doi:10.1006/geno.2002.6793, available online at http://www.idealibrary.com on IDEAL

TABLE 2: BMD and other characteristics of women (n = 1090) according to menopausal status and *CCR2* genotype

	Premenopausal (n = 277)				Postmenopausal (n = 813)			
	GG	*GA*	*GG + GA*	*AA*	*GG*	*GA*	*GG + GA*	*AA*
Number (%)	158 (57.0)	101 (36.5)	259 (93.5)	18 (6.5)	413 (50.8)	321 (39.5)	734 (90.3)	79 (9.7)
Age (years)	45.7 ± 0.3	46.1 ± 0.3	45.9 ± 0.2	46.1 ± 0.8	64.0 ± 0.4	64.0 ± 0.5	64.0 ± 0.3	64.0 ± 1.0
BMI (kg/m^2)	22.8 ± 0.3	22.9 ± 0.3	22.8 ± 0.2	22.2 ± 0.8	23.0 ± 0.2	22.8 ± 0.2	22.9 ± 0.1	23.0 ± 0.4
Smoking (%)	13.9	7.9	11.6	0	6.8	5.0	6.0	7.6
BMD values measured by pQCT (mg/cm^3)								
D50	246.2 ± 4.5	250.9 ± 5.5	248.1 ± 3.5	231.6 ± 12.9	160.5 ± 3.5	163.6 ± 3.9	161.8 ± 2.6	177.5 ± 7.8[a]
Adjusted D50	242.1 ± 5.9	246.8 ± 7.1	243.7 ± 5.4	228.3 ± 13.8	163.2 ± 5.1	167.3 ± 5.5	164.9 ± 4.7	180.1 ± 8.1[b]
D100	602.7 ± 6.3	616.2 ± 7.6	608.2 ± 4.9	602.5 ± 18.0	441.6 ± 5.4	439.8 ± 6.2	440.8 ± 4.1	457.3 ± 12.3
Adjusted D100	597.6 ± 8.4	611.5 ± 10.1	602.3 ± 7.7	597.0 ± 19.5	441.1 ± 7.4	440.6 ± 7.9	440.9 ± 6.8	456.6 ± 11.7
P100	1360.6 ± 9.7	1363.7 ± 11.7	1361.9 ± 7.5	1388.7 ± 27.7	1072.7 ± 9.5	1076.8 ± 10.7	1074.5 ± 7.1	1107.7 ± 21.3
Adjusted P100	1355.3 ± 12.8	1360.4 ± 15.4	1357.1 ± 11.7	1383.7 ± 29.9	1072.9 ± 12.2	1078.3 ± 13.1	1075.1 ± 11.3	1107.2 ± 19.3
BMD values measured by DEXA (g/cm^2)								
Total body	1.099 ± 0.006	1.097 ± 0.008	1.098 ± 0.005	1.078 ± 0.019	0.915 ± 0.005	0.921 ± 0.006	0.917 ± 0.004	0.931 ± 0.012
Adjusted total body	1.100 ± 0.009	1.098 ± 0.010	1.099 ± 0.008	1.082 ± 0.021	0.922 ± 0.007	0.929 ± 0.008	0.925 ± 0.006	0.939 ± 0.011
L2-L4	1.036 ± 0.010	1.024 ± 0.012	1.031 ± 0.007	0.988 ± 0.028	0.804 ± 0.008	0.810 ± 0.009	0.806 ± 0.006	0.830 ± 0.017
Adjusted L2-L4	1.029 ± 0.012	1.016 ± 0.015	1.025 ± 0.011	0.984 ± 0.030	0.816 ± 0.010	0.825 ± 0.011	0.820 ± 0.010	0.843 ± 0.017
Femoral neck	0.779 ± 0.008	0.773 ± 0.010	0.777 ± 0.006	0.742 ± 0.023	0.639 ± 0.005	0.643 ± 0.006	0.641 ± 0.004	0.666 ± 0.012[b]
Adjusted femoral neck	0.779 ± 0.010	0.774 ± 0.012	0.777 ± 0.009	0.749 ± 0.023	0.644 ± 0.007	0.651 ± 0.007	0.647 ± 0.006	0.672 ± 0.011[c]
Trochanter	0.665 ± 0.007	0.658 ± 0.009	0.662 ± 0.005	0.629 ± 0.021	0.535 ± 0.005	0.540 ± 0.006	0.537 ± 0.004	0.557 ± 0.012
Adjusted trochanter	0.663 ± 0.009	0.655 ± 0.011	0.660 ± 0.008	0.632 ± 0.021	0.539 ± 0.007	0.546 ± 0.007	0.542 ± 0.006	0.562 ± 0.010[b]
Ward's triangle	0.667 ± 0.010	0.662 ± 0.013	0.665 ± 0.008	0.629 ± 0.030	0.447 ± 0.007	0.454 ± 0.008	0.450 ± 0.005	0.473 ± 0.017
Adjusted Ward's triangle	0.668 ± 0.013	0.664 ± 0.015	0.665 ± 0.012	0.639 ± 0.030	0.452 ± 0.009	0.460 ± 0.010	0.455 ± 0.009	0.478 ± 0.015

Data are means ± SE. Adjusted BMD indicate BMD with adjustment for age, BMI, and smoking status by regression analysis.
[a] P = 0.05 versus *GG + GA*.
[b] P = 0.04 versus *GG + GA*.
[c] P = 0.02 versus *GG*, P = 0.01 versus *GG + GA*.

extension at 68°C for 30 seconds; and a final extension at 68°C for 2 minutes. Amplified DNA was denatured with 0.3 M NaOH and then subjected to hybridization at 37°C for 30 minutes in hybridization buffer containing 40% formamide with allele-specific capture probes (5′-AACATGCTG-GTCGTCCTCATC-3′ or 5′-AACATGCTGGTCATCCT-CATC-3′) fixed to the bottom of the wells of a 96-well plate. After thorough washing of the wells, alkaline-phosphatase-conjugated streptavidin was added to each and the plate was incubated at 37°C for 15 minutes with agitation. The wells were again washed, and, after the addition of a solution containing 0.8 mM of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (monosodium salt) and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, absorbance at 450 nm was measured.

To confirm the accuracy of genotyping for the 190G→A polymorphism by this method, we selected 50 DNA samples at random and subjected them to PCR-RFLP analysis as described [7]. In each instance, the genotype determined by the automated colorimetric allele-specific DNA probe assay system was identical to that determined by PCR-RFLP analysis.

Data were compared between two groups by the unpaired Student's *t*-test or among three groups by one-way analysis of variance and the Tukey–Kramer post hoc test. BMD values were compared among *CCR2* genotypes with adjustment for age, body mass index (BMI), and smoking status by regression analysis. Qualitative data were analyzed by the chi-square test. Allele frequencies were estimated by the gene-counting method, and the chi-square test was used to identify significant departures from Hardy–Weinberg equilibrium. A P value of ≤ 0.05 was considered statistically significant.

The distributions of *CCR2* genotypes with regard to the 190G→A polymorphism were in Hardy–Weinberg equilibrium both in men (Table 1) and in women (Table 2). Age, BMI, and smoking status did not differ among *CCR2* genotypes in men (Table 1) or in women (Table 2). There were also no differences in alcohol consumption, calcium intake, or physical activity among *CCR2* genotypes for men or for women (data not shown).

Among men aged < 60 years, BMD for the distal radius (D50 and D100), lumbar spine (L2–L4), or Ward's triangle was significantly greater in those with the AA genotype than in those with the GG or GA genotypes (Table 1). After adjustment for age, BMI, and smoking status, BMD for the distal radius (D50 and D100), L2–L4, femoral neck, or Ward's triangle was significantly greater in men aged < 60 years with the AA genotype than in those with the GG or GA genotypes (Table 1, shown as adjusted BMD). In contrast, for men aged ≥ 60 years, BMD was not associated with *CCR2* genotype (Table 1).

For postmenopausal women, BMD for the distal radius (D50) or femoral neck was significantly higher in those with the AA genotype than in those with the GG or GA genotypes (Table 2). After adjustment for age, BMI, and smoking status, BMD for the distal radius (D50), femoral neck, or trochanter was significantly greater in postmenopausal women with the AA genotype than in those with the GG or GA genotypes (Table 2, shown as adjusted BMD). In contrast, BMD was not associated with *CCR2* genotype in premenopausal women (Table 2).

Although polymorphisms of several candidate genes, including those encoding the vitamin D receptor [12,13], the estrogen receptor [14], collagen type Iα1 [15], interleukin-1β [16], interleukin-6 [17], and interleukin-1 receptor antagonist [18,19], have been associated with BMD, the prevalence of osteoporosis, or the risk of osteoporotic fracture, the genes that contribute to genetic susceptibility to osteoporosis remain to be identified definitively. We have now shown that the 190G→A (Val64Ile) polymorphism of *CCR2* is associated with BMD at various sites in community-dwelling, middle-aged Japanese men and postmenopausal women, and that the AA genotype represents a contributing factor to increased bone mass. We failed to detect an association of the 190G→A polymorphism of *CCR2* with BMD in men aged ≥ 60 years or premenopausal women. The reason for these differences related to age or menopausal status remains unclear, but differences in the concentrations of testosterone, estrogen, or other hormones between middle-aged and elderly men or between premenopausal and postmenopausal women might be contributing factors.

Given that selection bias can influence the results of association studies, it is important that study populations be genetically and ethnically homogeneous. Our study population was recruited randomly from individuals resident in Obu City and adjacent regions in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background [10]. We also showed that the distribution of *CCR2* genotypes in our study population was in Hardy–Weinberg equilibrium. We thus appeared to avoid admixture and selection bias, and our study population appeared genetically homogeneous.

Given that monocytes release factors that regulate bone formation or resorption, chemokines such as MCP-1 that initiate the recruitment of these cells are thought to be important in the regulation of bone remodeling [5]. The 190G→A polymorphism of *CCR2* has previously been shown not to affect the calcium responses of peripheral mononuclear cells to MCP-1 [20]. The molecular mechanism by which the A allele of this polymorphism protects against age-related bone loss thus remains unclear. Other ligands of CCR2, including MCP-2, -3, -4, or -5, might help to explain the *in vivo* effects of the 190G→A polymorphism. Alternatively, these effects may be mediated through intracellular interactions of variant CCR2 proteins with other chemokine receptors such as CCR5 and CXC chemokine receptor-4 [20]. It is also possible that the 190G→A polymorphism is in linkage disequilibrium with polymorphisms of other nearby genes located at chromosome 3p21 that are determinants of BMD. However, our results suggest that *CCR2* may be a new candidate for a susceptibility locus for bone mass, especially in middle-aged men and postmenopausal women.

doi:10.1006/geno.2002.6793, available online at http://www.idealibrary.com on IDEAL

ACKNOWLEDGMENTS

This work was supported in part by Research Grants for Longevity Sciences (12C-01) from the Ministry of Health, Labor, and Welfare of Japan (to Y.Y. and H.S.).

RECEIVED FOR PUBLICATION JANUARY 2;
ACCEPTED APRIL 25, 2002.

REFERENCES

1. Luster, A. D. (1998). Chemokines—chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338:** 436–445.
2. Rahimi, P., *et al.* (1995). Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse. *Endocrinology* **136:** 2752–2759.
3. Volejnikova, S., Laskari, M., Marks, S. C., and Graves, D. T. (1997). Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. *Am. J. Pathol.* **150:** 1711–1721.
4. Posner, L. J., *et al.* (1997). Monocyte chemoattractant protein-1 induces monocyte recruitment that is associated with an increase in numbers of osteoblasts. *Bone* **21:** 321–327.
5. Graves, D. T., Jiang, Y., and Valente, A. J. (1994). The expression of monocyte chemoattractant protein-1 and other chemokines by osteoblasts. *Front. Biosci.* **4:** D571–D580.
6. Frade, J. M. R., *et al.* (1997). The amino-terminal domain of the CCR2 chemokine receptor acts as coreceptor for HIV-1 infection. *J. Clin. Invest.* **100:** 497–502.
7. Smith, M. W., *et al.* (1997). Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. *Science* **277:** 959–965.
8. Hizawa, N., *et al.* (1999). The role of the C-C chemokine receptor 2 gene polymorphism V64I (CCR2-64I) in sarcoidosis in a Japanese population. *Am. J. Respir. Crit. Care Med.* **159:** 2021–2023.
9. Szalai, C., *et al.* (1999). Chemokine receptor CCR2 and CCR5 polymorphisms in children with insulin-dependent diabetes mellitus. *Pediatr. Res.* **46:** 82–84.
10. Shimokata, H., Ando, F., and Niino, N. (2000). A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). *J. Epidemiol.* **10:** S1–S9.
11. Yamada, Y., *et al.* (2001). Association of the $C^{-509} \rightarrow T$ polymorphism, alone or in combination with the $T^{869} \rightarrow C$ polymorphism, of the transforming growth factor-β1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women. *J. Mol. Med.* **79:** 149–156.
12. Morrison, N. A., *et al.* (1994). Prediction of bone density from vitamin D receptor alleles. *Nature* **367:** 284–287.
13. Gross, C., *et al.* (1996). The presence of a polymorphism at the translation initiation site of the vitamin D receptor is associated with low bone mineral density in postmenopausal Mexican-American women. *J. Bone Miner. Res.* **11:** 1850–1855.
14. Kobayashi, S., *et al.* (1996). Association of bone mineral density with polymorphism of the estrogen receptor gene. *J. Bone Miner. Res.* **11:** 306–311.
15. Uitterlinden, A. G., *et al.* (1998). Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. *N. Engl. J. Med.* **338:** 1016–1021.
16. Nemetz, A., *et al.* (2001). Allelic variation at the interleukin 1β gene is associated with decreased bone mass in patients with inflammatory bowel diseases. *Gut* **49:** 644–649.
17. Murray, R. E., McGuigan, F., Grant, S. F. A., Reid, D. M., and Ralston, S. H. (1997). Polymorphisms of the interleukin-6 gene are associated with bone mineral density. *Bone* **21:** 89–92.
18. Langdahl, B. L., Lokke, E., Carstens, M., Stenkjaer, L. L., and Eriksen, E. F. (2000). Osteoporotic fractures are associated with an 86-base pair repeat polymorphism in the interleukin-1-receptor antagonist gene but not with polymorphisms in the interleukin-1β gene. *J. Bone Miner. Res.* **15:** 402–414.
19. Keen, R. W., Woodford-Richens, K. L., Lanchbury, J. S., and Spector, T. D. (1998). Allelic variation at the interleukin-1 receptor antagonist gene is associated with early postmenopausal bone loss at the spine. *Bone* **23:** 367–371.
20. Lee, B., *et al.* (1998). Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4. *J. Virol.* **72:** 7450–7458.

ELSEVIER

Matrix Biology 21 (2002) 389–392

MATRIX BIOLOGY

www.elsevier.com/locate/matbio

Short note

Association of a polymorphism of the matrix metalloproteinase-1 gene with bone mineral density

Yoshiji Yamada[a,*], Fujiko Ando[b], Naoakira Niino[b], Hiroshi Shimokata[b]

[a]*Department of Gene Therapy, Gifu International Institute of Biotechnology and Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505-0116, Japan*

[b]*Department of Epidemiology, National Institute for Longevity Sciences, Obu, Aichi, Japan*

Received 20 December 2001; received in revised form 20 February 2002; accepted 5 April 2002

Abstract

Matrix metalloproteinase-1 (MMP-1) is a key mediator of the degradation of collagen, which is abundant in bone matrix. A nucleotide polymorphism (G→GG) at position −1607 in the promoter of the MMP-1 gene is associated with increased gene transcription. The possible relation of the −1607G→GG polymorphism of the MMP-1 gene to bone mineral density (BMD) was examined in a population-based study with 1095 women and 1127 men. BMD for the distal radius was significantly lower in postmenopausal women with the *GG/GG* genotype than in those with the *G/GG* or those with the *G/G* or *G/GG* genotype; these differences were not apparent in premenopausal women. No significant differences in BMD at other sites were detected among MMP-1 genotypes for women. Men did not exhibit any significant differences in BMD among MMP-1 genotypes. The MMP-1 gene may thus be a susceptibility locus for reduced BMD at the distal radius in postmenopausal women.

Keywords: Matrix metalloproteinase-1; Collagenase; Polymorphism; Bone mineral density; Osteoporosis; Population-based study

1. Introduction

Matrix metalloproteinase-1 (MMP-1) is the interstitial collagenase expressed most widely among tissues and therefore plays a prominent role in collagen degradation. Overexpression of MMP-1 is associated with several pathological conditions, including the irreversible degradation of cartilage, tendon, and bone in individuals with arthritis (Vincenti et al., 1996). A common single-nucleotide insertion polymorphism (G→GG) at nucleotide position −1607 in the promoter region of the MMP-1 gene results in the creation of a binding site for the Ets family of transcription factors as well as in increased transcription of the MMP-1 gene and increased enzyme activity (Rutter et al., 1998).

Osteoporosis, a common disease that is characterized by reduced bone mass and an increased risk of fracture, has a strong genetic component (Pocock et al., 1987). Given that interstitial collagenase is an important mediator of degradation of the bone matrix, we have now examined whether the −1607G→GG polymorphism of the MMP-1 gene is associated with bone mineral density (BMD) in a population-based study.

2. Experimental procedures

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 gender, and are randomly selected from resident registrations in the city of Obu and the town of Higashiura in central Japan. The lifestyle of residents of this area is typical of that of individuals in most regions of Japan. The numbers of men and women recruited are similar and age at the

Abbreviations: MMP-1, matrix metalloproteinase-1; BMD, bone mineral density; BMI, body mass index; pQCT, peripheral quantitative computed tomography; DEXA, dual-energy X-ray absorptiometry.

*Corresponding author. Tel.: +81-574-67-6616; fax: +81-574-67-6627.

E-mail address: yoyamada@giib.or.jp (Y. Yamada).

0945-053X/02/$ - see front matter
PII: S0945-053X(02)00030-6

baseline is 40–79 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. The NILS-LSA aims to determine the relations between various gene polymorphisms and geriatric diseases. We have now examined the association of the −1607G→GG polymorphism of the MMP-1 gene with BMD in 2222 subjects [1095 women (276 premenopausal and 819 postmenopausal) and 1127 men]. 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.

BMD at the radius was measured by peripheral quantitative computed tomography (pQCT) and 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 total body, lumbar spine (L2–L4), right femoral neck, right trochanter, and right Ward's triangle was measured by dual-energy X-ray absorptiometry (DEXA).

The MMP-1 genotype was determined with an automated colorimetric allele-specific DNA probe assay system (Toyobo Gene Analysis, Tsuruga, Japan) (Yamada et al., 2001b). In brief, the polymorphic region of the gene was amplified by the polymerase chain reaction with sense (5′-ATGAGGAAATTGTAGTTAAATAATTAGA) and biotin-labeled antisense (5′-TCAGTATATCTTGGATTGATTTGAGA) primers. The reaction mixture (25 μl) contained 50 ng of DNA, 5 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM $MgSO_4$ and 1 U of DNA polymerase (KODplus; Toyobo, Osaka, Japan) in KODplus buffer. The amplification protocol comprised initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 15 s, annealing at 52.5 °C for 30 s, and extension at 68 °C for 30 s; and a final extension at 68 °C for 2 min. Amplified DNA was denatured with 0.3 M NaOH and then subjected to hybridization at 37 °C for 30 min in hybridization buffer containing 33.8% formamide with allele-specific capture probes (5′-AAATAATTAGAAA*GATATGACTTATCTCA* or 5′-AATAATTAGAAA*GGATATGACTTATCTC*) fixed to the bottom of the wells of a 96-well plate. The wells were then washed thoroughly, alkaline phosphatase–conjugated streptavidin was added to each, and the plate was incubated at 37 °C for 15 min with agitation. After further washing of the wells, a solution containing 0.8 mM 2-(4-iodophenyl)- 3 -(4 - nitrophenyl) - 5 - (2,4 - disulfophenyl)-2*H*-tetrazolium (monosodium salt) and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt was added to each and absorbance at 450 nm was measured.

Data were compared between two groups by the unpaired Student's *t*-test or among three groups by one-way analysis of variance and the Tukey–Kramer post-hoc test. Qualitative data were analyzed by the Chi-square test. Allele frequencies were estimated by the gene-counting method, and the Chi-square test was used to identify significant departures from the Hardy–Weinberg equilibrium. A P value of <0.05 was considered statistically significant.

3. Results

The distributions of MMP-1 genotypes with regard to the −1607G→GG polymorphism were in Hardy–Weinberg equilibrium both in women (Table 1) and in men (Table 2). Age and body mass index (BMI) did not differ among MMP-1 genotypes in premenopausal or postmenopausal women (Table 1) or in men (Table 2). The frequency of smoking was lower in premenopausal women with the *GG/GG* genotype than in those with the *G/G* or *G/GG* genotype. No difference in smoking status was apparent among MMP-1 genotypes in postmenopausal women or men (Tables 1 and 2). There were also no differences in alcohol consumption, calcium intake, or physical activity among MMP-1 genotypes for premenopausal or postmenopausal women or for men (data not shown). Among postmenopausal women, BMD for the distal radius (D50 and D100) was significantly lower in those with the *GG/GG* genotype than in those with the *G/GG* genotype or those with the *G/G* or *G/GG* genotype; such differences were not detected for premenopausal women (Table 1). There were no significant differences in BMD for the proximal radius, total body, lumbar spine (L2–L4), femoral neck, trochanter, or Ward's triangle among MMP-1 genotypes for premenopausal or postmenopausal women. For men, no significant differences in BMD among MMP-1 genotypes were detected (Table 2).

4. Discussion

Given that collagens are the most abundant proteins in the body, collagenases play an important role in modeling and remodeling of the extracellular matrix. Cathepsin K, a lysosomal cysteine protease, is abundant in osteoclasts (Tezuka et al., 1994) and is the principal collagen-degrading enzyme in bone resorption (Drake et al., 1996). The interstitial collagenase MMP-1 also plays an important role in collagen degradation. We have now shown that the −1607G→GG polymorphism of the MMP-1 gene is associated with BMD for the

Table 1
BMD and other characteristics of women ($n=1095$) according to menstrual status and MMP-1 genotype

	Premenopausal women ($n=276$)				Postmenopausal women ($n=819$)			
	G/G	*G/GG*	*G/G+G/GG*	*GG/GG*	*G/G*	*G/GG*	*G/G+G/GG*	*GG/GG*
Number (%)	24 (8.7)	125 (45.3)	149 (54.0)	127 (46.0)	84 (10.3)	343 (41.9)	427 (52.1)	392 (47.9)
Age (years)	45.5±0.7	45.5±0.3	45.5±0.3	46.3±0.3	64.7±0.9	63.3±0.5	63.6±0.4	64.4±0.4
BMI (kg/m^2)	22.9±0.7	22.8±0.3	22.8±0.3	22.7±0.3	23.3±0.4	23.0±0.2	23.1±0.2	22.8±0.2
Smoking (%)	12.5	14.4	14.1	6.3*	7.1	6.4	6.6	5.6
BMD values measured by pQCT (mg/cm^3)								
D50	257.9±12.2	251.5±5.0	252.4±4.6	240.1±4.9	166.8±7.7	171.0±3.8	170.2±3.4	155.8±3.5†
D100	630.9±16.8	611.3±6.8	614.1±6.3	599.0±6.8	450.1±12.1	450.6±5.9	450.4±5.3	433.8±5.6*
P100	1408.7±26.1	1370.4±10.6	1375.8±9.8	1349.3±10.5	1080.7±21.1	1090.5±10.3	1088.6±9.3	1067.3±9.7
BMD values measured by DEXA (g/cm^2)								
Total body	1.081±0.017	1.105±0.007	1.101±0.007	1.092±0.007	0.932±0.012	0.922±0.006	0.924±0.005	0.912±0.006
L2–L4	1.014±0.025	1.027±0.011	1.025±0.010	1.032±0.011	0.822±0.017	0.815±0.008	0.816±0.007	0.800±0.008
Femoral neck	0.747±0.020	0.786±0.009	0.780±0.008	0.768±0.009	0.647±0.012	0.643±0.006	0.644±0.005	0.643±0.005
Trochanter	0.632±0.018	0.665±0.008	0.659±0.007	0.661±0.008	0.536±0.011	0.542±0.006	0.541±0.005	0.538±0.005
Ward's triangle	0.643±0.026	0.676±0.011	0.671±0.010	0.629±0.030	0.457±0.016	0.456±0.008	0.456±0.007	0.448±0.007

Data are means±S.E.
*$P=0.03$ vs. *G/G+G/GG*.
† $P=0.009$ vs. *G/GG*, $P=0.003$ vs. *G/G+G/GG*.

distal radius in community-dwelling postmenopausal Japanese women, and that the *GG/GG* genotype represents a risk factor for genetic susceptibility to reduced bone mass at this site. However, given that the maximal difference in BMD between postmenopausal women with the *G/GG* genotype and those with the *GG/GG* genotype was 8.9% of the larger value, the impact of the latter genotype on osteoporosis might be limited.

We failed to detect an association of the −1607G→GG polymorphism of the MMP-1 gene with BMD in men or premenopausal women. The reason for these differences related to gender or menstrual status remains unclear, but differences in the concentrations of estrogen or other hormones between men and women or between premenopausal and postmenopausal women might be contributing factors.

Our study population was recruited randomly from individuals resident in the city of Obu or the town of Higashiura in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background (Shimokata et al., 2000). We also showed that the distribution of MMP-1 genotypes in our study population was in Hardy–Weinberg equilibrium. Our study population thus appeared genetically homogeneous and we appeared to avoid admixture and selection bias.

We previously showed that a 869T→C (Leu10Pro) polymorphism of the transforming growth factor-β1

Table 2
BMD and other characteristics of men ($n=1127$) according to MMP-1 genotype

	−1607G→GG genotype			
	G/G	*G/GG*	*G/G+G/GG*	*GG/GG*
Number (%)	135 (12.0)	503 (44.6)	638 (56.6)	489 (43.4)
Age (years)	60.2±0.9	58.6±0.5	59.0±0.4	59.5±0.5
BMI (kg/m^2)	23.0±0.2	22.9±0.1	22.9±0.1	22.9±0.1
Smoking (%)	37.0	38.6	38.2	37.6
BMD values measured by pQCT (mg/cm^3)				
D50	269.0±6.2	269.4±3.2	269.3±2.8	263.6±3.2
D100	545.6±8.8	544.4±4.5	544.7±4.0	536.5±4.5
P100	1177.9±13.5	1190.6±6.9	1188.0±6.1	1182.7±6.9
BMD values measured by DEXA (g/cm^2)				
Total body	1.081±0.008	1.089±0.004	1.088±0.004	1.087±0.004
L2–L4	0.974±0.014	0.981±0.007	0.979±0.006	0.987±0.007
Femoral neck	0.754±0.010	0.753±0.005	0.753±0.005	0.753±0.005
Trochanter	0.666±0.009	0.668±0.005	0.667±0.004	0.670±0.005
Ward's triangle	0.551±0.012	0.553±0.006	0.552±0.006	0.552±0.006

Data are means±S.E.

gene was associated with BMD at the distal radius (D50) in elderly women of an overlapping study population (Yamada et al., 2001a). We thus compared BMD at various sites among MMP-1 genotypes in women and in men after adjustment for transforming growth factor-β1 genotype. The results obtained were essentially identical to the unadjusted data shown in Tables 1 and 2. Thus, D50 and D100 were associated with MMP-1 genotype in postmenopausal women but not in premenopausal women, whereas BMD for the other sites in women or BMD for all sites in men was not associated with MMP-1 genotype (data not shown).

Three polymorphisms in the 5′ region (nucleotides −524 to +52) of the MMP-1 gene have previously been shown not to be associated with osteoporosis (Thiry-Blaise et al., 1995). These polymorphisms have not been shown to be associated with transcriptional activity or other gene function. The −1607G→GG polymorphism results in the creation of an Ets binding site adjacent to an AP-1 site and markedly increases the transcriptional activity of the MMP-1 gene promoter (Rutter et al., 1998).

A G→T polymorphism at the first base of a consensus binding site for the transcription factor Sp1 in the first intron of the collagen type Iα1 gene was shown to be associated not only with BMD but with the risk of osteoporotic fracture in women (Grant et al., 1996; Uitterlinden et al., 1998). This Sp1 binding site polymorphism was recently shown to affect collagen gene regulation, resulting in abnormal production of the α1(I) collagen chain relative to that of the α2(I) chain as well as reduced bone strength by mechanisms partly independent of an effect on bone mass (Mann et al., 2001). These observations suggest that genetic variation that affects collagen metabolism is important in the development of osteoporosis and osteoporotic fracture. Our present observation that the largest reduction in BMD in postmenopausal women with the *GG/GG* genotype was apparent in cancellous bone is consistent with the previous observations of Grant et al. (1996), Uitterlinden et al. (1998).

It is possible that the −1607G→GG polymorphism of the MMP-1 gene is in linkage disequilibrium with polymorphisms of other nearby genes at chromosome 11q22–q23 that are determinants of BMD. Our present study, however, suggests that the MMP-1 gene may be a susceptibility locus for reduced BMD at the distal radius in postmenopausal women.

Acknowledgments

This work was supported in part by Research Grants for Longevity Sciences (12C-01) from the Ministry of Health, Labor, and Welfare of Japan (to Y.Y. and H.S.).

References

Drake, F.H., Dodds, R.A., James, I.E., et al., 1996. Cathepsin K, but not cathepsin B, L, or S, is abundantly expressed in human osteoclasts. J. Biol. Chem. 271, 12511–12516.

Grant, S.F., Reid, D.M., Blake, G., Herd, R., Fogelman, I., Ralston, S.H., 1996. Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type Iα1 gene. Nat. Genet. 14, 203–205.

Mann, V., Hobson, E.E., Li, B., et al., 2001. A *COLIA1* Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. J. Clin. Invest. 107, 899–907.

Pocock, N.A., Eisman, J.A., Hopper, J.L., Yeates, M.G., Sambrook, P.N., Eberl, S., 1987. Genetic determinants of bone mass in adults: a twin study. J. Clin. Invest. 80, 706–710.

Rutter, J.L., Mitchell, T.I., Buttice, G., et al., 1998. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res. 58, 5321–5325.

Shimokata, H., Ando, F., Niino, N., 2000. A new comprehensive study on aging—the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J. Epidemiol. 10, S1–S9.

Tezuka, K., Tezuka, Y., Maejima, A., et al., 1994. Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. J. Biol. Chem. 269, 1106–1109.

Thiry-Blaise, L.M., Taquet, A.N., Reginster, J.Y., Nusgens, B., Franchimont, O., Lapiere, C.M., 1995. Investigation of the relationship between osteoporosis and the collagenase gene by means of polymorphism of the 5′ upstream region of this gene. Calcif. Tissue Int. 56, 88–91.

Uitterlinden, A.G., Burger, H., Huang, Q., et al., 1998. Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N. Engl. J. Med. 338, 1016–1021.

Vincenti, M.P., White, L.A., Schroen, D.J., Benbow, U., Brinckerhoff, C.E., 1996. Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): mechanisms that control enzyme activity, transcription, and mRNA stability. Crit. Rev. Eukaryot. Gene Expr. 6, 391–411.

Yamada, Y., Ando, F., Niino, N., Shimokata, H., 2001a. Transforming growth factor-β1 gene polymorphism and bone mineral density. J. Am. Med. Assoc. 285, 167–168.

Yamada, Y., Miyauchi, A., Takagi, Y., Tanaka, M., Mizuno, M., Harada, A., 2001b. Association of the $C^{-509}\rightarrow T$ polymorphism, alone or in combination with the $T^{869}\rightarrow C$ polymorphism, of the transforming growth factor-β1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women. J. Mol. Med. 79, 149–156.

J Mol Med (2002) 80:452–460
DOI 10.1007/s00109-002-0348-0

ORIGINAL ARTICLE

Yoshiji Yamada · Fujiko Ando · Naoakira Niino
Shigeo Ohta · Hiroshi Shimokata

Association of polymorphisms of the estrogen receptor α gene with bone mineral density of the femoral neck in elderly Japanese women

Received: 10 January 2002 / Accepted: 2 April 2002 / Published online: 4 June 2002

YOSHIJI YAMADA received his M.D. degree in 1982 and his Ph.D. degree in 1990 in cardiovascular medicine from Nagoya University Graduate School of Medicine in Nagoya, Japan. He carried out postdoctoral research at the Eccles Institute of Human Genetics in Salt Lake City, Utah, USA. He is presently Deputy Director of the Department of Gene Therapy at Gifu International Institute of Biotechnology, Mitake, Gifu, Japan. His research interests include molecular genetics and epidemiology of osteoporosis and cardiovascular diseases.

HIROSHI SHIMOKATA received his M.D. degree in 1977 and his Ph.D. in 1983 in internal medicine from Nagoya University Graduate School of Medicine in Nagoya, Japan. He carried out postdoctoral research at the National Institute on Aging, NIH, Baltimore, Maryland, USA. He is presently Director of the Department of Epidemiology at National Institute for Longevity Sciences, Obu, Aichi, Japan. His research interests include molecular epidemiology and geriatrics.

Y. Yamada (✉)
Department of Gene Therapy,
Gifu International Institute of Biotechnology,
and Institute of Applied Biochemistry, Mitake,
Gifu 505-0116, Japan
e-mail: yoyamada@giib.or.jp

F. Ando · N. Niino · H. Shimokata
Department of Epidemiology,
National Institute for Longevity Sciences, Obu, Aichi, Japan

S. Ohta
Department of Biochemistry and Cell Biology,
Institute of Gerontology, Nippon Medical School,
Kawasaki, Japan

Abstract The estrogen receptor α gene is a candidate locus for genetic influence on bone mass. The possible association between two polymorphisms in the first intron of this gene, alone or in combination, and bone mineral density at various sites was examined in participants in the National Institute for Longevity Sciences Longitudinal Study of Aging, a population-based prospective cohort study of aging and age-related diseases. The relationship of the TC (*Pvu*II) and AG (*Xba*I) polymorphisms in the first intron of the estrogen receptor α gene to bone mineral density was determined in 2230 subjects (1120 men, 1110 women) and in 2238 subjects (1128 men, 1110 women), respectively, all of whom were community-dwelling individuals aged 40–79 years. Bone mineral density at the radius was measured by peripheral quantitative computed tomography and that for the lumbar spine, right femoral neck, right trochanter, right Ward's triangle, and total body was measured by dual-energy X-ray absorptiometry. Estrogen receptor α genotypes were determined with an automated fluorescent allele-specific DNA primer assay system. Analysis of the TC (*Pvu*II) polymorphism revealed that bone mineral density for the total body, femoral neck, and trochanter was significantly lower in women aged 60 years or over with the *CC* genotype than in those with the *TT* genotype, but statistical significance was not achieved after adjustment for age, body mass index, and smoking status. Analysis of the AG (*Xba*I) polymorphism revealed that bone mineral density for the femoral neck was significantly lower in women aged 60 years or over with the *GG* genotype than in those with the *AA* genotype. After adjustment for age, body mass index, and smoking status, bone mineral density for the femoral neck was significantly lower in women aged 60 years or over with the *GG* genotype than in those with the *AA* or *AG* genotypes. Analysis of combined genotypes in women aged 60 years or over revealed that bone mineral density for the femoral neck was significantly lower in women with the *CC/GG* genotype than in those with the *TT/AA* or *TC/AA* genotypes. After adjustment for age, body mass index, and smoking status, bone mineral density for the femoral neck was

significantly lower in women aged 60 years or over with the *CC/GG* genotype than in those with other genotypes. No differences in bone mineral density at the various sites were detected among TC (*Pvu*II), AG (*Xba*I), or combined genotypes in women aged under 60 years or in men. These results suggest that the estrogen receptor α gene is a susceptibility locus for bone mass, especially for the femoral neck, in elderly Japanese women.

Keywords Estrogen receptor · Single nucleotide polymorphism · Bone mineral density · Osteoporosis · Population-based study

Abbreviations *BMD*: Bone mineral density · *BMI*: Body mass index · *ERα*: Estrogen receptor α · *NILS*: National Institute for Longevity Sciences · *SNP*: Single nucleotide polymorphism

Introduction

Osteoporosis is characterized by a decrease in bone mineral density (BMD) and a deterioration in the microarchitecture of bone, which result in an increased susceptibility to fractures [1]. Although several environmental factors, such as diet and physical exercise, influence BMD, a genetic contribution to the etiology of osteoporosis has been recognized [2]. Genetic linkage studies [3, 4] and candidate gene association studies [5, 6] have implicated several loci and candidate genes in the regulation of bone mass and the pathogenesis of osteoporotic fractures. One such candidate gene is the estrogen receptor α (ERα) gene. The importance of ERα in the regulation of bone mass has been indicated by the occurrence of osteoporosis in a man with a nonsense mutation in the ERα gene [7] and by the observation that the BMD of mice lacking a functional ERα gene is 20–25% less than that of wild-type mice [8].

The human ERα gene is located on chromosome 6p25.1, comprises eight exons, and spans more than 140 kb [9]. Two single nucleotide polymorphisms (SNPs) have been identified in the first intron of the ERα gene: a TC polymorphism that is recognized by the restriction endonuclease *Pvu*II [*T* and *C* alleles correspond to the presence (*p* allele) and absence (*P* allele) of the restriction site, respectively], and an AG polymorphism that is recognized by *Xba*I [*A* and *G* alleles correspond to the presence (*x* allele) and absence (*X* allele) of the restriction site, respectively]. In some studies these SNPs, alone or in combination, have been associated with bone mass in postmenopausal women [10, 11] or premenopausal women [12, 13] or with the response to hormone replacement therapy [14]. However, other studies have not confirmed these observations [15, 16, 17, 18, 19, 20]. In addition, a microsatellite (TA repeat) polymorphism of the ERα gene, but not the TC and AG polymorphisms in the first intron, was shown to be associated with BMD and with the prevalence of fractures [11, 17, 18]. The reason for this discrepancy remains to be determined, but it may result from the limited sample size of many of the studies. Thus, large-scale population-based studies are required to clarify the role of polymorphisms of the ERα gene in determining BMD.

We have now examined whether the TC (*Pvu*II) and AG (*Xba*I) polymorphisms in the first intron of the ERα gene, alone or in combination, are associated with BMD in women and men in a population-based study.

Materials and methods

Study subjects

The National Institute for Longevity Sciences (NILS) Longitudinal Study of Aging is a population-based prospective cohort study of aging and age-related diseases [21, 22]. We examined the possible association of the TC (*Pvu*II) and AG (*Xba*I) SNPs of the ERα gene with BMD in 2230 participants (1120 men, 1110 women) and in 2238 participants (1128 men, 1110 women), respectively, all of whom were community-dwelling individuals aged 40–79 years and were randomly recruited from regions near to the NILS in central Japan. 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.

BMD measurement

BMD at the radius was measured by peripheral quantitative computed tomography and 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 lumbar spine (L2–L4), right femoral neck, right trochanter, right Ward's triangle, and total body was measured by dual-energy X-ray absorptiometry.

Determination of ERα genotype

ERα genotypes were determined with an automated fluorescent allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan). For determination of TC (*Pvu*II) genotype the polymorphic region of the gene was amplified by the polymerase

Table 1 Distribution of TC (*Pvu*II) and AG (*Xba*I) genotypes of the ERα gene among the study population

	AA		*AG*		*GG*		Total	
	n	%	*n*	%	*n*	%	*n*	%
TT	796	35.76	1	0.04	0	0.00	797	35.80
TC	587	26.37	466	20.93	5	0.22	1058	47.53
CC	123	5.53	175	7.86	73	3.28	371	16.67
Total	1506	67.65	642	28.84	78	3.50	2226	100.00

Table 2 Bone mineral density (*BMD*) and other characteristics of women according to age and TC (*PvuII*) genotype of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	Total (n=1110)			<60 years (n=551)			≥60 years (n=559)		
	TT	*TC*	*CC*	*TT*	*TC*	*CC*	*TT*	*TC*	*CC*
n	399 (36.0%)	518 (46.7%)	193 (17.4%)	211 (38.3%)	244 (44.3%)	96 (17.4%)	188 (33.6%)	274 (49.0%)	97 (17.4%)
Age (years)	58.4±0.5	59.8±0.5	59.7±0.8	50.0±0.4	49.6±0.3	49.5±0.5	67.9±0.4	68.9±0.3	69.8±0.6*
BMI	22.8±0.2	22.9±0.1	23.1±0.2	22.6±0.2	22.7±0.2	23.3±0.3	23.1±0.2	23.0±0.2	22.9±0.3
Smoking (%)	8.3	5.6	8.8	12.3	9.5	10.4	3.7	2.2	7.2
Fracture (%)	19.6	18.6	16.1	14.8	14.1	13.5	25.0	22.6	18.6
BMD values measured by pQCT (mg/cm³)									
D50	186.9±3.8	182.7±3.3	185.4±5.5	218.9±4.8	224.1±4.4	233.3±7.0	151.2±4.5	146.1±3.7	138.1±6.3
D100	491.7±6.4	480.3±5.5	483.6±9.1	560.3±7.3	564.2±6.7	568.4±10.8	415.0±6.7	406.2±5.5	399.7±9.3
P100	1165.9±10.9	1146.6±9.5	1136.4±15.6	1292.2±11.0	1295.0±10.2	1287.9±16.3	1024.7±12.3	1015.5±10.1	986.4±17.1
BMD values measured by DEXA (g/cm²)									
Total body	0.972±0.007	0.960±0.006	0.962±0.009	1.047±0.007	1.052±0.007	1.064±0.011	0.887±0.006	0.878±0.005	0.860±0.009**
Adjusted total body	0.970±0.006	0.970±0.006	0.969±0.008	1.055±0.008	1.056±0.008	1.064±0.011	0.882±0.010	0.878±0.010	0.865±0.011
L2–L4	0.871±0.009	0.865±0.008	0.856±0.013	0.951±0.010	0.965±0.010	0.970±0.015	0.779±0.010	0.774±0.008	0.743±0.014
Adjusted L2–L4	0.870±0.009	0.878±0.009	0.864±0.011	0.961±0.011	0.967±0.011	0.964±0.015	0.783±0.016	0.783±0.016	0.757±0.018
Femoral neck	0.688±0.006	0.673±0.005	0.668±0.009	0.748±0.007	0.741±0.007	0.747±0.011	0.620±0.006	0.612±0.005	0.589±0.009***
Adjusted femoral neck	0.688±0.006	0.682±0.006	0.673±0.008	0.756±0.008	0.745±0.008	0.744±0.011	0.618±0.010	0.616±0.010	0.599±0.011
Trochanter	0.579±0.006	0.567±0.005	0.563±0.008	0.630±0.007	0.632±0.006	0.635±0.010	0.520±0.007	0.509±0.006	0.491±0.009[4]**
Adjusted trochanter	0.577±0.006	0.573±0.006	0.567±0.007	0.636±0.007	0.634±0.007	0.631±0.010	0.516±0.010	0.510±0.010	0.499±0.011
Ward's triangle	0.514±0.008	0.504±0.007	0.499±0.012	0.613±0.010	0.615±0.009	0.624±0.015	0.403±0.008	0.404±0.007	0.375±0.012
Adjusted Ward's triangle	0.510±0.008	0.513±0.008	0.505±0.010	0.622±0.011	0.618±0.011	0.619±0.015	0.395±0.013	0.402±0.013	0.382±0.015

*P=0.014 vs. *TT*, **P=0.030 vs. *TT*, ***P=0.017 vs. *TT*, [4]**P=0.034 vs. *TT*

Table 3 Bone mineral density (*BMD*) and other characteristics of men (n=1120) according to TC (*Pvu*II) genotype of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	TC genotype		
	TT	*TC*	*CC*
n	399 (35.6%)	541 (48.35)	180 (16.15)
Age (years)	58.9±0.5	59.3±0.5	59.5±0.8
BMI	23.1±0.1	23.0±0.1	22.4±0.2*
Smoking (%)	38.6	37.0	41.7
Fracture (%)	27.1	24.4	27.4
BMD values measured by pQCT (mg/cm^3)			
D50	268.9±3.5	266.2±3.0	260.8±5.2
D100	544.0±5.0	539.1±4.3	537.6±7.4
P100	1190.8±7.7	1182.8±6.6	1181.0±11.5
BMD values measured by DEXA (g/cm^2)			
Total body	1.090±0.005	1.087±0.004	1.083±0.007
Lumbar spine (L2–L4)	0.985±0.008	0.983±0.007	0.977±0.012
Femoral neck	0.757±0.006	0.751±0.005	0.752±0.009
Trochanter	0.676±0.005	0.664±0.005	0.667±0.008
Ward's triangle	0.558±0.007	0.550±0.006	0.549±0.011

*P=0.009 vs. *TT*

chain reaction with allele-specific sense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-AGTTCCAAATGTCCCAGXTG-3′) or with Texas red (5′-AGTTCCAAATGTCCCAGXCG-3′) and an antisense primer labeled at the 5′ end with biotin (5′-TCTGGGAAACAGAGACAAAGC-3′). The reaction mixture (25 µl) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, and 1 U DNA polymerase (rTaq; Toyobo, Osaka, Japan) in rTaq 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 62.5°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 2 min. For determination of the AG (*Xba*I) genotype the polymorphic region of the gene was amplified by the polymerase chain reaction with a sense primer labeled at the 5′ end with biotin (5′-CTGTTTCCCAGAGACCCTGAG-3′) and allele-specific antisense primers labeled at the 5′ end either with fluorescein isothiocyanate (5′-CCAATGCTCATCCCAACTXTA-3′) or with Texas red (5′-CCAATGCTCATCCCAACTXCA-3′). The reaction mixture (with the exception of the primers) and the amplification protocol (with the exception that the annealing temperature was 65°C) were identical to those used for genotyping of the TC (*Pvu*II) polymorphism.

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 placed on a magnetic stand, and the supernatants were then collected from each well, transferred to the wells of a 96-well plate containing 0.01 M NaOH, and 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.

Statistical analysis

Quantitative data were compared by one-way analysis of variance and the Tukey-Kramer post hoc test. BMD values were analyzed with adjustment for age, body mass index (BMI), and smoking status by the least squares method in a general linear model. Qualitative data were analyzed by the χ^2 test. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify significant departures from Hardy-Weinberg equilibrium. A P value of 0.05 or less was considered statistically significant.

Results

The distributions of ERα genotypes with regard to the TC and AG SNPs were in Hardy-Weinberg equilibrium for the study population overall (Table 1) as well as in women (Tables 2, 4) and men (Tables 3, 5) separately. The distribution of haplotypes for the TC and AG polymorphisms in all study subjects was as follows: *T/A* 61.9%, *T/G* 0.2%, *C/A* 28.6%, and *C/G* 9.3%. The TC and AG SNPs were in linkage disequilibrium [pairwise linkage disequilibrium coefficient, $D'(D/D_{max})$, of 0.97; standardized linkage disequilibrium coefficient, r, of 0.40; $P<0.0001$, χ^2 test].

The relationship of the TC (*Pvu*II) SNP in the first intron of the ERα gene to BMD was examined. For all women, age, BMI, smoking status, and the frequency of fracture did not differ among TC genotypes (Table 2). Furthermore, there were no differences in BMD at the various sites examined among TC genotypes in women overall. To examine the possible influence of age on the relationship between TC genotype and BMD we analyzed BMD and other characteristics independently in women aged under 60 years and those aged 60 years or over. In women aged under 60 years, BMD was not associated with TC genotype (Table 2). In contrast, in women aged 60 years or over, BMD for the total body, femoral neck, and trochanter was significantly lower in those with the *CC* genotype than in those with the *TT* genotype. After adjustment for age, BMI, and smoking status, however, statistical significance was not achieved for the differences in BMD values at these sites among TC genotypes in women aged 60 years or over. BMD at the sites studied did not differ among TC genotypes either in men overall (Table 3) or in men aged under 60 years or aged 60 years or over (data not shown).

We next examined the relationship of the AG (*Xba*I) SNP in the first intron of the ERα gene to BMD. For all women, age, smoking status, and the frequency of fracture did not differ among AG genotypes, whereas BMI was significantly greater in women with the *AG* genotype than in those with the *AA* genotype (Table 4). No differences in BMD at the various sites examined were detected among AG genotypes in women overall. Also, in women aged under 60 years, BMD was not associated with AG genotype (Table 4). In contrast, in women aged 60 years or over, BMD for the femoral neck was significantly lower in those with the *GG* genotype than in those with the *AA* genotype (Table 4). After adjustment for

Table 4 Bone mineral density (*BMD*) and other characteristics of women according to age and AG (*XbaI*) genotype of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	Total (n=1110)			<60 years (n=554)			≥60 years (n=556)		
	AA	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*
n	739 (66.6%)	332 (29.9%)	39 (3.5%)	377 (68.1%)	158 (28.5%)	19 (3.4%)	362 (65.1%)	174 (31.3%)	20 (3.6%)
Age (years)	58.9±0.4	59.9±0.6	60.3±1.7	49.7±0.3	49.8±0.4	50.1±1.2	68.5±0.3	69.1±0.4	69.9±1.2
BMI	22.7±0.1	23.3±0.2*	23.1±0.5	22.4±0.2	23.4±0.3**	24.4±0.7***	23.0±0.2	23.3±0.3	21.8±0.7
Smoking (%)	7.7	6.0	5.1	11.7	9.5	5.3	3.6	2.9	5.0
Fracture (%)	18.2	19.7	12.8	14.9	12.8	10.5	21.6	25.9	15.0
BMD values measured by pQCT (mg/cm^3)									
D50	185.6±2.8	185.3±4.2	169.9±12.1	222.3±3.6	228.3±5.5	220.6±15.9	147.7±3.2	146.1±4.7	124.4±13.5
D100	486.8±4.7	486.0±6.9	454.2±20.1	562.7±5.4	567.3±8.4	549.0±24.3	408.4±4.8	411.9±6.9	368.9±20.0
P100	1156.8±8.0	1147.2±11.9	1115.8±34.5	1293.1±8.2	1292.8±12.6	1295.1±36.8	1016.3±8.8	1014.7±12.8	954.4±36.9
BMD values measured by DEXA (g/cm^2)									
Total body	0.970±0.005	0.959±0.007	0.948±0.021	1.053±0.005	1.050±0.008	1.042±0.024	0.882±0.004	0.874±0.007	0.858±0.019
Adjusted total body	0.972±0.005	0.967±0.007	0.964±0.015	1.059±0.007	1.053±0.009	1.046±0.022	0.877±0.009	0.871±0.010	0.869±0.020
L2–L4	0.870±0.006	0.861±0.010	0.842±0.028	0.961±0.008	0.961±0.012	0.964±0.034	0.774±0.007	0.769±0.011	0.726±0.031
Adjusted L2–L4	0.875±0.008	0.869±0.010	0.859±0.022	0.968±0.009	0.958±0.012	0.955±0.030	0.776±0.015	0.771±0.017	0.751±0.033
Femoral neck	0.680±0.004	0.677±0.006	0.647±0.019	0.743±0.005	0.749±0.008	0.736±0.024	0.614±0.005	0.610±0.007	0.563±0.020[5]*
Adjusted femoral neck	0.685±0.005	0.683±0.007	0.656±0.015	0.751±0.007	0.748±0.009	0.728±0.022	0.616±0.009	0.614±0.010	0.576±0.020[5]*
Trochanter	0.572±0.004	0.573±0.006	0.545±0.018	0.628±0.005	0.641±0.008	0.626±0.022	0.513±0.005	0.509±0.007	0.468±0.020
Adjusted trochanter	0.574±0.005	0.575±0.006	0.550±0.014	0.633±0.006	0.638±0.008	0.617±0.021	0.510±0.009	0.507±0.011	0.478±0.020
Ward's triangle	0.509±0.006	0.508±0.009	0.482±0.027	0.611±0.007	0.627±0.011	0.604±0.033	0.401±0.006	0.398±0.009	0.367±0.026
Adjusted Ward's triangle	0.511±0.007	0.516±0.009	0.490±0.020	0.619±0.009	0.626±0.012	0.596±0.030	0.395±0.012	0.395±0.014	0.363±0.027

*P=0.008 vs. *AA*, **P=0.002 vs. *AA*, ***P=0.018 vs. *AA*, [4]*P=0.033 vs. *AA*, [5]*P=0.036 vs. *AA* + *AG*

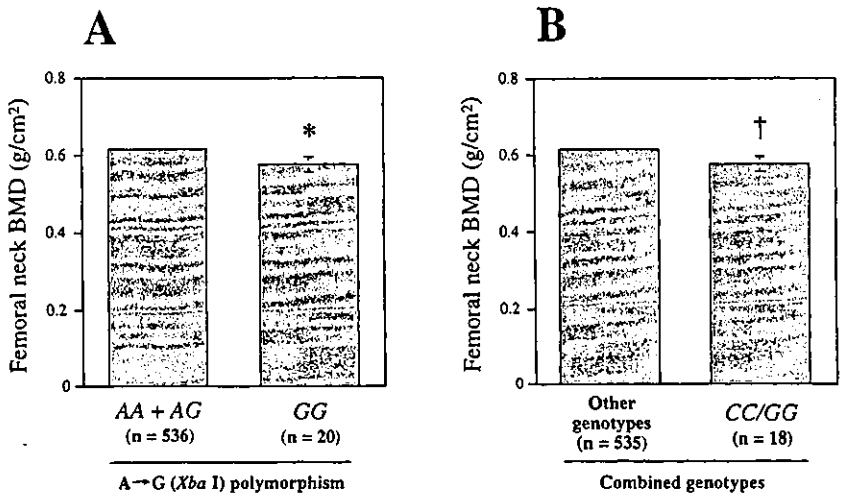

Fig. 1A, B Association of BMD for the femoral neck with the AG (*Xba*I) polymorphism (**A**) or with combined genotypes of the TC (*Pvu*II) and AG (*Xba*I) polymorphisms (**B**) in the ERα gene in women aged 60 years or over. Data are expressed as means ±SE. *P=0.036 vs. *AA + AG*, †P=0.037 vs. other genotypes

Table 5 Bone mineral density (*BMD*) and other characteristics of men (n=1128) according to AG (*Xba*I) genotype of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	AG genotype		
	AA	*AG*	*GG*
n	775 (68.7%)	314 (27.8%)	39 (3.5%)
Age (years)	59.2±0.4	59.0±0.6	59.2±1.8
BMI	23.0±0.1	22.7±0.2	23.2±0.4
Smoking (%)	38.2	37.9	35.9
Fracture (%)	24.9	28.6	25.6
BMD values measured by pQCT (mg/cm³)			
D50	267.3±2.5	265.9±4.0	255.6±11.2
D100	541.2±3.6	539.4±5.6	538.3±15.9
P100	1186.2±5.6	1180.1±8.7	1199.7±24.6
BMD values measured by DEXA (g/cm²)			
Total body	1.086±0.004	1.092±0.006	1.072±0.016
Lumbar spine (L2–L4)	0.987±0.006	0.973±0.009	0.966±0.026
Femoral neck	0.753±0.004	0.751±0.006	0.757±0.018
Trochanter	0.670±0.004	0.662±0.006	0.672±0.017
Ward's triangle	0.554±0.005	0.551±0.008	0.539±0.023

age, BMI, and smoking status statistical significance was not achieved for the differences in BMD at the femoral neck among AG genotypes in women aged 60 years or over; however, adjusted BMD for the femoral neck was significantly lower in women aged 60 years or over with the *GG* genotype than in those with the *AA* or *AG* genotypes [0.576±0.020 g/cm² (*GG*) vs. 0.616±0.009 g/cm² (*AA + AG*); P=0.036, unpaired Student's t test; Fig. 1A]. There were no differences in BMD at the various sites among AG genotypes either in men overall (Table 5) or in men aged under 60 years or aged 60 years or over (data not shown).

To determine whether the TC and AG SNPs exert an additive or synergistic effect on BMD we examined the relationship between the combined genotype and BMD. Given the small number of subjects with the *TT/AG* (n=1), *TT/GG* (n=0), and *TC/GG* (n=5) genotypes, these genotypes were excluded from the analysis of combined genotype. For women overall the distribution of combined genotypes was as follows: *TT/AA* 36.1%, *TC/AA* 24.4%, *TC/AG* 22.3%, *CC/AA* 6.3%, *CC/AG* 7.6%, and *CC/GG* 3.4%. In women aged under 60 years there were no differences in BMD at the sites examined among combined genotypes (Table 6). In women aged 60 years or over BMD for the femoral neck was significantly lower in those with the *CC/GG* genotype than in those with the *TT/AA* or *TC/AA* genotypes (Table 7). After adjustment for age, BMI, and smoking status statistical significance was not achieved for the differences in BMD at the femoral neck among the six genotypes in women aged 60 years or over; however, femoral neck BMD was significantly lower in women aged 60 years or over with the *CC/GG* genotype than in those with the other five genotypes [0.577±0.019 g/cm² (*CC/GG*) vs. 0.615± 0.009 g/cm² (other genotypes); P=0.037, unpaired Student's t test; Fig. 1B]. The differences in adjusted BMD for the femoral neck in women aged 60 years or older between the *TT* and *CC* genotypes, between the *AA* and *GG* genotypes, and between the *TT/AA* and *CC/GG* genotypes (expressed as a percentage of the corresponding larger value) were 3.1%, 6.5%, and 6.9%, respectively. No differences in BMD at the various sites were observed among combined genotypes in men (Table 8).

Discussion

We have shown that the AG (*Xba*I) SNP in the first intron of the ERα gene, alone or in combination with the TC (*Pvu*II) SNP, is associated with BMD for the femoral neck in community-dwelling elderly women, and that the *CC/GG* genotype is a genetic factor for predisposition to reduced bone mass, although the contribution of these

Table 6 Bone mineral density (*BMD*) and other characteristics of women aged under 60 years (n=551) according to combined TC and AG genotypes of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	TT/AA	*TC/AA*	*TC/AG*	*CC/AA*	*CC/AG*	*CC/GG*
n	211 (38.3%)	129 (23.4%)	115 (20.9%)	35 (6.4%)	42 (7.6%)	19 (3.5%)
Age (years)	50.0±0.4	49.4±0.5	49.8±0.5	49.1±0.9	49.6±0.8	50.1±1.2
BMI	22.6±0.2	22.2±0.3	23.3±0.3	22.1±0.5	23.7±0.5	24.4±0.7*
Smoking (%)	12.3	10.2	8.7	11.4	11.9	5.3
Fracture (%)	14.8	14.7	13.3	17.1	11.9	10.5
BMD values measured by pQCT (mg/cm^3)						
D50	218.9±4.8	225.4±6.1	222.6±6.4	230.6±11.6	241.4±10.6	220.6±15.9
D100	560.3±7.3	570.4±9.3	557.3±9.8	548.8±17.7	593.8±16.3	549.0±24.3
P100	1292.2±11.0	1303.8±14.1	1285.2±14.8	1253.8±26.7	1313.8±24.7	1295.1±36.8
BMD values measured by DEXA (g/cm^2)						
Total body	1.047±0.007	1.062±0.009	1.041±0.010	1.062±0.018	1.076±0.016	1.042±0.024
Adjusted total body	1.055±0.008	1.066±0.010	1.044±0.010	1.060±0.017	1.076±0.015	1.046±0.022
L2–L4	0.951±0.010	0.973±0.013	0.957±0.014	0.973±0.026	0.970±0.023	0.964±0.034
Adjusted L2–L4	0.961±0.011	0.978±0.013	0.956±0.014	0.972±0.023	0.960±0.020	0.954±0.030
Femoral neck	0.748±0.007	0.739±0.009	0.743±0.010	0.730±0.018	0.765±0.016	0.736±0.024
Adjusted femoral neck	0.756±0.008	0.747±0.010	0.743±0.010	0.736±0.017	0.759±0.015	0.727±0.022
Trochanter	0.630±0.007	0.629±0.008	0.636±0.009	0.612±0.017	0.658±0.015	0.626±0.022
Adjusted trochanter	0.636±0.007	0.635±0.009	0.634±0.009	0.615±0.016	0.651±0.014	0.617±0.021
Ward's triangle	0.613±0.010	0.609±0.013	0.621±0.013	0.611±0.024	0.643±0.022	0.604±0.033
Adjusted Ward's triangle	0.621±0.011	0.616±0.013	0.620±0.014	0.613±0.023	0.635±0.021	0.596±0.030

*P=0.049 vs. *TC/AA*

Table 7 Bone mineral density (*BMD*) and other characteristics of women aged 60 years or over (n=553) according to combined TC and AG genotypes of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	TT/AA	*TC/AA*	*TC/AG*	*CC/AA*	*CC/AG*	*CC/GG*
n	187 (33.8%)	140 (25.3%)	131 (23.7%)	35 (6.3%)	42 (7.6%)	18 (3.3%)
Age (years)	67.8±0.4	68.8±0.5	69.0±0.5	70.3±0.9	69.1±0.8	70.6±1.3
BMI	23.1±0.2	22.9±0.3	23.2±0.3	23.1±0.6	23.4±0.5	21.6±0.8
Smoking (%)	3.8	2.1	2.3	8.6	4.8	5.6
Fracture (%)	24.6	19.3	25.9	14.3	23.8	16.7
BMD values measured by pQCT (mg/cm^3)						
D50	150.4±4.5	148.6±5.2	142.9±5.3	130.2±10.2	153.4±9.6	116.4±14.2
D100	414.3±6.7	405.1±7.7	408.0±7.9	391.3±15.1	421.3±14.3	365.1±21.1
P100	1023.3±12.4	1020.1±14.2	1012.7±14.6	965.9±27.9	1014.7±26.4	954.2±38.8
BMD values measured by DEXA (g/cm^2)						
Total body	0.887±0.006	0.881±0.007	0.875±0.008	0.851±0.015	0.869±0.013	0.855±0.020
Adjusted total body	0.880±0.010	0.880±0.011	0.874±0.011	0.856±0.016	0.868±0.015	0.870±0.020
L2–L4	0.779±0.010	0.773±0.012	0.777±0.012	0.749±0.024	0.747±0.021	0.718±0.033
Adjusted L2–L4	0.779±0.017	0.779±0.018	0.781±0.018	0.760±0.025	0.748±0.024	0.753±0.033
Femoral neck	0.620±0.006	0.612±0.007	0.611±0.008	0.587±0.015	0.609±0.014	0.550±0.021*
Adjusted femoral neck	0.620±0.010	0.619±0.011	0.616±0.011	0.598±0.015	0.612±0.015	0.577±0.019**
Trochanter	0.520±0.007	0.506±0.008	0.512±0.008	0.499±0.016	0.500±0.014	0.454±0.021
Adjusted trochanter	0.514±0.010	0.507±0.011	0.511±0.011	0.506±0.016	0.497±0.015	0.478±0.020
Ward's triangle	0.403±0.008	0.403±0.010	0.402±0.010	0.378±0.020	0.387±0.018	0.342±0.027
Adjusted Ward's triangle	0.394±0.014	0.402±0.015	0.401±0.015	0.384±0.021	0.384±0.019	0.364±0.027

*P=0.017 vs. *TT/AA*, P=0.05 vs. *TC/AA*, **P=0.037 vs. other genotypes

SNPs to bone mass appears relatively small. Given that femoral neck fracture is the most serious complication of osteoporosis, our results may have clinical implications for the prevention of this condition.

Since selection bias can influence the results of association studies, it is important that study populations be genetically and ethnically homogeneous. Our study population was recruited randomly from individuals resident

Table 8 Bone mineral density (*BMD*) and other characteristics of men (n=1116) according to combined TC and AG genotypes of the ERα gene (*pQCT* peripheral quantitative computed tomography, *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, *P100* proximal radius BMD for the entire cross-sectional area consisting mostly of cortical bone, *DEXA* dual-energy X-ray absorptiometry)

	TT/AA	*TC/AA*	*TC/AG*	*CC/AA*	*CC/AG*	*CC/GG*
n	398 (35.7%)	318 (28.5%)	220 (19.7%)	53 (4.8%)	91 (8.2%)	36 (3.2%)
Age (years)	58.9±0.5	59.5±0.6	59.0±0.7	60.2±1.5	59.1±1.1	59.3±1.8
BMI	23.1±0.1	23.0±0.2	22.9±0.2	22.4±0.4	22.1±0.3*	23.0±0.5
Smoking (%)	38.7	38.1	35.5	39.6	45.1	36.1
Fracture (%)	27.1	21.8	28.4	24.5	28.9	27.8
BMD values measured by pQCT (mg/cm^3)						
D50	268.5±3.5	266.8±3.9	264.9±4.7	261.8±9.8	263.3±7.3	253.1±11.5
D100	543.5±5.0	539.9±5.6	537.5±6.7	535.2±14.0	540.1±10.4	534.6±16.5
P100	1190.7±7.8	1184.1±8.7	1180.0±10.4	1176.9±21.7	1177.7±16.2	1195.4±25.6
BMD values measured by DEXA (g/cm^2)						
Total body	1.089±0.005	1.081±0.005	1.093±0.007	1.090±0.013	1.088±0.010	1.061±0.016
L2–L4	0.984±0.008	0.987±0.009	0.975±0.011	1.011±0.022	0.968±0.017	0.951±0.027
Femoral neck	0.757±0.006	0.749±0.006	0.752±0.008	0.754±0.016	0.749±0.012	0.755±0.019
Trochanter	0.675±0.005	0.664±0.006	0.692±0.007	0.674±0.015	0.663±0.011	0.668±0.018
Ward's triangle	0.558±0.007	0.549±0.008	0.552±0.010	0.561±0.019	0.549±0.015	0.531±0.024

*P=0.029 vs. *TT/AA*

in Obu City and adjacent regions in central Japan, where the population is thought to share the same ethnic ancestry and to possess a homogeneous genetic background. We also showed that the genotype distributions of the two SNPs of the ERα gene were in Hardy-Weinberg equilibrium both in women and in men in our study population. We thus appeared to avoid admixture and selection bias, and our study population appeared genetically homogeneous.

For the TC (*Pvu*II) SNP of the ERα gene, BMD tended to be lower in women aged 60 years or over with the *CC* genotype than in those with the *TT* or *TC* genotypes, consistent with a previous observation in postmenopausal Japanese women [10]. For the AG (*Xba*I) SNP of the ERα gene, however, BMD tended to be lower in women aged 60 years or over with the *GG* genotype than in those with the *AA* or *AG* genotypes. This finding differs from the previous observation for postmenopausal Japanese women that individuals with the *GG* genotype showed the highest BMD for the lumbar spine or total body and those with the *AA* genotype showed the lowest BMD, although statistical significance for these differences was not achieved [10]. In women aged under 60 years we detected no association between TC (*Pvu*II) or AG (*Xba*I) SNPs and BMD at various sites. In contrast, ERα genotype was previously shown to be associated with BMD for the femoral neck or lumbar spine in women aged 24–44 years [12] and to be an independent predictor of heel stiffness index determined by quantitative bone ultrasound in women aged 18–35 years [13]. However, the association of ERα genotype with BMD differed between these two previous studies: Individuals with the *TT* genotype or *AA* genotype showed the lowest BMD in the former study, whereas the individuals with these genotypes showed the highest heel stiffness index in the latter study. Our present data are consistent with the results of the latter study [13]. Several studies have not detected an association of these two SNPs of the ERα gene, either alone or in combination, with BMD [15, 16, 17, 18, 19, 20]. Albagha et al. [11] demonstrated an association of BMD for the lumbar spine or femoral neck with haplotype for these SNPs, although no association was detected between BMD and each SNP alone. An association between estrogen responsiveness of BMD and ERα genotype was not apparent in postmenopausal Korean women who had undergone hormone replacement therapy [20], whereas Salmén et al. [14] suggested that the *TT* genotype of the *Pvu*II polymorphism is a relatively estrogen-insensitive genotype, and that women with the *C* allele may benefit more from the protective effect of hormone replacement therapy on fracture risk than women with the *TT* genotype.

The molecular mechanism that underlies the association of the TC (*Pvu*II) and AG (*Xba*I) SNPs of the ERα gene with age-related bone loss remains unclear. It is possible that these SNPs are in linkage disequilibrium with polymorphisms of other nearby genes located at chromosome 6p25 that are determinants of BMD. However, our present results suggest that the ERα gene is a susceptibility locus for bone mass, especially at the femoral neck, in elderly Japanese women.

Acknowledgements This work was supported in part by Research Grants for Longevity Sciences (12C-01) from the Ministry of Health, Labor, and Welfare of Japan (to Y.Y. and H.S.).

References

1. Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141
2. Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S (1987) Genetic determinations of bone mass in adults: a twin study. J Clin Invest 80:706–710
3. Devoto M, Shimoya K, Caminis J, Ott J, Tenenhouse A, Whyte MP, Sereda L, Hall S, Considine E, Williams CJ, Tromp G, Kuivaniemi H, Ala-Kokko L, Prockop DJ (1998) First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4p. Eur J Hum Genet 6:151–157
4. Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB (1997) Linkage of a gene causing high bone mass to human chromosome 11 (11q12–13). Am J Hum Genet 60:1309–1311
5. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA (1994) Prediction of bone density from vitamin D receptor alleles. Nature 367:284–287
6. Uitterlinden AG, Burger H, Huang Q, Yue F, McGuigan FEA, Grant SFA, Hofman A, van Leeuwen JPTM, Pols HAP, Ralston SH (1998) Relation of alleles of the collagen type Iα1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. N Engl J Med 338:1016–1021
7. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
8. Korach KS (1994) Insights from the study of animals lacking functional estrogen receptor. Science 266:1524–1527
9. Ponglikitmongkol M, Green S, Chambon P (1988) Genomic organization of the human oestrogen receptor gene. EMBO J 7:3385–3388
10. Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H (1996) Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res 11: 306–311
11. Albagha OME, McGuigan FEA, Reid DM, Ralston SH (2001) Estrogen receptor α gene polymorphisms and bone mineral density: haplotype analysis in women from the United Kingdom. J Bone Miner Res 16:128–134
12. Willing M, Sowers M, Aron D, Clark MK, Burns T, Bunten C, Crutchfield M, D'Agostino D, Jannausch M (1998) Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. J Bone Miner Res 13:695–705
13. Patel MS, Cole DEC, Smith JD, Hawker GA, Wong B, Trang H, Vieth R, Meltzer P, Rubin LA (2000) Alleles of the estrogen receptor α-gene and an estrogen receptor cotranscriptional activator gene, amplified in breast cancer-1 (*AIB1*), are associated with quantitative calcaneal ultrasound. J Bone Miner Res 15:2231–2239
14. Salmén T, Heikkinen A-M, Mahonen A, Kröger H, Komulainen M, Saarikoski S, Honkanen R, Mäenpää P (2000) The protective effect of hormone-replacement therapy on fracture risk is modulated by estrogen receptor α genotype in early postmenopausal women. J Bone Miner Res 15:2479–2486
15. Gennari L, Becherini L, Masi L, Mansani R, Gonnelli S, Cepollaro C, Martini S, Montagnani A, Lentini G, Becorpi AM, Brandi ML (1998) Vitamin D and estrogen receptor allelic variants in Italian postmenopausal women: evidence of multiple gene contribution to bone mineral density. J Clin Endocrinol Metab 83:939–944
16. Vandevyver C, Vanhoof J, Declerck K, Stinissen P, Vandervorst C, Michiels L, Cassiman JJ, Boonen S, Raus J, Geusens P (1999) Lack of association between estrogen receptor genotypes and bone mineral density, fracture history, or muscle strength in elderly women. J Bone Miner Res 14: 1576–1582
17. Becherini L, Gennari L, Masi L, Mansani R, Massart F, Morelli A, Falchetti A, Gonnelli S, Fiorelli G, Tanini A, Brandi ML (2000) Evidence of a linkage disequilibrium between polymorphisms in the human estrogen receptor α gene and their relationship to bone mass variation in postmenopausal Italian women. Hum Mol Genet 9:2043–2050
18. Langdahl BL, Lokke E, Carstens M, Stenkjaer LL, Eriksen EF (2000) A TA repeat polymorphism in the estrogen receptor gene is associated with osteoporotic fractures but polymorphisms in the first exon and intron are not. J Bone Miner Res 15:2222–2230
19. Brown MA, Haughton MA, Grant SFA, Gunnell AS, Henderson NK, Eisman JA (2001) Genetic control of bone density and turnover: role of the collagen 1αI, estrogen receptor, and vitamin D receptor genes. J Bone Miner Res 16:758–764
20. Han KO, Moon IG, Kang YS, Chung HY, Min HK, Han IK (1997) Nonassociation of estrogen receptor genotypes with bone mineral density and estrogen responsiveness to hormone replacement therapy in Korean postmenopausal women. J Clin Endocrinol Metab 82:991–995
21. Shimokata H, Ando F, Niino N (2000) A new comprehensive study on aging – the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J Epidemiol 10:S1–S9
22. Yamada Y, Ando F, Niino N, Shimokata H (2001) Transforming growth factor-β1 gene polymorphism and bone mineral density. JAMA 285:167–168

発達心理学研究
2002, 第13巻, 第1号, 42-50

原　著

中高年のストレスおよび対人交流と抑うつとの関連：家族関係の肯定的側面と否定的側面

福川　康之（国立長寿医療研究センター）
坪井さとみ（国立長寿医療研究センター）
新野　直明（国立長寿医療研究センター）
安藤富士子（国立長寿医療研究センター）
小杉正太郎（早稲田大学文学部）
下方　浩史（国立長寿医療研究センター）

本研究は，中高年者を対象として，家族との肯定的・否定的な対人交流と，ストレスおよび抑うつとの関連を検討したものである。対象は国立長寿医療研究センター“老化に関する長期縦断疫学研究”（NILS-LSA）の第一次調査に参加した40代から70代の地域住民2,010名である。階層的重回帰分析の結果，肯定的交流による抑うつ低減効果と，否定的交流による抑うつ増大効果がそれぞれ示され，肯定的交流の抑うつ低減効果は，否定的交流の抑うつ増大効果よりも強いことが明らかとなった。さらに，否定的交流とストレス体験との交互作用が有意となり，否定的交流の抑うつ増大効果は，ストレスレベルが中ないし低度の場合にのみ示され，ストレスレベルが高い場合には示されなかった。これらの結果は，家族との肯定的交流と否定的交流が，中高年者のストレスないし心理的健康とそれぞれ特徴的な関連をもつことを示唆するものである。

【キー・ワード】中高年，ストレス，対人交流，抑うつ，家族

対人関係が心理的健康に及ぼす影響に関しては，これまで肯定的な側面が重視され，ソーシャルサポートの健康増進効果が多く実証されてきた（Cohen, & Willis, 1985; Finch, Okun, Pool, & Ruehlman, 1999）。これに対してRook (1984) は，対人関係には肯定的な側面とともに否定的な側面があることを指摘し，高齢寡婦を対象とした研究により，否定的な対人関係（problematic social ties）が健康増悪効果をもつことを明らかにした。Rook (1984) の知見は，対人交流の肯定的側面に偏重していた従来の研究動向に大きな影響を与え，以後，社会的葛藤（social conflict; Lepore, 1992），否定的相互作用（negative interaction; Krause, & Jay, 1991），否定的交流（negative exchanges; Okun & Keith, 1998）といった用語で，プライバシーの侵害や約束の反故など，対人ネットワークから受ける否定的な作用と，心理的健康との関連が検討されるようになった。本邦でも，坂田・Liang・前田 (1990) が，日本の高齢者研究において，家族との同居がソーシャルサポートの受容と同義に扱われてきた点を挙げ，対人交流の否定的側面に関する考察がこれまで不十分であったことを指摘している。彼らは60歳以上を対象とした全国調査により，肯定的交流と否定的交流とがそれぞれライフイベントや抑うつとどのように関連するかを検討し，否定的交流が抑うつを増大させる効果は，肯定的交流が抑うつを低減する効果よりも強いこと，また，肯定的交流は“身近な人や自身の疾病”が抑うつに及ぼす影響を緩和するが，否定的交流はこの種のライフイベントとは無関係に抑うつを増大させることなど，対人交流のこれら2側面が，ストレスや抑うつとそれぞれ特徴的な関連をもつことを明らかにしている。

これら先行研究の知見は，高齢者の日常生活における適応を考えるうえで，対人交流の否定的側面が心理的健康に及ぼす効果の強さやメカニズムを明らかにする必要を示している。例えば，否定的交流の健康増悪効果と肯定的交流の健康増進効果とを明確に区別することで，従来企図されてきたような対人交流の活性や社会的支援の促進だけでなく，否定的交流の除去を目的とした介入方略の検討が可能となろう（Krause, 1995; Okun, Melichar, & Hill, 1990）。

対人交流が心理的健康に影響を及ぼすメカニズムに関しては，これまで，直接効果（direct effect），結合効果（joint effect），ストレス緩衝・増幅効果（stress-buffering/stress-amplifying effect）の3種の効果が指摘されている。

直接効果は，対人交流とストレスとがそれぞれ独自に心理的健康に及ぼす効果を指す。したがって，ストレス（ストレッサー）の有無や程度に関わらず，肯定的交流が多いほど心理的に健康であること，あるいは，否定的交流が多いほど心理的に不健康であることが示されれば，対人交流の直接効果があるとみなされる。先行研究では，

このような直接効果を検討することで，各対人交流が心理的健康に及ぼす効果の強さが比較されてきた（e.g., Chogahara, 1999; Finch, Okun, Barrera, Zautra, & Reich, 1989; Lepore, 1992; Rook, 1984）。レビュー研究によれば（Finch et al., 1999; Okun, & Keith, 1998），分析対象や使用尺度による違いはあるものの，否定的交流の直接効果は肯定的交流の直接効果よりも強いとの結果が得られる場合が多い。

結合効果は，対人交流の2側面が心理的健康に及ぼす効果を互いに抑制する作用である。結合効果に関する従来の研究モデルの多くは，否定的交流をストレッサーととらえ，肯定的交流がその影響を緩和する効果を明らかにしている（e.g., Lepore, 1992; Okun, & Keith, 1998; Pagel, Erdly, & Becker, 1987; Rhodes, & Woods, 1995）。これに対して，Krause（1995）は，批判や過度の要求などの否定的交流が，ソーシャルサポート受容の満足感を低下させることを示した。Krause（1995）の研究は，心理的健康を外的基準としたものではないが，肯定的交流のもたらす影響を否定的交流が緩衝する効果を部分的に検討したモデルとみなすことができよう。

ストレス緩衝ないし増幅効果では，ストレスが心理的健康に及ぼす影響が，対人交流のレベルによって異なることを仮定している。従来のソーシャルサポート研究が明らかにしてきたように（e.g., Comijs, Penninxs, Knipscheer, & van Tilburg, 1999; Dalgard, Bjørk, & Tambs, 1995），ストレス緩衝効果とは，社会的資源の防護的作用であり，良好な肯定的交流がある場合は，そうでない場合と比べて，強いストレス下でも心理的不健康が生じないことにより，その効果が確認される。これは，肯定的交流が，ストレッサーに対する脅威の知覚を弱め，適切な対処行動を促進するためといわれる（Cohen, & Willis, 1985）。これに対してストレス増幅効果は，否定的交流がストレッサーの影響を強める効果といえる（Shinn, Lehmann, & Wong, 1984）。ストレス下にある人物は，周囲の不安や脅威の感情を喚起するため，不適切で非援助的な言動を受けたり，ストレスの原因となる出来事が生じた責任について，批判や非難を受けたりすることがある（Krause, & Jay, 1991; Wortman, & Lehman, 1985）。このような場合，否定的な対人交流は，ストレスが心理的健康を低下させる効果を増幅すると考えられる。

ストレス，対人交流と心理的健康との関連における上記の諸効果は，相互排除的なものではなく，複数の効果が同時に見いだされることも考えられる（Comijs et al., 1999; Shinn et al., 1984）。しかしながら，先行研究は，肯定的交流と否定的交流のいずれかに焦点を当てるか，複数の仮説の部分的な検討にとどまっているため，対人交流の様々な効果の比較や統制が不十分である。加えて，これらの研究は，特定のストレッサーと対人交流との関連のみに焦点を当てていることや，小規模な対象を用いている点で問題が指摘される（Ingersoll-Dayton, Morgan, & Antonucci, 1997; Okun, & Keith, 1998）。そこで本研究では，中高年の地域住民を対象に大規模調査を行い，ストレスや肯定的・否定的な対人交流が抑うつに及ぼす影響を包括的に検討することとした。本研究の仮説は以下の3つである。1）肯定的交流は抑うつと負の関連をもち，否定的交流は抑うつと正の関連をもつ（直接効果）。2）肯定的交流と否定的交流とは交互作用をもち，抑うつに対する互いの効果を緩衝する（結合効果）。3）肯定的交流ならびに否定的交流は，それぞれストレス体験と交互作用をもち，肯定的交流が多い場合はストレス体験と抑うつとの関連が弱まり（ストレス緩衝効果），否定的交流が多い場合はストレス体験と抑うつとの関連が強まる（ストレス増幅効果）。

方　法

調査および分析対象者

本研究の分析データは，国立長寿医療研究センターによる，“老化に関する長期縦断疫学研究（NILS-LSA; Shimokata, Ando, & Niino, 2000）”から得られた。NILS-LSAの調査対象は，性および年代ごとに層化無作為抽出した40歳から79歳の地域住民である。1997年11月から2000年4月の間に行われたNILS-LSAの第一次調査に参加した2,267名のうち，データに欠損のある者を除く2,010名を本研究の分析対象とした（男性1,065名：平均年齢58.9歳，*SD*10.9，女性945名：同58.2歳，*SD*10.7）。

調査項目

本研究のデータは，ストレス体験に関して面接調査したほかは，すべて自記式調査票により収集した。調査票は自宅で記入したうえで面接時に持参するよう依頼した。面接は国立長寿医療研究センターで行った。本研究で用いた変数を以下に示す。

抑うつ：Radloff（1977）によるCenter for Epidemiologic Studies Depression Scale（CES-D）の日本語版（20項目：島・鹿野・北村・浅井，1985）を用いて測定した。各項目について，最近一週間の自身の状態について“ほとんどなかった”から“たいていそうだった”の4件法で回答させ，順に0点から3点を与えた（逆転項目については順に3点から0点を与えた）。得点が高いほど強い抑うつ傾向にあることを示す。本研究の対象について，Cronbachのα信頼性係数を算出したところ，.86となり，十分安定した尺度と考えられた。

対人交流：野口（1991）により開発された尺度を用いて家族との対人交流を測定し，これを肯定的・否定的対

人交流の指標とした[1]。本尺度は，肯定的交流の因子として“情緒的サポート”および“道具的サポート”，否定的交流の因子として“ネガティブサポート”の計3因子からなる。本研究では“情緒的サポート”因子を肯定的交流の指標として用いた[2]。情緒的サポート因子は“家族のなかに，心配事や悩み事を聞いてくれる人がいますか”，“家族のなかに，あなたを元気づけてくれる人はいますか”などの4項目からなる。否定的交流因子は“家族のなかに，あなたに文句や小言を言う人はいますか”，“家族のなかに，あなたの世話をやきすぎたり余計なお世話をする人はいますか”などの4項目からなる。対象者に，別居を含む家族全般を想定させ，各項目に該当する人物の有無を尋ねた。該当人物がいない場合は0点，いる場合は1点を与え，因子ごとに総計を算出した。したがって，得点が高いほど肯定的ないし否定的交流が多いことを示す。2件法尺度の信頼性評価には，Kuder-Richardson formula-20の利用が推奨されている（Carmines, & Zeller, 1979/1983）。そこで本研究の対象に関して，この方法で信頼性係数を求めたところ，肯定的交流は.72，否定的交流は.65となり，ともに分析に耐える内的一貫性をもつと判断された。

ストレス体験：チェックリストを用いた半構造化面接により測定した。チェックリストは，既存の尺度（Holms, & Rahe, 1967; 三川・中西, 1985; 宗像・川野, 1994）を参考にNILS-LSAで作成した63項目からなり，各項目について最近2年間の体験の有無を尋ねる形式をもつ。本研究では，肯定的な意味を含む項目（“子どもの誕生”など）や，家族との否定的交流と概念的に混同する項目（“子とのトラブル”など）を分析から除くこととし，ライフイベントや慢性的なストレッサーに関する，30項目のストレス体験に関して分析を行った（Appendix参照）。各項目について，体験がなかった場合は0点，体験があった場合は1点を与え，30項目の合計得点を算出し，ストレス体験が多いほど高得点となるよう指標化した。

このほか，対象者の基本的属性（性・年齢・学歴・収入・主観的健康度）を分析上の調整変数として用いた。これらの指標はいずれも対人交流ないし抑うつの関連要因とされている（Dimond, Lund, & Caserta, 1987; Eckenrode, 1983; Roskin, 1984）。性についてはダミー変数を作成し，男性を0，女性を1とした。学歴（小学校もしくは新制中学卒＝1～大学卒業以上＝4）および収入（家計を同じくする者の合計年収150万円以下＝1～同2,000万円以上＝11）は，調査票の当該質問項目への選択肢を連続変数とみなし，高得点ほど高学歴ないし高収入となるよう指標化した。主観的健康度に関しては，NILS-LSA調査票に含まれている単一項目の質問（“あなたの健康状態はいかがですか”）に対して，“非常に悪い”から“非常に良い”までの5件法で回答させ，順に1点から5点を与えた。

分　析

本研究では，抑うつを基準変数とした階層的重回帰分析により，上述の3仮説を検討した。はじめに，基本的属性のみを説明変数として，それらの抑うつに対する効果を確認した（モデルⅠ）。ついで，これらの基本的属性を共変量としたうえで，肯定的交流，否定的交流，ストレス体験を説明変数としたモデル（モデルⅡ）により，対人交流の直接効果を検討した。さらに，モデルⅡに肯定的交流と否定的交流の交互作用項を説明変数として加えたモデル（モデルⅢ）により，結合効果を検討した。最後に，モデルⅢに肯定的交流とストレス体験，ならびに否定的交流とストレス体験の交互作用項を説明変数として加えたモデル（モデルⅣ）により，ストレス緩衝効果とストレス増幅効果を検討した。説明変数の追加に伴う決定係数（ΔR^2）の上昇をあわせて検討することで，加えた変数の効果を確認した。なお，分析には全て統計プログラムパッケージSAS（Ver. 6.12）を使用した。

結　果

変数間の相関分析

Table 1に本研究で用いた変数の平均値と単相関行列を示す。対人交流と基本的属性との相関については，肯定的交流は性（r＝.05），収入（r＝.08），主観的健康度（r＝.05）と有意な相関を示し，女性，高収入，健康度が高い場合は肯定的交流が強い傾向がみられた。これに対して否定的交流は，年齢（r＝−.19），収入（r＝.07），学歴（r＝.10），主観的健康度（r＝−.05）との間に有意な相関が認められ，若齢，高収入，高学歴，健康度が低いほど，否定的交流が強いことが明らかとなった。他方，肯定的交流は抑うつ（r＝−.22）と負の相関を示し，否定的交流はストレス体験（r＝.13）および抑うつ（r＝.14）と

1) NILS-LSA第一次調査では，家族以外との対人交流に関して，否定的交流の項目を含まない一般的なソーシャルサポート尺度により測定している。本研究は，肯定的交流と否定的交流の双方を取り上げて検討することを目的としていることから，家族との対人交流に焦点を当てた分析を行った。もちろん，異なる対人領域に同一の尺度を施行することで，対人交流と心理的健康との関連をより詳細に検討することが可能となろう。NILS-LSA第二次調査（2000年4月より継続中）では，このような方法を用いて血縁者と非血縁者それぞれとの肯定的・否定的交流を測定しており，データの分析を始めている（福川ほか, 2001）。

2) 予備的分析により，“情緒的サポート”因子と“道具的サポート”因子との間に比較的強い正の相関が示された（r＝.52, p＜.001）。両因子を同時に分析することで多重共線性の問題が生じる可能性があること，また，先行研究では情緒的サポートのみを肯定的交流の指標として用いる場合もあることから（e.g. Okun, & Keith, 1998），本研究では情緒的サポートを分析に採用した。なお，道具的サポート”因子を“情緒的サポート”因子のかわりに用いて分析した場合も，本研究と同様の結果が得られた。

Table 1　*分析変数の平均値および相関係数*

	平均値(*SD*)	性	年齢	収入	学歴	主観的健康度	肯定的交流	否定的交流	ストレス体験	CES-D
性	—	1.00								
年齢	58.57(10.78)	-.03	1.00							
収入	6.26(2.46)	-.08***	-.43***	1.00						
学歴	2.35(1.34)	-.15***	-.33***	.23***	1.00					
主観的健康度	3.18(0.67)	-.03	-.17***	.15***	.12***	1.00				
肯定的交流	3.72(0.75)	.05*	.01	.08***	-.01	.05*	1.00			
否定的交流	1.95(1.33)	-.02	-.19***	.07**	.10***	-.05*	-.02	1.00		
ストレス体験	2.09(1.77)	-.04	-.07**	-.01	.05*	-.13***	-.04	.13***	1.00	
CES-D	7.18(6.65)	-.03	.06*	-.16***	-.07**	-.29***	-.22***	.14***	.22***	1.00

*p<.05, **p<.01, ***p<.001

正の相関を示した。これらの結果は，対人交流の2側面が，他の変数とそれぞれ異なる関連をもつことを示唆している。また，肯定的交流と否定的交流との相関係数は有意とならず（r=-.02, n.s.），両変数が互いに独立していることが示された。

ストレスおよび対人交流と抑うつとの関連

抑うつ得点を基準変数とした階層的重回帰分析を行った（Table 2）。

はじめに，モデルⅠとして，性，年齢，学歴，収入，主観的健康度を説明変数とした分析を行ったところ，年齢（β=-.06），収入（β=-.14），主観的健康度（β=-.28）の効果が有意となり，若齢，低収入，不健康の自覚による抑うつ増大効果が示された。また，モデルⅠの決定係数（R^2）は.102であった。

続いて，モデルⅠで投入した基本的属性の効果を調整したうえで，肯定的交流，否定的交流，ストレス体験がそれぞれ抑うつに及ぼす直接効果を検討した（モデルⅡ）。結果，3変数の主効果は全て有意となり，肯定的交流は抑うつ低減効果をもつ一方で，否定的交流とストレス体験は抑うつ増大効果をもつことが示された。肯定的交流の標準偏回帰係数(β)は-.20，否定的交流のβは.11であり，肯定的交流の抑うつ低減効果は，否定的交流の抑うつ増大効果よりも強いと考えられた。また，これら3変数は，モデルⅡのモデルⅠに対する決定係数の上昇にそれぞれ有意な寄与を示した（肯定的交流の追加によるΔR^2=.044, F(1,2001)=101.55, p<.001; 否定

Table 2　*ストレス体験および対人交流が抑うつに及ぼす影響*

	モデルⅠ		モデルⅡ		ΔR^2	モデルⅢ		ΔR^2	モデルⅣ		ΔR^2
	b[a]	β[b]	b	β		b	β		b	β	
性（男性=0，女性=1）	.10	.01	.39	.03		.39	.03		.37	.03	
年齢	-.03	-.06*	-.00	-.01		-.00	-.01		-.00	-.01	
学歴	-.09	-.02	-.16	-.03		-.16	-.03		-.17	-.03	
収入	-.37	-.14***	-.29	-.11***		-.29	-.11***		-.29	-.11***	
主観的健康度	-2.80	-.28***	-2.37	-.24***		-2.37	-.24***		-2.36	-.24***	
肯定的交流			-1.75	-.20***	.044	-1.74	-.20***		-1.75	-.20***	
否定的交流			.55	.11***	.011	.55	.11***		.54	.11***	
ストレス体験			.62	.16***	.030	.62	.17***		.65	.17***	
肯定的交流×否定的交流						.10	.02	.000	.09	.01	
肯定的交流×ストレス体験									.05	.01	.000
否定的交流×ストレス体験									-.12	-.04*	.002*
決定係数（R^2）		.102***		.184***			.184***			.186***	

*p<.05, ***p<.001

[a] 偏回帰係数

[b] 標準偏回帰係数