The alleles of the −1997G → T polymorphism associated with reduced

Table 4. Effects of the −1997G → T Genotype of COL1A1 on BMD for All Women (n = 1,110) or Postmenopausal Women (n = 815)

Site	R^2	P
All women		
L2–L4	0.0061	0.0102
Femoral neck	0.0047	0.0243
Trochanter	0.0062	0.0093
Ward's triangle	0.0046	0.0262
Postmenopausal women		
L2–L4	0.0061	0.0263
Femoral neck	0.0101	0.0044
Trochanter	0.0076	0.0137
Ward's triangle	0.0071	0.0172

The R^2 and P values were derived from multivariate regression analysis including age, BMI, and COL1A1 genotype (0 = **G/*G*, 1 = **G/*T* = **T/*T*).

Table 5. Biochemical Markers of Bone Turnover for Women or Men According to the −1997G → T Genotype of COL1A1

Marker	**G/*G*	**G/*T*	**T/*T*
Premenopausal women			
Osteocalcin (ng/mL)	6.35 ± 0.29	6.46 ± 0.24	6.93 ± 0.42
Bone-specific alkaline phosphatase (U/L)	19.6 ± 0.5	20.3 ± 0.5	19.0 ± 0.8
dPyr (pmol/μmol Cr)	5.54 ± 0.15	5.35 ± 0.12	5.50 ± 0.22
NTx (pmol BCE/μmol Cr)	33.5 ± 1.5	33.6 ± 1.3	38.3 ± 2.3
Postmenopausal women			
Osteocalcin (ng/mL)	10.53 ± 0.21	10.30 ± 0.19	10.25 ± 0.32
Bone-specific alkaline phosphatase (U/L)	31.6 ± 0.6	31.5 ± 0.6	30.5 ± 0.9
dPyr (pmol/μmol Cr)	4.08 ± 0.06	4.01 ± 0.05	3.99 ± 0.10
NTx (pmol BCE/μmol Cr)	60.4 ± 1.6	60.2 ± 1.5	59.4 ± 2.5
Men			
Osteocalcin (ng/mL)	7.67 ± 0.11	7.64 ± 0.11	7.64 ± 0.20
Bone-specific alkaline phosphatase (U/L)	26.3 ± 0.4	25.6 ± 0.4	26.2 ± 0.7
dPyr (pmol/μmol Cr)	4.08 ± 0.06	4.01 ± 0.05	3.99 ± 0.10
NTx (pmol BCE/μmol Cr)	36.6 ± 0.7	36.2 ± 0.7	36.4 ± 1.2

Data are means ± SE. dPyr, deoxypyridinoline; Cr, creatinine; NTx, cross-linked N-telopeptides of type I collagen; BCE, bone collagen equivalents.

BMD thus differ between the present study (**G* allele) and the previous study (**T* allele) (Garcia-Giralt et al. 2002). Although the reason for this apparent discrepancy is unclear, there are three major differences between the two studies. First, the subjects were older in our study (mean age of 64 years for postmenopausal women) than in the previous study (mean age, 51 years), and years since menopause were significantly greater in our study (mean, 15.0 years) than in the previous study (mean, 3.6 years). Given that bone resorption markedly increases during 10 years after menopause, genetic effects on BMD might differ between women for short and long time after menopause. Second, the number of subjects in which the association was detected was greater in our study ($n = 815$ for postmenopausal women) than in the previous study ($n = 256$). The results of association studies with small sample sizes are prone to bias compared with those with large sample sizes. Finally, the distribution of −1997G → T genotypes differed significantly ($P < 0.0001$; chi-square test) between our study (postmenopausal women: **G/*G*, 38%; **G/*T*, 46%; **T/*T*, 16%) and the previous study (**G/*G*, 76%; **G/*T*, 22%; **T/*T*, 2%), possibly reflecting the difference in ethnicity. The difference in genetic influences on BMD between different ethnic groups might be attributable, at least in part, to the difference in the distribution of genotypes. It is also possible that the −1997G → T SNP of COL1A1 is in linkage disequilibrium with other polymorphisms of COL1A1 or with polymorphisms of other nearby genes that are actually responsible for the observed association with BMD. Given the multiple comparisons of genotype performed, we cannot completely exclude the possible occurrence of statistical errors such as

false positives, although we observed a significant association of this SNP with BMD at different sites.

Evidence suggests that the −1997G → T SNP of COL1A1 may affect promoter function (Garcia-Giralt et al. 2002). A double-stranded oligonucleotide containing the −1997G → T site bound osteoblast nuclear factors; however, the extent of factor binding was even more pronounced with a single-stranded antisense DNA probe, suggesting the involvement of a protein selective for single-stranded DNA. The extent of factor binding observed with a probe corresponding to the **G* allele was greater than that apparent with a probe based on the **T* allele. The effect of this SNP on COL1A1 transcription, however, remains to be determined.

In conclusion, our present results suggest that the −1997G → T SNP of COL1A1 is associated with BMD for the femoral neck and trochanter in postmenopausal Japanese women and that the alleles associated with reduced BMD differ between postmenopausal Japanese (**G* allele) and Spanish (**T* allele) women, although the contribution of this SNP to bone mass appears relatively small.

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Association of polymorphisms in *CYP17A1, MTP,* and *VLDLR* with bone mineral density in community-dwelling Japanese women and men

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Abstract

We examined whether a −34T → C polymorphism of the gene for cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*), a −493G → T polymorphism of the microsomal triglyceride transfer protein gene (*MTP*), and a CGG repeat polymorphism of the very low density lipoprotein receptor gene (*VLDLR*) were associated with bone mineral density (BMD) in community-dwelling Japanese women and men. The −34T → C polymorphism of *CYP17A1* was associated with BMD in postmenopausal women, with the *CC* genotype being related to increased BMD. The −493G → T polymorphism of *MTP* was associated with BMD in premenopausal women, with the *TT* genotype being related to increased BMD. The CGG repeat polymorphism of *VLDLR* was associated with BMD in men, with two $(CGG)_{n \geq 8}$ alleles being related to increased BMD. These results suggest that *CYP17A1* and *MTP* are susceptibility loci for increased BMD in postmenopausal and premenopausal Japanese women, respectively, and that *VLDLR* constitutes such a locus in Japanese men.

Keywords: Polymorphism, genetic; Genetics; Osteoporosis; Bone density; Steroid 17α-hydroxylase; Microsomal triglyceride transfer protein; VLDL receptor

Osteoporosis, a major health problem of the elderly, is characterized by a reduction in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, both of which result in predisposition to fractures [1]. Although reproductive, nutritional, and lifestyle factors influence BMD, family and twin studies have suggested that BMD is largely heritable and under the control of multiple genes [2–4]. Personalized prevention and treatment of osteoporosis and osteoporotic fractures are important public health goals, one approach to which is to identify disease susceptibility genes. Although genetic linkage analyses [5–7] and candidate gene association studies [7–10] have implicated various loci and genes in predisposition to osteoporosis or fractures, the genes that confer genetic susceptibility to this condition remain to be identified definitively. In addition, because of both ethnic divergence of gene polymorphisms and gene–environment interactions, it is important to examine polymorphisms related to BMD in each ethnic group.

Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) is an enzyme with both 17α-hydroxylase and 17,20-lyase activities that is essential for the production of gonadal and adrenal androgens [11]. Loss-of-function mutations in *CYP17A1,* which is located at chromosome 10q24.3, result in reduced skeletal growth and diffuse osteoporosis [12]. The 5′ untranslated region of *CYP17A1* contains a common polymorphism (−34T → C) located 34 bp upstream of the translation initiation site [13]. The presence of C at this site creates a potential Sp1-like binding motif (CCACC box) that has been proposed to increase both *CYP17A1* expression and consequent androgen biosynthesis [13]. In vitro experiments, however, failed to detect a difference in transcriptional activity between the two allelic variants [14,15]. Men homozygous for the *C* allele of this polymorphism were found to have higher bioavailable testosterone concentrations as well as an increased adult stature and femoral size compared with those homozygous for the *T* allele [16]. In contrast, postmenopausal women with the *CC* genotype had a

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lower BMD at the femoral neck compared with those harboring a *T* allele [17]. In a study of Danish women, although lean individuals homozygous for the *C* allele had a lower BMD for the lumbar spine or femoral neck than did those with the *T* allele, this association was not detected in overweight women [18]. Given the apparently inconsistent results of previous studies, the effect of the −34T → C polymorphism of *CYP17A1* on BMD remains unclear.

Recent observations suggest that lipid and bone metabolism are closely related and that an atherogenic lipid profile has adverse effects on bone remodeling [19–24]. We thus hypothesized that polymorphisms in genes that play a role in lipid metabolism, such as those for microsomal triglyceride transfer protein (*MTP*) and the very low density lipoprotein receptor (*VLDLR*), might affect BMD. MTP is a heterodimeric lipid transfer protein that is essential for both the assembly of apolipoprotein B-containing lipoproteins and their secretion from the liver and intestine [25]. Mutations in the coding region of *MTP* prevent the production of apolipoprotein B-containing lipoproteins, resulting in the rare genetic disorder abetalipoproteinemia [26]. *MTP* is located at chromosome 4q22–q24 and is polymorphic, with several genetic variants existing in linkage disequilibrium [27]. A common polymorphism (−493G → T) has been identified in the promoter region of *MTP* (located 493 bp upstream of the transcription start site), with the less prevalent *T* variant having been associated with a reduced plasma concentration of low density lipoprotein (LDL)-cholesterol [28]. Functional analysis of the −493G → T polymorphism with the use of promoter constructs revealed that the promoter activity of the *T* variant was greater than that of the *G* variant [28]. The −493G → T polymorphism was also recently shown to be associated with the prevalence of coronary heart disease [29]. Furthermore, a haplotype marker of *MTP* was associated with longevity, suggesting that *MTP* might modify human life span [30]. The effect of the −493G → T polymorphism of *MTP* on BMD, however, has not been examined.

The very low density lipoprotein receptor (VLDL-R) binds apolipoprotein E-containing lipoproteins such as VLDL, intermediate density lipoprotein, and β-VLDL and is expressed in heart, muscle, and adipose tissue, but not in the liver, suggesting that it might contribute to the metabolism of triglyceride-rich lipoproteins [31]. The structure of *VLDLR*, which is located at chromosome 9p24, is similar to that of the LDL-R gene [32]. The 5′ untranslated region of *VLDLR* contains a polymorphic triplet (CGG) repeat sequence, with 4 to 11 repeat units having been identified in population samples [32–34]. This polymorphism has been related to plasma concentrations of lipoprotein E:B and lipoprotein A-I [33] as well as to the prevalence of sporadic Alzheimer disease in Japanese [34]. The possible effect of the CGG repeat polymorphism of *VLDLR* on BMD has not been examined, however.

We have been attempting to identify genes significantly associated with BMD in Japanese women or men in a population-based study. *CYP17A1*, *MTP*, and *VLDLR* are candidates for genes that might affect BMD. In the present study, we examined the relations of polymorphisms of these three genes to BMD, even though there is no apparent biological link among them. Our aim was to identify a single polymorphism significantly associated with BMD for each gene. Among several polymorphisms previously identified, the −34T → C polymorphism of *CYP17A1*, the −493G → T polymorphism of *MTP*, and the CGG repeat polymorphism of *VLDLR* have been shown to have the potential to affect gene function. We thus examined the relations of these polymorphisms with BMD in Japanese women and men in a population-based study.

Results

Association of the −34T → C polymorphism of CYP17A1 with BMD

The distribution of −34T → C genotypes of *CYP17A1* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women (Table 1), premenopausal women (data not shown), or postmenopausal women (Table 2). Among all women, BMD for the femoral neck, with adjustment for age, height, and body weight, was significantly ($p < 0.01$) greater in individuals with the *CC* genotype than in those in the combined group of *TT* and *TC* genotypes (Table 1). To examine the possible influence of menopause on the relation between *CYP17A1* genotype and BMD, we analyzed premenopausal and postmenopausal women independently. Because of their small number ($n = 16$), perimenopausal women were excluded from this analysis. *CYP17A1* genotype was not associated with BMD in premenopausal women (data not shown). For postmenopausal women, however, BMD for the femoral neck was significantly greater in individuals with the *CC* genotype than in those in the combined group of *TT* and *TC* genotypes (Table 2). The difference in BMD for the femoral neck between individuals with the *CC* genotype and those in the combined group of *TT* and *TC* genotypes (expressed as a percentage of the larger value) was 2.9%.

The distribution of −34T → C genotypes of *CYP17A1* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for men (data not shown). No significant association was detected between *CYP17A1* genotype and BMD in men (data not shown).

Association of the −493G → T polymorphism of MTP with BMD

The distribution of −493G → T genotypes of *MTP* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women

Table 1
BMD and other characteristics for all women (n = 1108) according to *CYP17A1* genotype

Characteristic	*TT*	*TC*	*CC*	*TT* + *TC*
Number (%)	307 (27.7)	544 (49.1)	257 (23.2)	851 (76.8)
Age (years)	58.3 ± 0.6	59.6 ± 0.5	59.7 ± 0.7	59.1 ± 0.4
Height (cm)	151.6 ± 0.3	151.4 ± 0.3	150.8 ± 0.4	151.5 ± 0.2
Body weight (kg)	52.1 ± 0.5	52.9 ± 0.4	52.7 ± 0.5	52.6 ± 0.3
BMD measured with pQCT (mg/cm^3)				
D50	183.7 ± 3.5	185.7 ± 2.7	186.3 ± 3.9	185.0 ± 2.1
D100	488.5 ± 5.1	485.3 ± 3.8	485.1 ± 5.6	486.5 ± 3.1
P100	1161.3 ± 8.2	1145.1 ± 6.2	1161.7 ± 8.9	1151.0 ± 4.9
BMD measured with DXA (g/cm^2)				
Total body	0.965 ± 0.005	0.962 ± 0.004	0.972 ± 0.005	0.963 ± 0.003
L2–L4	0.861 ± 0.007	0.862 ± 0.005	0.879 ± 0.008	0.861 ± 0.004[a]
Femoral neck	0.676 ± 0.005	0.673 ± 0.004[b]	0.691 ± 0.005	0.674 ± 0.003[c]
Trochanter	0.569 ± 0.005	0.569 ± 0.004	0.578 ± 0.005	0.569 ± 0.003

BMD is adjusted for age, height, and body weight. Data are means ± SE.
[a] p = 0.047 versus *CC*.
[b] p = 0.019 versus *CC*.
[c] p = 0.006 versus *CC*.

(Table 3), premenopausal women (Table 4), or postmenopausal women (data not shown). There were no differences in BMD among *MTP* genotypes for all women (Table 3). For premenopausal women, however, BMD for the distal radius (D100) was significantly greater in individuals with the *TT* genotype than in those with the *GG* genotype or those in the combined group of *GG* and *GT* genotypes (Table 4). The difference in BMD for D100 between individuals with the *TT* genotype and those in the combined group of *GG* and *GT* genotypes (expressed as a percentage of the larger value) was 12.5%. *MTP* genotype was not associated with BMD in postmenopausal women (data not shown).

The distribution of −493G → T genotypes of *MTP* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for men (data not shown). No relation was detected between *MTP* genotype and BMD for men (data not shown).

Association of the CGG repeat polymorphism of VLDLR with BMD

The number of CGG repeats in *VLDLR* ranged from 5 to 10 (6.9 ± 1.5, mean ± SD; n = 4410 alleles) for all women and men (Table 5). The number of CGG repeats tended to be related ($p < 0.05$) to BMD for the distal radius (D100) or trochanter for women and to BMD for the distal radius (D100), lumbar spine, femoral neck, or trochanter for men, with the polymorphism explaining 0.2 to 0.3% of the variance in BMD (Table 6). At each of these sites, BMD increased as the number of CGG repeats increased. Given that the mean and median numbers of CGG repeats were 6.9 and 8,

Table 2
BMD and other characteristics for postmenopausal women (n = 813) according to *CYP17A1* genotype

Characteristic	*TT*	*TC*	*CC*	*TT* + *TC*
Number (%)	218 (26.8)	401 (49.3)	194 (23.9)	619 (76.1)
Age (years)	63.2 ± 0.6	64.3 ± 0.4	63.9 ± 0.6	63.9 ± 0.4
Height (cm)	150.3 ± 0.4	150.3 ± 0.3	149.8 ± 0.4	150.3 ± 0.3
Body weight (kg)	51.2 ± 0.6	52.2 ± 0.4	52.6 ± 0.6	51.8 ± 0.3
BMD measured with pQCT (mg/cm^3)				
D50	164.7 ± 4.3	162.5 ± 3.2	165.6 ± 4.6	163.3 ± 2.6
D100	448.2 ± 6.2	441.1 ± 4.6	443.8 ± 6.6	443.6 ± 3.7
P100	1089.4 ± 10.3	1067.9 ± 7.5	1094.4 ± 10.9	1075.4 ± 6.1
BMD measured with DXA (g/cm^2)				
Total body	0.919 ± 0.006	0.915 ± 0.004	0.927 ± 0.006	0.916 ± 0.003
L2–L4	0.810 ± 0.009	0.803 ± 0.006	0.818 ± 0.009	0.806 ± 0.005
Femoral neck	0.639 ± 0.006[a]	0.640 ± 0.004[b]	0.659 ± 0.006	0.640 ± 0.003[c]
Trochanter	0.538 ± 0.006	0.537 ± 0.004	0.548 ± 0.006	0.537 ± 0.003

BMD is adjusted for age, height, and body weight. Data are means ± SE.
[a] p = 0.045 versus *CC*.
[b] p = 0.030 versus *CC*.
[c] p = 0.006 versus *CC*.

Table 3
BMD and other characteristics for all women (n = 1108) according to *MTP* genotype

Characteristic	*GG*	*GT*	*TT*	*GG* + *GT*
Number (%)	763 (68.9)	313 (28.2)	32 (2.9)	1076 (97.1)
Age (years)	59.0 ± 0.4	60.1 ± 0.6	57.3 ± 1.9	59.3 ± 0.3
Height (cm)	151.3 ± 0.2	151.4 ± 0.3	152.4 ± 1.1	151.3 ± 0.2
Body weight (kg)	52.5 ± 0.3	52.6 ± 0.5	54.1 ± 1.4	52.5 ± 0.2
BMD measured with pQCT (mg/cm^3)				
D50	185.4 ± 2.2	185.4 ± 3.5	184.8 ± 11.0	185.4 ± 1.9
D100	483.8 ± 3.2	489.5 ± 5.0	507.5 ± 15.9	485.5 ± 2.7
P100	1151.2 ± 5.2	1161.1 ± 8.1	1154.5 ± 25.7	1154.1 ± 4.4
BMD measured with DXA (g/cm^2)				
Total body	0.968 ± 0.003	0.961 ± 0.005	0.964 ± 0.015	0.966 ± 0.003
L2–L4	0.866 ± 0.005	0.863 ± 0.007	0.885 ± 0.022	0.866 ± 0.004
Femoral neck	0.679 ± 0.003	0.674 ± 0.005	0.703 ± 0.015	0.677 ± 0.003
Trochanter	0.573 ± 0.003	0.566 ± 0.005	0.581 ± 0.014	0.571 ± 0.003

BMD is adjusted for age, height, and body weight. Data are means ± SE.

respectively, we designated $(CGG)_n \leq 7$ and $(CGG)_n \geq 8$ alleles as short (*S*) and long (*L*) alleles, respectively.

The distribution of *SS*, *SL*, and *LL* genotypes of *VLDLR* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes, for all women, premenopausal women, or postmenopausal women (data not shown). There was no significant relation between *VLDLR* genotype and BMD for all women, premenopausal women, or postmenopausal women (data not shown).

For men, the distribution of *SS*, *SL*, and *LL* genotypes of *VLDLR* was in Hardy–Weinberg equilibrium, and age, height, and body weight did not differ among genotypes (Table 7). Among men, BMD for the lumbar spine was significantly greater in individuals with the *LL* genotype than in those with the *SL* genotype or those in the combined group of *SS* and *SL* genotypes (Table 7). The difference in BMD for the lumbar spine between individuals with the *LL* genotype and those in the combined group of *SS* and *SL* genotypes (expressed as a percentage of the larger value) was 2.8%.

Effects of genotypes for CYP17A1, MTP, and VLDLR on BMD

The effects of the −34T → C genotype of *CYP17A1*, the −493G → T genotype of *MTP*, and the CGG repeat genotype of *VLDLR* on BMD at various sites were evaluated by multiple regression analysis (Table 8). The analysis revealed that the −34T → C genotype of *CYP17A1* significantly affected BMD for the femoral neck with an R^2 variance of 0.6% in postmenopausal women, the −493G → T genotype of *MTP* affected BMD for the distal radius (D100) with an R^2 variance of 2.9% in premenopausal women, and the CGG repeat genotype of *VLDLR* affected BMD for the lumbar spine with an R^2 variance of 0.7% in men.

Table 4
BMD and other characteristics for premenopausal women (n = 279) according to *MTP* genotype

Characteristic	*GG*	*GT*	*TT*	*GG* + *GT*
Number (%)	200 (71.7)	72 (25.8)	7 (2.5)	272 (97.5)
Age (years)	46.1 ± 0.3	46.6 ± 0.5	46.3 ± 1.7	46.2 ± 0.3
Height (cm)	154.4 ± 0.3	154.8 ± 0.6	155.1 ± 1.8	154.5 ± 0.3
Body weight (kg)	54.3 ± 0.6	54.3 ± 1.0	53.8 ± 3.1	54.3 ± 0.5
BMD measured with pQCT (mg/cm^3)				
D50	245.7 ± 3.9[a]	240.6 ± 6.5[b]	294.8 ± 20.1	244.3 ± 3.3[c]
D100	601.1 ± 5.4[d]	610.1 ± 9.1[e]	689.9 ± 28.3	603.5 ± 4.6[f]
P100	1353.5 ± 8.1	1367.5 ± 13.7	1445.5 ± 42.5	1357.2 ± 7.0[g]
BMD measured with DXA (g/cm^2)				
Total body	1.097 ± 0.006	1.080 ± 0.010	1.135 ± 0.030	1.093 ± 0.005
L2–L4	1.028 ± 0.008	1.016 ± 0.013	1.033 ± 0.043	1.025 ± 0.007
Femoral neck	0.772 ± 0.006	0.766 ± 0.010	0.838 ± 0.034	0.770 ± 0.005[h]
Trochanter	0.660 ± 0.006	0.647 ± 0.010	0.701 ± 0.031	0.657 ± 0.005

BMD is adjusted for age, height, and body weight. Data are means ± SE.
[a] p = 0.044 versus *TT*.
[b] p = 0.028 versus *TT*.
[c] p = 0.013 versus *TT*.
[d] p = 0.006 versus *TT*.
[e] p = 0.020 versus *TT*.
[f] p = 0.002 versus *TT*.
[g] p = 0.041 versus *TT*.
[h] p = 0.047 versus *TT*.

Table 5
Distribution of the number of CGG repeats in *VLDLR*

	Repeat number					
	5	6	7	8	9	10
Women (n = 1097; 2194 alleles)	851 (38.79%)	1 (0.05%)	0 (0%)	1226 (55.88%)	98 (4.47%)	18 (0.82%)
Men (n = 1108; 2216 alleles)	823 (37.14%)	1 (0.05%)	1 (0.05%)	1260 (56.86%)	108 (4.87%)	23 (1.04%)

Relation of serum lipid profile to genotypes for CYP17A1, MTP, or VLDLR or to BMD

Finally, the relation of serum lipid profile to *CYP17A1*, *MTP*, or *VLDLR* genotypes or to BMD was examined. There were no significant differences in the serum concentrations of total cholesterol, high density lipoprotein (HDL)-cholesterol, LDL-cholesterol, or triglycerides among *CYP17A1*, *MTP*, or *VLDLR* genotypes for women or men (data not shown). For women, BMD for the distal (D100) or proximal (P100) radius or total body was significantly related to the serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides, and BMD for the distal radius (D50), lumbar spine, femoral neck, or trochanter was significantly related to the serum concentrations of total cholesterol, LDL-cholesterol, and triglycerides (Table 9). For men, BMD for D50 or D100 was significantly related to the serum concentrations of LDL-cholesterol or total cholesterol, respectively, and BMD for the femoral neck or trochanter was significantly related to the serum concentration of HDL-cholesterol (Table 9).

Discussion

We have examined the relations of the −34T → C polymorphism of *CYP17A1*, the −493G → T polymorphism of *MTP*, and the CGG repeat polymorphism of *VLDLR* to BMD at various sites in community-dwelling Japanese women and men. Our results now show that the *CC* genotype of *CYP17A1* and the *TT* genotype of *MTP* are associated with increased BMD in postmenopausal and premenopausal women, respectively, and that two $(CGG)_{n \geq 8}$ alleles are associated with increased BMD in men.

Table 6
Relation between the CGG repeat number of *VLDLR* and BMD

	Women (n = 1097; 2194 alleles)		Men (n = 1108; 2216 alleles)	
	p	R^2	p	R^2
BMD measured with pQCT (mg/cm^3)				
D50	0.279		0.075	
D100	0.038	0.002	0.045	0.002
P100	0.064		0.267	
BMD measured with DXA (g/cm^2)				
Total body	0.085		0.168	
L2–L4	0.161		0.035	0.002
Femoral neck	0.085		0.013	0.003
Trochanter	0.048	0.002	0.028	0.002

Data were analyzed by simple regression analysis.

Association of the −34T → C polymorphism of CYP17A1 with BMD

The −34T → C polymorphism of *CYP17A1* has been associated with polycystic ovary syndrome [13] and breast cancer [35,36], with the *C* allele representing a risk factor for these diseases. The *C* variant was suggested to create a novel Sp1 recognition site that might increase *CYP17A1* gene expression [13]. In vitro experiments, however, did not detect a difference in binding of recombinant Sp1 to, or in transcriptional activity of, DNA sequences corresponding to the two allelic variants of *CYP17A1* [14,15]. We have now shown that the *CC* genotype of the −34T → C polymorphism of *CYP17A1* was associated with increased BMD for the femoral neck in postmenopausal women.

Several lines of evidence suggest that this polymorphism might influence endogenous sex hormone levels [37,38]. Premenopausal nulliparous women with the *CC* genotype were thus found to have higher concentrations of serum estradiol than those with the *TT* genotype [37], and postmenopausal women with the *CC* genotype had higher levels of serum estrone and estradiol than did those with the *TT* genotype [38]. In addition, postmenopausal women with the *CC* genotype were shown to be less likely to be current users of hormone replacement therapy [39]. These previous observations showing a relation between the *CC* genotype and higher serum concentrations of estrogen support our present data, given that estrogen exhibits beneficial effects on bone remodeling by inhibiting bone resorption and stimulating bone formation [40].

Men with the *CC* genotype were previously found to exhibit a 20% increase in bioavailable testosterone concentrations, a 3-cm increase in height, and a 5% increase in cross-sectional area of the femoral neck compared with men with the *TT* genotype, although *CYP17A1* genotype was not related to BMD at the femoral neck [16]. In our study, BMD was not related to *CYP17A1* genotype in men. We also did not detect a relation between *CYP17A1* genotype and the serum concentrations of total testosterone or free testosterone in men (data not shown). The lack of an association between *CYP17A1* genotype and BMD for men in the present study may thus be attributable, at least in part, to the absence of a relation between *CYP17A1* genotype and serum testosterone concentrations.

Table 7
BMD and other characteristics for men (n = 1108) according to *VLDLR* genotype

Characteristic	*SS*	*SL*	*LL*	*SS* + *SL*
Number (%)	154 (13.9)	517 (46.7)	437 (39.4)	671 (60.6)
Age (years)	59.4 ± 0.9	59.6 ± 0.5	58.6 ± 0.5	59.6 ± 0.4
Height (cm)	163.6 ± 0.5	164.6 ± 0.3	164.8 ± 0.3	164.4 ± 0.2
Body weight (kg)	61.1 ± 0.7	62.6 ± 0.4	62.7 ± 0.4	62.3 ± 0.4
BMD measured with pQCT (mg/cm^3)				
D50	261.8 ± 5.4	266.5 ± 2.9	269.2 ± 3.2	265.4 ± 2.6
D100	533.6 ± 7.5	539.7 ± 4.0	546.3 ± 4.4	538.3 ± 3.6
P100	1174.7 ± 11.3	1187.6 ± 6.1	1188.4 ± 6.6	1184.7 ± 5.4
BMD measured with DXA (g/cm^2)				
Total body	1.090 ± 0.007	1.083 ± 0.004	1.092 ± 0.004	1.085 ± 0.003
L2–L4	0.989 ± 0.012	0.968 ± 0.007[a]	1.000 ± 0.007	0.972 ± 0.006[a]
Femoral neck	0.745 ± 0.008	0.748 ± 0.004	0.760 ± 0.005	0.747 ± 0.004[b]
Trochanter	0.667 ± 0.008	0.663 ± 0.004	0.675 ± 0.005	0.664 ± 0.004[c]

BMD is adjusted for age, height, and body weight. Data are means ± SE.
[a] p = 0.002 versus *LL*.
[b] p = 0.032 versus *LL*.
[c] p = 0.048 versus *LL*.

In contrast to our observations for women, in a previous study [17] postmenopausal women with the *CC* genotype had a lower BMD at the femoral neck compared with those with the *TT* or the *TC* genotype. In a study of Danish women, among those with a body mass index of <25 kg/m^2, individuals with the *CC* genotype had a reduced BMD, bone mineral content, and cross-sectional area for the lumbar spine or femoral neck compared with those with the *T* allele, although these associations were not detected among women with a body mass index of ≥25 kg/m^2 [18]. In the present study, the *CC* genotype for the −34T → C polymorphism of *CYP17A1* was associated with increased BMD for postmenopausal women. The alleles of the −34T → C polymorphism associated with increased BMD thus differ between the present study (*C* allele) and previous studies (*T* allele) [17,18]. The reason for this apparent discrepancy is unclear. The distribution of −34T → C genotypes differed significantly between the postmenopausal Japanese women in our study (*TT*, 27%; *TC*, 49%; *CC*, 24%; n = 813) and the postmenopausal Caucasian women in the previous studies (*TT*, 42%; *TC*, 50%; *CC*, 8%; n = 252; $p < 0.0001$, χ^2 test [17]; and *TT*, 36%; *TC*, 49%; *CC*, 15%; n = 1788; $p < 0.0001$ [18]), possibly reflecting the difference in ethnicity. The difference in the effects of *CYP17A1* genotype on BMD between ethnic groups might be attributable either to a difference in lifestyle factors or gene–environment interactions or to linkage disequilibrium with other BMD susceptibility genes.

Association of the −493G → T polymorphism of MTP and the CGG repeat polymorphism of VLDLR with BMD

Bone loss is associated with an expansion of adipose tissue in bone marrow [19], and osteoblasts and adipocytes share a

Table 8
Effects of genotypes for *CYP17A1*, *MTP*, and *VLDLR* on BMD

Genotype	D50	D100	P100	Total body	L2–L4	Femoral neck	Trochanter
All women							
CYP17A1	0.761	0.827	0.291	0.223	0.041 (0.002)	**0.006** (0.004)	0.108
MTP	0.955	0.171	0.988	0.922	0.730	0.091	0.495
VLDLR	0.499	0.040 (0.002)	0.057	0.154	0.471	0.205	0.116
Premenopausal women							
CYP17A1	0.855	0.435	0.243	0.838	0.107	0.550	0.862
MTP	0.019 (0.019)	**0.003** (0.029)	0.039 (0.013)	0.172	0.843	0.045 (0.012)	0.157
VLDLR	0.708	0.553	0.411	0.906	0.552	0.500	0.333
Postmenopausal women							
CYP17A1	0.655	0.968	0.127	0.222	0.192	**0.005** (0.006)	0.085
MTP	0.334	0.759	0.580	0.846	0.115	0.207	0.618
VLDLR	0.421	0.098	0.174	0.118	0.794	0.242	0.178
Men							
CYP17A1	0.748	0.486	0.025 (0.004)	0.173	0.241	0.150	0.119
MTP	0.670	0.951	0.481	0.600	0.609	0.807	0.592
VLDLR	0.355	0.159	0.666	0.232	**0.003** (0.007)	0.034 (0.003)	0.044 (0.003)

Data were analyzed by multiple regression analysis including age, height, body weight, and genotype for *CYP17* (*TT* = *TC* = 0, *CC* = 1), *MTP* (*GG* = *GT* = 0, *TT* = 1), or *VLDLR* (*SS* = *SL* = 0, *LL* = 1). Data are p values (R^2). p values <0.01 are shown in bold.

Table 9
Effects of serum lipid profile on BMD

Serum lipid profile	D50	D100	P100	Total body	L2–L4	Femoral neck	Trochanter
Women							
Total cholesterol	**<0.001** (0.022)	**<0.001** (0.020)	**<0.001** (0.021)	**<0.001** (0.041)	**<0.001** (0.031)	**<0.001** (0.028)	**<0.001** (0.014)
HDL-cholesterol	0.124	**<0.001** (0.013)	**<0.001** (0.015)	**<0.001** (0.015)	0.383	0.251	0.309
LDL-cholesterol	**<0.001** (0.017)	**<0.001** (0.018)	**<0.001** (0.021)	**<0.001** (0.042)	**<0.001** (0.025)	**<0.001** (0.019)	**0.002** (0.008)
Triglycerides	**<0.001** (0.013)	**<0.001** (0.032)	**<0.001** (0.031)	**<0.001** (0.035)	**0.003** (0.008)	**<0.001** (0.012)	**<0.001** (0.009)
Men							
Total cholesterol	0.014 (0.005)	**0.006** (0.007)	0.090	0.704	0.350	0.981	0.507
HDL-cholesterol	0.775	0.855	0.893	0.204	0.028 (0.004)	**<0.001** (0.010)	**0.002** (0.008)
LDL-cholesterol	**0.007** (0.007)	0.012 (0.006)	0.041 (0.004)	0.397	0.838	0.117	0.046 (0.004)
Triglycerides	0.518	0.513	0.953	0.274	0.624	0.333	0.273

Data were analyzed by simple regression analysis. Data are p values (R^2). p values <0.01 are shown in bold.

common progenitor derived from stromal cells in bone marrow [20]. Products of lipoprotein oxidation and an atherogenic diet also inhibit preosteoblast differentiation and result in reduced bone mineralization [20,21]. In addition, lipid-lowering agents (statins) stimulate bone formation and inhibit bone resorption, resulting in the prevention of both bone loss and osteoporotic fractures [22]. Early postmenopausal women with an atherogenic serum lipid profile (total cholesterol of ≥240 mg/dl, LDL-cholesterol of ≥160 mg/dl, or lipoprotein(a) of ≥25 mg/dl) had a lower BMD for the lumbar spine or femoral neck and an increased risk of osteopenia compared with those with a normal lipid profile [23]. Postmenopausal women with increased plasma concentrations of LDL-cholesterol have also been shown to be at greater risk of developing osteopenia than are those with normal concentrations, suggesting that an increased plasma level of LDL-cholesterol is a risk factor for reduced BMD [24]. These observations suggest the existence of a close relation between lipid and bone metabolism as well as demonstrating adverse effects of an atherogenic lipid profile on bone remodeling.

Although hyperlipidemia has been shown to be related to reduced BMD or osteoporosis [23,24], the mechanism that underlies this relation remains unknown. A contributing factor might be the fact that estrogen deficiency results both in impairment of lipid metabolism and in acceleration of bone resorption [23]. Alternatively, bone formation might be impaired by ischemia of bone tissue caused by dyslipidemia, given that osteoblast progenitors are located adjacent to the subendothelial matrix of bone vessels and atherosclerosis may influence the function of these bone-forming cells [23]. Another possibility relates to the potential interaction between cholesterol synthesis and regulation of bone metabolism, given that statins have beneficial effects on bone mass [22].

MTP mediates the transport of triglycerides, cholesteryl esters, and phospholipids between phospholipid surfaces [26]. The −493G → T polymorphism of *MTP* has been associated with the plasma concentration of LDL-cholesterol [28], with the *T* allele being related to low concentrations. However, other studies have failed to detect an association between this polymorphism and the plasma concentration of LDL-cholesterol [41] or have detected an adverse effect of the *T* allele [42]. This discrepancy might be related to the observation that the phenotype associated with the −493G → T polymorphism of *MTP* is modulated by visceral obesity and hyperinsulinemia [43]. In the present study, BMD for the distal radius was significantly greater in premenopausal women with the *TT* genotype than in those with the *G* allele. *MTP* genotype was not, however, associated with the serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, or triglycerides in women. It is thus unlikely that the association of this polymorphism with BMD in premenopausal women was attributable to an effect on serum lipid concentrations. The mechanism by which the −493G → T polymorphism of *MTP* affects BMD thus remains unclear.

VLDL-R is a member of the LDL-R family of proteins, is abundant in fatty acid-metabolizing tissues, binds triglyceride-rich lipoproteins (but not LDL), and functions as a peripheral receptor for remnant lipoproteins [44]. VLDL-R-deficient mice manifest a reduced adipose tissue mass compared with wild-type mice [45]. The CGG repeat polymorphism of *VLDLR* has been associated with the prevalence of sporadic Alzheimer disease in Japanese [34] and with that of vascular dementia [46]. It has also been shown to be related to the plasma concentrations of lipoproteins, with the $(CGG)_{n=9}$ allele being associated with lower levels of lipoprotein E:B and higher levels of lipoprotein A-I [33]. The frequencies of the $(CGG)_{n=9}$ and $(CGG)_{n=5}$ alleles were lower and higher, respectively, in subjects with dyslipidemia treated with lipid-lowering drugs than in control subjects, although the statistical significance of this difference was marginal ($p = 0.054$) [33]. In the present study, the combination of two $(CGG)_{n\geq 8}$ alleles was significantly associated with increased BMD for the lumbar spine in men. There were, however, no differences in the serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, or triglycerides among *VLDLR* genotypes in men. It is thus unlikely that the association of the CGG repeat polymorphism with BMD in men was attributable to an effect on serum lipid concentrations. The effects of the CGG repeat polymorphism of *VLDLR* on gene expression or the function of the encoded protein have not been determined.

The molecular mechanism of the effect of this polymorphism on BMD thus remains unclear.

There are some limitations of the present study: (1) Serum concentrations of vitamin D were not measured in the study population. Although no subjects with clinical vitamin D deficiency, such as osteomalacia, were included, the National Nutrition Survey in 2001 suggested that vitamin D intake was smaller than the daily requirement (100 IU) in ~25% of Japanese individuals. The serum concentration of free thyroxine slightly exceeded the normal range (0.77 to 1.93 ng/dl) in three subjects. It is thus possible that individuals with subclinical vitamin D deficiency or thyrotoxicosis were included in the present study. (2) Given that the effects of single polymorphisms on BMD were small, the association between a polymorphism and BMD might be influenced by age, gender, or status of sex hormones. The associations observed in the present study were thus not apparent in the entire population, only in subgroups. (3) Given the multiple comparisons of genotypes with BMD, we adopted a strict criterion ($p < 0.01$) for statistical significance. However, we did not perform Bonferroni's correction. It is therefore not possible to rule out completely the occurrence of potential statistical errors such as false positives. (4) It is possible that the polymorphisms examined in our study are in linkage disequilibrium with polymorphisms of nearby genes that are actually responsible for regulating BMD.

Despite these various limitations, our present results suggest that *CYP17A1* and *MTP* are susceptibility loci for increased BMD in postmenopausal and premenopausal Japanese women, respectively, and that *VLDLR* constitutes such a locus for Japanese men.

Materials and methods

Study population

The National Institute for Longevity Sciences Longitudinal Study of Aging is a population-based prospective cohort study of aging and age-related diseases [47]. The subjects are stratified by both age and gender and are randomly selected from resident registrations in the city of Obu and town of Higashiura in central Japan [48,49]. The lifestyle of residents of this area is typical of that of Japanese individuals. The numbers of men and women recruited are similar and age at the baseline is 40 to 79 years, with similar numbers of participants in each decade (40s, 50s, 60s, 70s). The subjects will be followed up every 2 years. All participants are subjected at a special center to a detailed examination, which includes not only medical evaluation but also assessment of exercise physiology, body composition, nutrition, and psychology. Individuals who had disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, chronic renal failure, rheumatoid arthritis, as well as parathyroid, thyroid, adrenal, and other endocrine diseases, or who had taken drugs that affect bone metabolism, such as estrogen, glucocorticoids, bisphosphonates, vitamin D, and statins, were excluded from the present study. Individuals whose genotypes were not successfully determined (two individuals for *CYP17A1*, 27 individuals for *VLDLR*) were also excluded from the analysis.

We examined the relations of BMD at various sites to the −34T → C polymorphism of *CYP17A1* (NCBI, dbSNP, rs743572) in 2230 individuals (1108 women, 1122 men), to the −493G → T polymorphism of *MTP* (NCBI, dbSNP, rs1800591) in 2232 individuals (1108 women, 1124 men), and to the triplet repeat [$(CGG)_n$] polymorphism of *VLDLR* (GenBank Accession No. D16495) in 2205 individuals (1097 women, 1108 men). In addition, to uncover potential differences between women according to menopausal status, we conducted all analyses separately for premenopausal and postmenopausal women. Menopausal status was evaluated with a detailed questionnaire, and menopause was defined as complete cessation of menstruation. The study protocol complies with the Declaration of Helsinki and was approved by the Committee on Ethics of Human Research of the National Institute for Longevity Sciences. Written informed consent was obtained from each subject.

Measurement of BMD

BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) (Desiscan 1000; Scanco Medical, Bassersdorf, Switzerland) and was expressed as D50 (distal radius BMD for the inner 50% of the cross-sectional area, comprising mostly cancellous bone), D100 (distal radius BMD for the entire cross-sectional area, including both cancellous and cortical bone), and P100 (proximal radius BMD for the entire cross-sectional area, consisting mostly of cortical bone). BMD for the total body, lumbar spine (L2–L4), right femoral neck, and right trochanter was measured by dual-energy X-ray absorptiometry (DXA) (QDR 4500; Hologic, Bedford, MA, USA). The coefficients of variation of the pQCT instrument for BMD values were 0.7 (D50), 1.0 (D100), and 0.6% (P100), and those of the DXA instrument were 0.9 (total body), 0.9 (L2–L4), 1.3 (femoral neck), and 1.0% (trochanter).

Determination of genotype

Genotypes for *CYP17A1* and *MTP* were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) [50]. The polymorphic region of *CYP17A1* was amplified by the polymerase chain reaction (PCR) with sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-GCCACAGCTCTTCTACTCCAX*C*G-3′) or Texas red (5′-TGCCACAGCTCTTCTACTCCAX*T*G-3′) and with an antisense primer labeled at the 5′ end with biotin (5′-ATAAGC-TAGGGTAAGCAGCAAGAGA-3′). The polymorphic region of *MTP* was amplified with allele-specific sense

primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-ACATTATTTTGAAGTGATTGGX*T*G-3′) or Texas red (5′-ACATTATTTTGAAGTGATTGGX-*G*G-3′) and with an antisense primer labeled at the 5′ end with biotin (5′-AATTCACACTGAATTTTAGGATTTA-3′). The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 3 (for *CYP17A1*) or 4.5 (for *MTP*) mmol/L $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 65 (for *CYP17A1*) or 62.5°C (for *MTP*) for 30 s, and extension at 72 (for *CYP17A1*) or 70°C (for *MTP*) for 30 s; and a final extension at 72 (for *CYP17A1*) or 70°C (for *MTP*) for 2 min. The amplified DNA was then incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a 96-well plate at room temperature, and the plate was placed on a magnetic stand. The supernatants from each well were transferred to the wells of a 96-well plate containing 0.01 mol/L NaOH and were measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red.

The triplet repeats $[(CGG)_n]$ in the 5′ untranslated region of *VLDLR* were amplified by PCR with a sense primer (5′-CTCCCTTCCCCCGCCAACTC-3′) and with an antisense primer labeled at the 5′ end with 6-carboxyfluorescein (5′-GCCCAGAGCGCGGACGTG-3′). The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 1 mmol/L $MgSO_4$, and 0.4 U of KODplus DNA polymerase (Toyobo) in polymerase buffer. The amplification protocol comprised initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 2 min. The size of triplet-repeat-containing DNA fragments amplified by PCR was determined with a Prism 3100 DNA sequencer with GeneScan and Genotyper software (Applied Biosystems, Foster City, CA, USA).

Measurement of serum lipid profile

Venous blood was collected in the early morning after the subjects had fasted overnight. Blood samples were centrifuged at 1600 *g* for 15 min at 4°C, and serum was separated and stored at −30°C until analysis. The serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured by standard methods.

Statistical analysis

Statistical analysis was performed with SAS software (SAS Institute, Cary, NC, USA). Data were compared among three groups by one-way analysis of variance and the Tukey–Kramer post hoc test and between two groups by the unpaired Student *t* test. BMD values were compared among genotypes of each polymorphism with adjustment for age, height, and body weight by the least-squares method in a general linear model. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departure from Hardy–Weinberg equilibrium. The relation of the number of CGG repeats in *VLDLR* to BMD was analyzed by simple regression analysis. The relation of serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, or triglycerides to BMD was also analyzed by simple regression analysis. The effect of genotype of each polymorphism on BMD was evaluated by multivariable regression analysis; *p* values and R^2 were calculated from analyses including age, height, body weight, and genotype for *CYP17A1* (*TT* = *TC* = 0, *CC* = 1), *MTP* (*GG* = *GT* = 0, *TT* = 1), or *VLDLR* (*SS* = *SL* = 0, *LL* = 1). A *p* value of <0.01 was considered statistically significant.

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