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Association of radial bone mineral density with CA repeat polymorphism at the interleukin 6 locus in postmenoposal Japanese women

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Abstract Twin studies have shown strong correlations between bone mass and genetic factors. Some of the genes involved could regulate bone metabolism and bone formation and resorption, all processes that determine bone mass. One candidate gene, interleukin 6 (*IL-6*), has been implicated in the pathogenesis of bone loss because it stimulates osteoclasts. We investigated a possible association between the CA repeat polymorphism at the *IL-6* gene locus and the bone mineral density (BMD) of radial bone in 472 postmenopausal Japanese women. Genotypes were classified into six groups according to the number of CA repeats present, from 13 to 18. BMD was expressed as adjusted BMD, which was the body mass index (BMI)- and age-adjusted average BMD. The 73 women who possessed an A1 allele (134 bp, containing 18 repeats of CA) had significantly lower adjusted BMD than those participants ($n = 399$) who did not carry an allele of that size (mean ± SD values, 0.294 ± 0.064 vs 0.312 ± 0.061 g/cm^2; $P = 0.0221$). This result suggests that genetic variation at the *IL-6* gene locus is associated with some determinants of BMD in postmenopausal women.

Key words Interleukin 6 · Bone mineral density · Osteoporosis · Association study · Microsatellite marker

Introduction

The reduction in bone mass per unit volume that is characteristic of osteoporosis is not accompanied by any qualitative abnormalities in bone mineral content or the organic matrix. This indicates that the rate of bone resorption must exceed that of bone formation, and, in fact, the rate of bone formation tends to be lower than normal in patients with osteoporosis, particularly in women after menopause. However, the degree of reduction varies among postmenopausal women, ranging from "normal" to "rapid". As twin studies have shown strong correlations between bone mass and genetic factors (Pocock et al. 1987; Slemenda et al. 1991), it follows that some of the genes in question are likely to act as regulators of bone formation or resorption. Therefore the heterogeneity in bone mass may reflect genetic variations within the population.

Calcium levels in serum are maintained in homeostasis through balanced interactions among calcitonin, parathyroid hormone, vitamin D, steroid hormones, and cytokines, and their receptors and modulators. One of the most likely candidates for determining bone mass is interleukin 6 (*IL-6*). *IL-6* is a pleiotropic cytokine that has also been called interferon beta-2 (IFNB2), B-cell differentiation factor (BSF2), hepatocyte stimulatory factor (HSF), and hybridoma growth factor (HGF), according to the biological activities by which this peptide was identified by independent researchers (Zilberstein et al. 1986, Hirano et al. 1986, Sehgal et al. 1987). *IL-6* has been implicated in the pathogenesis of bone loss because the gene product stimulates osteoclasts. To investigate a possible relationship between genetic variation at the human *IL-6* locus and osteoporosis, we determined the genotypes of a large panel of Japanese women at this polymorphic locus, and correlated their genotypes with bone mineral density.

Subjects and methods

Subjects

DNA samples were obtained from the peripheral blood of 472 postmenopausal Japanese women living in the rural Akita area, whose ages ranged from 66 to 92 years (mean,

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73.2 ± 5.8 years). All were non-related volunteers and gave their informed consent prior to the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary disease, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal disease, or collagen disease, and none was receiving estrogen replacement therapy.

Measurement of bone mineral density (BMD)

The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual-energy X-ray absorptiometry (DPX-L; Lunar, Madison, WI, USA). This parameter was recorded as the adjusted BMD, to correct for differences in age, height, and weight. The formulas used were:

$$\text{Body mass index (BMI)} = (\text{body weight})(\text{kg})/(\text{body height})^2\,(\text{m})$$

$$\text{Adjusted BMD} = \text{BMD} - 0.0052432908 \times (73.1716102 - \text{age}) + 0.0088382998 \times (23.2271299 - \text{BMI})$$ (Kleinbaum et al. 1988).

Determination of microsatellite polymorphism by polymerase chain reaction (PCR)

Each PCR amplification of the CA repeat polymorphism at the *IL6* locus was performed in a volume of 10 μl containing 20 ng of genomic DNA obtained from peripheral blood, 10 mM of Tris HCl (pH 8.4), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.01% gelatin, 200 μM of dNTP, 2.5 pmol of [^{32}P] end-labeled IL6.6F and of non-labeled IL6.7R (Tsukamoto et al. 1998a), and 0.25 units of Taq polymerase. Cycle conditions were 94°C for 4 min, then 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, with a final extension step of 5 min at 72°C, in a Gene Amp PCR9600 System (Perkin Elmer Cetus, Norwalk, CT, USA). The PCR products were electrophoresed for 2 h at 2000 V in 0.3-mm-thick denaturing 6% polyacrylamide gels containing 36% formamide and 8 M urea. The gels were transferred to filter papers, dried at −80°C, and autoradiographed. The size of each allele was determined by comparison with a sequencing ladder of control DNAs (Tsukamoto et al. 1998a).

Statistical analysis

Adjusted BMD values were compared in individuals who possessed one allele of a given genotype and individuals who did not, using non-parametric (Student-Newman-Keuls) analysis. Differences in means were considered statistically significant for P values <0.05.

Results

The 472 postmenopausal Japanese women in our panel were genotyped for the CA repeat polymorphism at the *IL-6* locus. The polymorphic PCR products had been classified into six alleles; A1 (134 bp, 18 CA repeats) to A6 (124 bp, 13 CA repeats) The distribution of the six alleles in this population is shown in Fig. 1. The distribution among these women living in the rural Akita area was similar to that observed previously in the general Japanese population (Tsukamoto et al. 1998a).

We sought to correlate genotype(s) at the *IL-6* microsatellite locus with the adjusted BMD of radial bone. The 73 women who possessed the A1 allele (134 bp, 18 repeats) showed significantly lower adjusted BMD values than those women (n = 399) without this allele (mean ± SD, 0.294 ± 0.064 vs 0.312 ± 0.061 g/cm^2; P = 0.0221) (Table 1). Figure 2 shows the lowering effect on adjusted BMD of the A1 allele at the *IL-6* locus.

The background data of the groups with and without the A1 allele are summarized in Table 1. We found no significant differences between the two groups in mean age, height, or weight. Of the 73 women possessing the A1 allele,

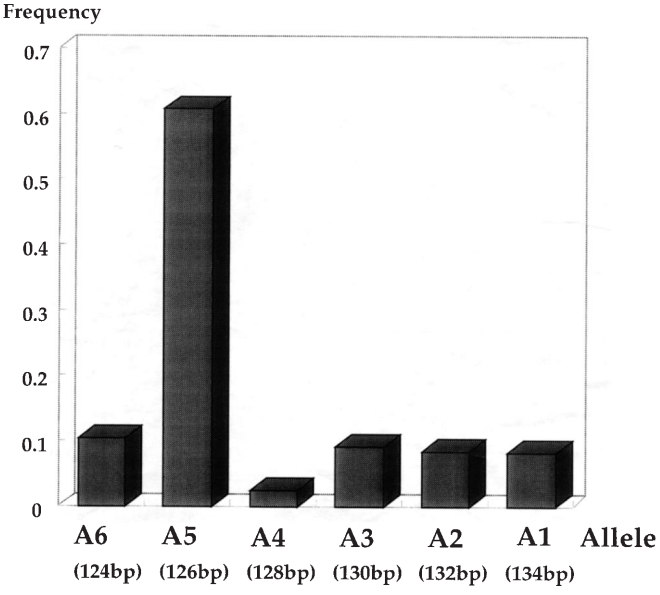

Fig. 1. Frequency distribution of alleles containing the CA repeat polymorphism at the *interleukin-6* (*IL*)*-6* gene locus in 472 postmenopausal Japanese women

Table 1 Comparison of age, body height, and weight in Japanese postmenopausal women with and without a 134-bp allele at the *IL-6* gene locus

	134-bp allele (+); n = 73	134-bp allele (−); n = 399	Statistical significance
Age (years)	72.3 ± 5.1	73.3 ± 5.9	NS
Body height (cm)	145.0 ± 5.8	144.8 ± 6.1	NS
Body weight (kg)	48.4 ± 6.9	49.7 ± 8.7	NS
ADJBMD (g/cm^2)	0.294 ± 0.064	0.312 ± 0.061	P = 0.0221

NS, Not significant; IL, interleukin; ADJBMD, mean adjusted bone mineral density (see text for explanation)

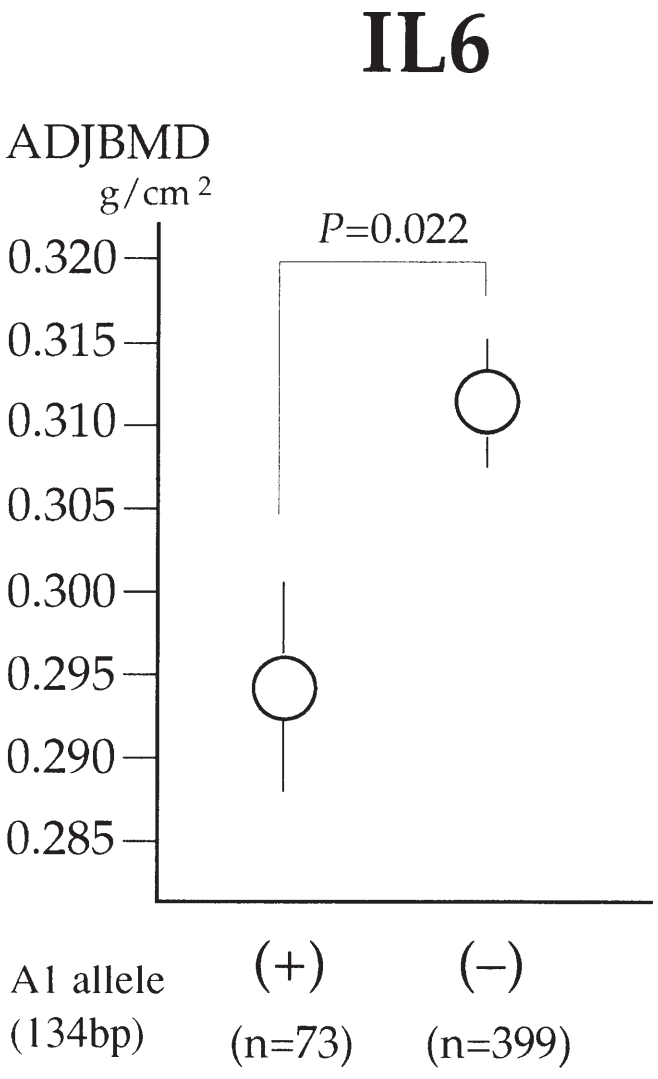

Fig. 2. Comparison of mean adjusted bone mineral density (*ADJBMD*) of radial bone in the group of individuals that carried an A1 allele (+) and the group that did not (−)

5 were homozygous at this locus. However, no significant differences in adjusted BMD were detected between women who were homozygous for the A1 allele and those who were heterozygous (data not shown).

Discussion

Interleukin 6 is a multifunctional cytokine essential to the regulation of bone resorption, as well as the immune response and hematopoiesis. It exerts its actions through binding to its cell-surface receptor, IL6 receptor. As IL-6 and its receptor stimulate osteoclast development and, thereby, the process of bone resorption, they are pathogenic factors in bone loss, especially that triggered by an estrogen-deficiency state (Manolagas et al. 1995). Osteoclast activity is essential for bone remodeling, and imbalances in the regulation of this process may lead to metabolic bone diseases. Postmenopausal osteoporosis is a common disease that is due to increased osteoclast activity. In particular, overproduction of IL-6 has been implicated in osteoporotic bone loss (Manolagas et al. 1995). *IL-6* is a nuclear factor kappa-beta (NFKB)-regulated gene, and its activation is blocked by the estrogen receptors in an NFKB-dependent way. In this connection, it is intriguing that NFkappaB-knockout mice developed osteopetrosis because of a defect in osteoclast differentiation (Iotsova et al. 1997).

For the study reported here, we genotyped a large panel of postmenopausal Japanese women at a newly isolated microsatellite at the *IL-6* locus. One genotype, A1 (134 bp, 18 CA repeats) was associated with lower BMD in these women. The data presented here suggest that variations or some mutations in or adjacent to the *IL-6* gene may affect bone metabolism and eventually cause variations in BMD. The postmenopausal Japanese women in our panel who carried the A1 allele at the *IL-6* locus showed lower adjusted BMD than those who carried only alleles of other sizes. Lowered BMD in postmenopausal women could be a result of abnormally rapid bone loss and/or lower peak bone mass that had occurred when they were young adults. To clarify these issues it will be necessary to carry out a further genetic study or to investigate the genomic structure of the entire region containing the *IL-6* gene and its control elements in affected individuals.

Investigations of genetic variations in humans have been attempted at the vitamin D receptor locus (Morrison et al. 1994), the estrogen receptor alpha / beta loci (Sano et al. 1995; Kobayashi et al. 1996, Tsukamoto et al. 1998b), the matrix Gla protein locus (Watanabe et al. 1998), and the collagen type I alpha 1 locus (Grant et al. 1996) to determine their role in bone metabolism. Murray et al. (1997) studied the relationship of *IL-6* polymorphism to BMD in women living in the northeast of Scotland; their results were similar for menopausal and post-menopausal women. The 3′ flanking polymorphism targeted in their study lies within 110 kb of the polymorphism targeted in the present study. Murray et al. measured BMD at the lumbar spine, a useful method for measuring bone mass in younger women. We measured BMD at the radius, following the 1995 Guidelines for Osteoporosis Screening in a Health Check-up Program for the Elderly conducted by the Ministry of Health and Welfare of Japan. This method was recommended for BMD measurement in elderly people, who often have osteoarthritis of the spine. Given that our results in postmenopausal Japanese women are similar to those of Murray et al., we suggest that the polymorphic microsatellite at the *IL-6* locus may be a useful marker for predicting future bone loss and for allowing early therapeutic intervention in women at high risk of osteoporosis.

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