Association of genetic variants of *MAOA* and *SH2B1* with bone mineral density in community-dwelling Japanese women

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Received November 23, 2007; Accepted December 27, 2007

Abstract. Although bone mineral density (BMD) is a complex trait that is influenced by both genetic and environmental factors, heritability studies in twins and families have shown that genetic factors account for 60-85% of its variance. We examined the relation of the variable number of tandem repeats (VNTR) polymorphism of the monoamine oxidase A gene (MAOA) and the $A \rightarrow G$ (Thr484Ala) polymorphism of the SH2B adaptor protein 1 gene (SH2B1) to BMD in communitydwelling Japanese women and men. The 2235 subjects (1107 women, 1128 men) were aged 40-79 years and were randomly recruited for a population-based prospective cohort study of aging and age-related diseases in Japan. BMD at the distal and proximal radius was measured by peripheral quantitative computed tomography, and the BMD of the total body, lumbar spine (L2-L4), right femoral neck and right trochanter was measured by dual-energy X-ray absorptiometry. The genotypes of the VNTR polymorphism of MAOA were determined by DNA fragment analysis, and those of the $A \rightarrow G$ (Thr484Ala) polymorphism of SH2B1 by melting curve analysis. The VNTR polymorphism of MAOA was associated with the BMD of the distal radius, total body, lumbar spine and trochanter in all women, and with the BMD of the total body and trochanter in postmenopausal ones, with the L (four repeats) and S (two or three repeats) alleles reflecting increased and decreased BMD, respectively. The A→G (Thr484Ala) polymorphism of SH2B1 was associated with the BMD of the lumbar spine in all women, with the BMD of the proximal radius in premenopausal women and with the BMD of the lumbar spine, femoral neck and trochanter in postmenopausal women, with the variant G allele being related to increased BMD. These results suggest that MAOA and SH2B1 are determinative loci for bone mass in Japanese women, especially in postmenopausal ones.

Introduction

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 the bone, both resulting in a predisposition to fractures (1). Although reproductive, nutritional and lifestyle factors influence BMD, family and twin studies have suggested that it is largely (60-85%) heritable and controlled by multiple genes (2-4). Personalized prevention of osteoporosis and osteoporotic fractures is an important public health goal and can be approached by identifying disease susceptibility genes. Although genetic linkage analyses (5-7) and candidate gene association studies (7-10) have implicated various loci and genes in the predisposition to osteoporosis or fractures, the genes that confer susceptibility to this condition have yet to be definitively identified. In addition, because of ethnic differences in gene polymorphisms as well as in lifestyle and other environmental factors, it is important to examine polymorphisms in relation to BMD in individual ethnic groups.

We have been attempting to identify, with a candidate gene approach, the genetic variants associated with BMD in Japanese women or men recruited for a population-based prospective cohort study. In the present study, we selected the monoamine oxidase A gene (MAOA) and SH2B adaptor protein 1 gene (SH2B1) as ones that might contribute to bone remodeling (Table I), and examined the relation between the polymorphisms of these genes and BMD, even though there is no apparent biological link between them. Our aim was to identify a single polymorphism significantly associated with BMD in each gene. Of the polymorphisms previously identified, we selected those that might be expected to affect gene function. We then examined the relation between these polymorphisms and BMD in community-dwelling Japanese women and 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, the details of which have been described previously (11-15). Individuals with disorders known to cause abnormalities of bone metabolism, including diabetes mellitus, chronic renal failure, rheumatoid arthritis, as well as thyroid, parathyroid,

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Key words: polymorphism, genetics, osteoporosis, bone density, MAOA, SH2B1

Locus	Gene	Symbol	Polymorphism	NCBI database
Xp11.3	Monoamine oxidase A	MAOA	$\label{eq:VNTR} \begin{split} & \text{VNTR} \left[(\text{ACCGGCACCGGCACCAGTACCCGCACCAGT})_n \right] \\ & \text{A} \! \! \! \! \! \! \! \! \! \! \! \! \! \! \! \! \! \! \!$	M89636 (nt 208-327)
16p11.2	SH2B adaptor protein 1	SH2B1		rs7498665

Table I. The two gene polymorphisms examined in the study.

adrenal and other endocrine diseases, or those who had taken drugs that affect bone metabolism such as estrogen, glucocorticoids, bisphosphonates and vitamin D, were excluded from the present study. We thus examined the relation between gene polymorphisms and BMD in 2235 individuals (1107 women, 1128 men). Individuals whose genotypes were not successfully determined were also excluded from the analysis. In addition, to uncover potential differences between women according to menopausal status, we conducted all determinations for associations in premenopausal and postmenopausal women separately. Menopausal status was evaluated by a detailed questionnaire, with menopause defined as complete cessation of menstruation. Because of their small number (n=17), perimenopausal women were excluded from the study. The study protocol complied with the Declaration of Helsinki and was approved by the Committee on Ethics of Human Research of the National Institute for Longevity Sciences. Written and informed consent was obtained from each subject.

Measurement of BMD. BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) with a Desiscan 1000 instrument (Scanco Medical, Bassersdorf, Switzerland) and was expressed as D50 (BMD for the inner 50% of the cross-sectional area of the distal radius, comprising mostly cancellous bone), D100 (BMD for the entire crosssectional area of the distal radius, including both cancellous and cortical bone) and P100 (BMD for the entire cross-sectional area of the proximal radius, consisting mostly of cortical bone). The BMD of the total body, lumbar spine (L2-L4), right femoral neck and right trochanter was measured by dualenergy X-ray absorptiometry (DXA) with a QDR 4500 instrument (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 0.9% (total body), 0.9% (L2-L4), 1.3% (femoral neck) and 1.0% (trochanter).

Determination of genotype. Genotypes for the variable number of tandem repeats (VNTR) polymorphism in the promoter region of *MAOA* were determined by DNA fragment analysis. The polymorphic region of *MAOA* was amplified by polymerase chain reaction (PCR) with a sense primer (5'-CCCA GGCTGCTCCAGAAAC-3') labeled at the 5' end with 6carboxyfluorescein and with an antisense primer (5'-GGA CCTGGGCAGTTGTGC-3'). The reaction mixture (25 μ l) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l MgSO₄ and 1.25 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 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 2 min. The fragment size of VNTR was determined with a PRISM 3100 DNA sequencer and with GeneScan and Genotyper software (Applied Biosystems, Foster City, CA, USA).

Genotypes for the A→G (Thr484Ala) polymorphism of SH2B1 were determined by melting curve analysis (intercalater-mediated fluorescence resonance energy transfer probe method). The polymorphic region of SH2B1 was amplified by PCR in a reaction mixture (25 μ l) containing 20 ng of DNA, 5 pmol each of sense (5'-TGGAACTGC TTCCCCCAGAGTTG-3') and antisense (5'-TACCTG TGGCTGTTTCCGGAGTGTC-3') primers, 0.2 mmol/l of each deoxynucleoside triphosphate, 2 mmol/l MgCl₂ and 1.25 U of rTaq DNA polymerase in polymerase buffer. The amplification protocol comprised initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 2 min. A solution $(2 \mu l)$ containing 10 pmol of probe (5'-GAACTGTCCCTG CTGGGG-3') labeled at the 5' end with Texas red and 1/400 diluted SYBR Green I was added to the PCR products, which were then transferred to a PRISM 7700 instrument (Applied Biosystems) for measurement of melting temperature. The program for analytic melting comprised incubation at 95°C for 30 sec, 40°C for 1 min, and temperatures increasing to 80°C over 10 min. The fluorescence signals were detected at excitation and emission wavelengths of 485 and 612 nm, respectively.

Statistical analysis. Data were presented as means \pm SE or \pm SD, as indicated. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC, USA). Data from three genotype groups were compared by one-way analysis of variance and the Tukey-Kramer post hoc test, and between two groups (dominant or recessive model) by the unpaired Student's t-test. The BMD values of genotypes for each polymorphism were compared with adjustment for age, height and body weight by the least squares method in a general linear model. The relation of the number of repeats in the VNTR polymorphism of MAOA to BMD was analyzed by multiple regression analysis with adjustment for age, height and body weight. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify a significant departure from Hardy-Weinberg equilibrium. A P-value of <0.05 was considered statistically significant.

Results

Relation between the VNTR polymorphism of MAOA and BMD. The number of repeats in the VNTR polymorphism of

Characteristic	SS	SL	LL	SS + SL	SL + LL
Number (%)	420 (38.2)	499 (45.4)	180 (16.4)	919 (83.6)	679 (61.8)
Age (years)	59.4±0.5	58.9±0.5	59.6±0.8	59.1±0.4	59.1±0.4
Height (cm)	150.8±0.3	151.5±0.3	152.1±0.5 ^b	151.1±0.2	151.6±0.2°
Body weight (kg)	52.2±0.4	52.9±0.4	52.7±0.6	52.6±0.3	52.9±0.3
BMD measured with	pQCT (mg/cm ³)				
D50	181.2±3.0	187.7±2.8	191.1±4.7	184.7±2.0	188.5±2.4
D100	478.9±4.4	490.8±4.0	493.8±6.7	485.4±3.0	491.6±3.4 ^d
P100	1157.0±7.1	1154.8±6.5	1148.1±10.9	1155.8±4.8	1153.1±5.6
BMD measured with	DXA (g/cm ²)				
Total body	0.953±0.004	0.975±0.004 ^e	0.971±0.006	0.965±0.003	0.974 ± 0.003^{f}
L2-L4	0.856±0.006	0.875±0.006	0.865±0.009	0.866±0.004	0.873±0.005 ^g
Femoral neck	0.672±0.004	0.681±0.004	0.684±0.006	0.677±0.003	0.682±0.003
Trochanter	0.564 ± 0.004	0.574±0.004	0.582 ± 0.006^{h}	0.569 ± 0.003	0.576 ± 0.003^{i}

Table II. BMD and other characteristics of all women (n=1099) according to MAOA genotype.^a

^aBMD is adjusted for age, height and body weight. Data are the means \pm SE. ^bP=0.0434, ^cP=0.0270, ^dP=0.0235, ^eP=0.004, ^fP=0.0001, ^gP=0.0343, ^hP=0.0385, ⁱP=0.0166 versus SS.

Characteristic	SS	SL	LL	SS + SL	SL + LL
Number (%)	319 (39.5)	347 (43.0)	141 (17.5)	666 (82.5)	488 (60.5)
Age (years)	63.4±0.5	64.4±0.5	63.7±0.7	63.9±0.3	64.2±0.4
Height (cm)	149.7±0.3	150.2±0.3	151.1±0.5	150.0±0.2 ^b	150.4±0.3
Body weight (kg)	51.5±0.5	52.2±0.4	52.5±0.7	51.9±0.3	52.3±0.4
BMD measured with	pQCT (mg/cm ³)				
D50	159.7±3.6	164.8±3.4	172.5±5.4	162.3±2.5	167.0±2.9
D100	436.9±5.2	446.6±4.9	453.1±7.7	441.9±3.6	448.5±4.2
P100	1084.6±8.5	1079.9±8.2	1071.7±12.7	1082.1±5.9	1077.5±6.9
BMD measured with	$DXA (g/cm^2)$				
Total body	0.907±0.005	0.926±0.005°	0.931±0.007 ^d	0.917±0.003	0.927±0.004
L2-L4	0.799±0.007	0.818±0.007	0.814±0.011	0.809 ± 0.005	0.817±0.006
Femoral neck	0.640±0.005	0.645 ± 0.005	0.655±0.007	0.642±0.003	0.648±0.004
Trochanter	0.534±0.005	0.541±0.004	0.553 ± 0.007^{f}	0.537 ± 0.003^{g}	0.544±0.004

Table III. BMD and other characteristics of postmenopausal women (n=807) according to MAOA genotype.^a

^aBMD is adjusted for age, height and body weight. Data are the means \pm SE. ^bP=0.0444, ^gP=0.0337 versus *LL*; ^eP=0.0103, ^dP=0.0169, ^eP=0.0009, ^fP=0.0454 versus *SS*.

MAOA was two, three or four for all women (mean \pm SD, 3.4 \pm 0.5; n=2198 alleles) and men (3.4 \pm 0.5; n=1096 alleles). The number of repeats in this polymorphism was related to the BMD in terms of the distal radius (D100, P=0.0102), total body (P=0.0103) or trochanter (P=0.0210) in all women, whereas no relation was detected in men. At each of these sites, the BMD was greater in women with four repeats than in those with three repeats. Given that the mean number of repeats was 3.4 for men and women, we designated alleles containing two or three repeats as short (*S*) and those containing four repeats as long (*L*).

Age and body weight did not differ among all women (Table II), premenopausal women (data not shown), and postmenopausal women (Table III) with the SS, SL and LL genotypes of MAOA. Height was greater in individuals with the LL genotype and in the combined group of SL and LL genotypes than in individuals with the SS genotype for all women, and greater in individuals with the LL genotype than in the combined group of SS and SL genotypes for postmenopausal women. Height did not differ among the premenopausal women with MAOA genotypes. In all women, the BMD of D100 and the lumbar spine was greater in the combined

Characteristic	AA	AG	GG	AA + AG	AG + GG
Number (%)	820 (74.1)	272 (24.6)	15 (1.4)	1092 (98.6)	287 (25.9)
Age (years)	59.5±0.4	58.7±0.7	56.8 ± 2.8	59.3±0.3	58.6±0.6
Height (cm)	151.2±0.2	151.5±0.4	153.3±1.6	151.3±0.2	151.6±0.4
Body weight (kg)	52.7±0.3	52.3±0.5	55.6±2.1	52.6±0.2	52.5±0.5
BMD measured with	pQCT (mg/cm ³)				
D50	184.7±2.2	186.1±3.7	192.3±15.6	185.1±1.9	186.5±3.6
D100	485.1±3.1	486.8±5.4	507.6±22.6	485.6±2.7	487.9±5.2
P100	1151.5±5.1	1157.8±8.6	1194.4±36.4	1153.1±4.4	1159.8±8.4
BMD measured with	DXA (g/cm ²)				
Total body	0.964±0.003	0.965 ± 0.005	0.998±0.022	0.965 ± 0.003	0.967±0.005
L2-L4	0.867±0.004	0.857 ± 0.008^{b}	0.938±0.033	0.864±0.004°	0.861±0.007
Femoral neck	0.676±0.003	0.680 ± 0.005	0.711±0.022	0.677±0.003	0.682±0.005
Trochanter	0.569±0.003	0.573±0.005	0.601±0.021	0.570 ± 0.002	0.575±0.005
^a BMD is adjusted for as	e height and body weig	wht Data are the means -	- SE ^b P=0.0432 ^c P=0.0	260 versus GG	

Table IV. BMD and other characteristics of all women (n=1107) according to SH2B1 genotype.^a

group of SL and LL genotypes than in individuals with the SS genotype (Table II). BMD for the total body was greater in individuals with the SL genotype and in the combined group of SL and LL genotypes than in individuals with the SS genotype. BMD for the trochanter was greater in individuals with the LL genotype or the combined group of SL and LL genotypes than in individuals with the SS genotype. The differences in the BMD of D100, total body and lumbar spine between the combined group of SL and LL genotypes and individuals with the SS genotype (expressed as a percentage of the larger value) were 2.6, 2.2, and 1.9%, respectively, and the difference in the BMD of the trochanter between individuals with the LL genotype and those with the SS genotype was 3.1%. In postmenopausal women, the BMD of the total body was greater in individuals with the LL genotype, SL genotype and the combined group of SL and LL genotypes than in individuals with the SS genotype (Table III). The BMD of the trochanter was greater in individuals with the LL genotype than in those with the SS genotype or in the combined group of SS and SL genotypes. The differences in the BMD of the total body and trochanter between individuals with the LL and SS genotype were 2.6 and 3.4%, respectively. In premenopausal women or men, the BMD did not differ among the MAOA genotypes (data not shown).

Relation of the $A \rightarrow G$ (Thr484Ala) polymorphism of SH2B1 to BMD. The distribution of $A \rightarrow G$ genotypes of SH2B1 was in Hardy-Weinberg equilibrium. Age, height and body weight did not differ among genotype groups for all women (Table IV), premenopausal women (data not shown) or postmenopausal women (Table V). In all the women, the BMD of the lumbar spine was greater in individuals with the GG genotype than in those with the AG genotype or the combined group of AA and AG genotypes. The difference in the BMD of the lumbar spine between individuals with the GG genotype and the combined group of AA and AG genotypes was 7.9%. In premenopausal women, the BMD of P100 in individuals with the AG genotype and in the combined group of AG and GG genotypes was greater than in individuals with the AA genotype (data not shown). In postmenopausal women, the BMD of the lumbar spine was greater in individuals with the GG genotype than in the combined group of AA and AG genotypes (Table V). The BMD of the femoral neck was greater in the combined group of AG and GG genotypes than in individuals with the AA genotype. The BMD of the trochanter was greater in individuals with the GG genotype than in the combined group of AA and AG genotypes, and greater in the combined group of AG and GG genotypes than in individuals with the AA genotype. The differences in the BMD of the lumbar spine and trochanter between individuals with the GG genotype and the combined group of AA and AG genotypes was 9.2 and 8.5%, respectively, and the difference in the BMD of the femoral neck between the combined group of AG and GG genotypes and individuals with the AA genotype was 2.4%. For men, the distribution of SH2B1 genotypes was in Hardy-Weinberg equilibrium; there was no difference in BMD among SH2B1 genotypes (data not shown).

Discussion

We examined the relation of the VNTR polymorphism of MAOA and the A \rightarrow G (Thr484Ala) polymorphism of SH2B1 to BMD at various sites in community-dwelling Japanese women and men. Our results showed that the polymorphisms of MAOA and SH2B1 were associated with BMD in women, especially in postmenopausal individuals, suggesting that MAOA and SH2B1 are determinative loci for bone mass in Japanese women.

MAOA is an important catabolic enzyme that regulates levels of monoamine neurotransmitters, including serotonin, dopamine and noradrenaline, in the central nervous system. The VNTR polymorphism in the promoter region of MAOAconsists of a 30-bp repeated sequence that is present in 3, 3.5, 4 or 5 copies (16), and has been shown to affect the tran-

Characteristic	AA	AG	GG	AA + AG	AG + GG
Number (%)	605 (74.3)	198 (24.3)	11 (1.4)	803 (98.6)	209 (25.7)
Age (years)	64.0±0.3	63.6±0.6	60.5±2.6	63.9±0.3	63.4±0.6
Height (cm)	150.1±0.2	150.4±0.4	152.0±1.8	150.2±0.2	150.5±0.4
Body weight (kg)	52.1±0.3	51.7±0.6	54.5±2.4	52.0±0.3	51.8±0.6
BMD measured with	pQCT (mg/cm ³)				
D50	162.4±2.6	166.9±4.5	179.7±18.8	163.5±2.3	167.6±4.4
D100	441.6±3.7	446.3±6.4	481.8±26.9	442.8±3.2	448.2±6.3
P100	1079.0 ± 6.2	1078.9±10.7	1057.1±44.6	1079.0±5.3	1083.1±10.4
BMD measured with	DXA (g/cm ²)				
Total body	0.916±0.004	0.925±0.006	0.963±0.026	0.918±0.003	0.927±0.006
L2-L4	0.808±0.005	0.810±0.009	0.890±0.038	0.808 ± 0.004^{b}	0.814±0.009
Femoral neck	0.640±0.003	0.655±0.006	0.683±0.025	0.644±0.003	0.656±0.006°
Trochanter	0.537±0.003	0.547 ± 0.006	0.589±0.024	0.539 ± 0.003^{d}	0.550±0.006e
^a BMD is adjusted for an	e height and hody weigh	nt. Data are the means + 9	SE ^b P-0.0335 ^d P-0.041	8 versus CC: °P-0.0182	P=0.0471 versus AA

Table V. BMD and other characteristics of postmenopausal women (n=814) according to SH2B1 genotype.^a

scriptional activity of the gene in vitro (16-18). Transcription of VNTR alleles with 3.5 or 4 repeats is more efficient than in the allele with 3 repeats in various cell lines and human male skin fibroblasts (16-18). This polymorphism was also shown to affect the expression and activity of MAOA in the brain of individuals with Alzheimer's disease (19). In addition, it has been associated with various pathological behavioral traits, such as mood disorders, autism, aggression and impulsivity (20-23). We have now shown that the VNTR polymorphism of MAOA was associated with BMD in postmenopausal women, with the L and S alleles reflecting increased and decreased BMD, respectively. As far as we are aware, this is the first demonstration of the association of this polymorphism of MAOA with BMD, although the underlying molecular mechanism remains to be elucidated. This association may be attributable, however, to the effects of the polymorphism on the neuroendocrine systems, given that neuroendocrine disorders can result in a decrease in the concentration of growth hormone and sex steroids and thus accelerate the development of osteoporosis (24).

SH2B1 is a widely-expressed cytoplasmic protein that simultaneously binds, via its Src homology 2 (SH2) domain, to both Janus kinase 2 (JAK2) and insulin receptor substrate 2 (IRS2), thereby promoting the leptin-induced activation of the phosphoinositide 3-kinase signaling pathway in cultured cells (25,26). SH2B1-deficient mice develop insulin resistance and type 2 diabetes mellitus (27) as well as severe leptin resistance, hyperphagia and obesity (28). SH2B1 is thus a key cytoplasmic signaling molecule that acts as a positive regulator of leptin and insulin signal transduction in mice. The $A \rightarrow G$ (Thr484Ala) polymorphism of SH2B1 (rs7498665) is a tag single nucleotide polymorphism (SNP) that represents five common SNPs in complete linkage disequilibrium within a 16-kb region encompassing SH2B1 (29). This polymorphism was associated with the serum concentration of leptin, total body fat, waist circumference and body weight in Caucasian female twins, although it is predicted to not affect protein structure or function and is likely in linkage disequilibrium with an as yet unidentified functional variant of SH2B1 (29). We have now shown that the A-G (Thr484Ala) polymorphism of SH2B1 is associated with BMD in women, especially in postmenopausal women, with the variant G allele being related to increased BMD. Given that leptin plays an important role in bone remodeling (30-32), the association of this polymorphism of SH2B1 with BMD may be attributable to effects on leptin signaling.

Given the multiple comparisons of genotypes with BMD at various sites in the present study, it is not possible to exclude potential type I errors (false positives). It is also possible that the polymorphisms associated with BMD in our study are in linkage disequilibrium with other polymorphisms in the same gene or with polymorphisms of nearby genes that are actually the determinants of BMD. Furthermore, the relevance of the polymorphisms to gene transcription or to protein structure or function and their effects on bone remodeling were not determined in the present study.

In conclusion, our present results suggest that *MAOA* and *SH2B1* are determinative loci for BMD in Japanese women. Determination of genotypes for these polymorphisms may prove informative for the assessment of the genetic risk of reduced BMD. Given that multiple variants, each having a small effect, will likely be found to be responsible for a large fraction of the genetic component of osteoporosis, identification of additional osteoporosis susceptibility genes will allow for a more accurate assessment of the genetic component of this condition.

Acknowledgements

This work was supported in part by a Research Grant for Comprehensive Research on Aging and Health (H17-Chojyu-039) from the Ministry of Health, Labor, and Welfare of Japan.

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