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ORIGINAL ARTICLE

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A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density

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Abstract Interleukin-6 (IL6) has come to be regarded as a potential osteoporotic factor because it has stimulatory effects on cells of the osteoclast lineage, and, thus, may play a role in the pathogenesis of bone loss associated with estrogen deficiency. We previously described association of the IL6 microsatellite with bone mineral density (BMD), as well as genetic linkage of the IL6 locus to human osteoporosis, by means of sib-pair analysis. However, the molecular mechanism by which this locus regulates BMD remains unknown. Accordingly, we searched for polymorphisms in the 5′ and 3′ flanking regions and in all five exons of the *IL6* gene in a Japanese population sample. We identified three single-nucleotide sequence variations: a C/G substitution at nucleotide (nt) −634 in the promoter region, a G/A substitution at nt 4391 in the 3′ noncoding region, and a variation in the AnTn tract around nt −447. The last of these had already been observed in Caucasians, as well as in Japanese. The single-nucleotide polymorphism at −634 created a restriction site for the *Bsr*BI endonuclease, and the frequency of the minor (G) allele was 0.184. Five haplotypes were constructed among three variations examined in the population. Linkage disequilibrium was observed between the variation at −634 and the variation at 4391, as well as between the variation at −634 and the AnTn tract variation. We found a significant correlation, in 470 subjects, between the presence of the G allele and decreased BMD, by analysis of variance. When BMD values were compared among the three genotypic groups (G/G, G/C, C/C) at nt −634, BMD was lowest among the G/G homozygotes (mean ± SD; 0.284 ± 0.062 g/cm^2), highest among the C/C homozygotes (0.314 ± 0.059 g/cm^2), and intermediate among the heterozygotes (0.303 ± 0.066 g/cm^2; $P < 0.05$).Given the several lines of evidence from different genetic studies, we suggest that *IL6* is, indeed, one of the genes affecting bone metabolism, in which variations can lead to osteoporosis.

Key words Interleukin-6 gene · Bone mineral density · Osteoporosis · Single-nucleotide polymorphism (SNP)

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Introduction

Osteoporosis is a common human disease that is considered to result from the interplay of multiple genetic and environmental factors. Twin and family studies have yielded strong correlations between bone mass and genetic factors. Certain genes (e.g., cytokines such as interleukin-1, interleukin-6, or tumor necrosis factor-alpha) are capable of regulating the metabolism, formation, and resorption of bone, processes that determine bone mass. The most important predictor of fracture is bone mineral density (BMD), a measurement that reflects many genetic and lifestyle elements. Knowing the genetic risk factors for an individual would assist in the diagnosis, prevention, and therapy of osteoporosis. Population-based association studies have suggested that genetic effects can be ascribed to polymorphisms of a number of genes involved in bone metabolism, although the genetic basis of osteoporosis is not well understood. Polymorphisms of genes encoding vitamin D receptor (VDR), estrogen receptor (ER), collagen type I alpha (COL1A1), calcitonin receptor (CTR), osteocalcin, transforming growth factor-beta (TGF-beta), interleukin-1 receptor antagonist (IL-1RN), insulin-like growth factor-I (IGF-I), calcitonin receptor (CTR), parathyroid hormone (PTH), peroxisome proliferator-activated receptor gamma (PPAR gamma), calcitonin (CT), and interleukin-6 (IL6)

have all been implicated as genetic markers for BMD (Morrison et al. 1994; Sano et al. 1995; Grant et al. 1996; Dohi et al. 1998; Keen et al. 1998; Masi et al. 1998; Miyao et al. 1998; Taboulet et al. 1998; Yamada et al. 1998; Hosoi et al. 1999; Ogawa et al. 1999; Tsukamoto et al. 1999; Miyao et al. 2000).

Interleukin-6 (IL-6) is a multifunctional cytokine essential to the regulation of the immune response, hematopoiesis, and bone resorption. It exerts its actions through binding to its cell-surface receptor, IL-6 receptor. As IL6 and its receptor stimulate osteoclast development, and, thereby, the process of bone resorption, they are likely pathogenic factors in conditions associated with bone loss, especially those triggered by estrogen deficiency (Monologas and Jilka 1995). IL6 also has been implicated as a mediator for the effects of IL1, a potent stimulator of bone resorption. IL6 is a possible mediator of estrogen-deficient bone loss in mice (Poli et al. 1994), and clinical studies have shown that IL-6 mRNA expression in bone, as detected by reverse transcription-polymerase chain reaction (RT-PCR) assay, is enhanced in 95% of patients with osteoporotic vertebral fracture, as compared with such enhancement in 50% of postmenopausal controls (Ralston 1994).

We previously carried out association and sib-pair linkage analyses, using both qualitative and quantitative methods, in which a marker located at the IL6 locus showed evidence for association and linkage (Ota et al. 1999; Tsukamoto et al. 1999). However, the molecular mechanism by which BMD is regulated by IL6 is uncertain. We have proposed that the regulation of *IL6* expression or activity in osteoporosis patients differs from that in unaffected individuals, possibly as the result of variations in its 5′ regulatory region, or mutations that alter the function of the gene product. Several well documented nucleotide polymorphisms occur within the regulatory regions of other cytokine genes, and some of them are associated with an altered rate of expression of the genes in question (Wilson et al. 1997; Fishman et al. 1998; Olomolaiye et al. 1998; Linker-Israeli et al. 1999). In the present study, we describe a sequence variation within the 5′ regulatory region of the *IL6* gene that is associated with lower BMD in a gene dose-dependent manner.

Subjects and methods

Subjects

DNA samples for association study were obtained from peripheral blood of 470 postmenopausal Japanese women, living in northern Japan, whose ages ranged from 66 to 92 years (mean, 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 and criteria 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 adjusted BMD (ADJBMD), in order to correct for differences in age, height, and weight. The formulas used were as follows: body mass index (BMI) = (body weight) (kg)/(body height)^2 (m); adjusted BMD (ADJBMD) = BMD − 0.0052432908 × (73.1716102 − age) + 0.0088382998 × (23.2271299 − BMI) (Kleinbaum et al. 1988). Although many researchers measure BMD in the lumbar spine, we measure BMD in the radius, following the Guidelines for Osteoporosis Screening in the health checkup program conducted by the Ministry of Health and Welfare of Japan (Orimo et al. 1996). This method is recommended for measuring BMD in elderly women, in whom osteoporosis is often associated with osteoarthritis of the spine. Higher BMD averages reported in some studies may simply reflect differences in methods of measurement.

Single-strand conformational polymorphism (SSCP) analysis

As genetic linkage results had suggested that variations in *IL6* could be involved in the pathogenesis of osteoporosis, 40 postmenopausal Japanese women were initially screened for molecular variants by SSCP analysis, performed on PCR-amplified segments spanning all five exons of the *IL6* gene, 154 bp of intron 1, and 821 bp of the genomic sequence upstream of the transcriptional start site. A total of 14 primer sets (Table 1) allowed complete coverage of these genomic regions. The lengths of the PCR products subjected to SSCP analysis ranged from 177 bp to 239 bp. Each segment was amplified according to procedures described previously (Tsukamoto et al. 1998); SSCP analysis was also carried out according to procedures described elsewhere (Hirayama et al. 1998). Briefly, each PCR product was mixed with loading buffer, heated rapidly, cooled on ice, and applied to a 5% polyacrylamide gel containing 5% glycerol in 0.5 × Tris-borate + ethylenediamiretetraacetic acid (TBE) buffer. Electrophoresis was performed under two different conditions: 150 V for 16 h at room temperature, and 240 V for 16 h at 4°C. Each variant PCR product was sequenced according to methods previously described (Hopkins et al. 1999), in an ABI 377 system (Foster, CA, USA), using 3.2 pmol of the appropriate primer and 0.1 pmol/μl of PCR product. Sequencing took place at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, for 25 cycles.

Genotyping for molecular variants in the *IL6* gene

The A/T variation in the composition of the AnTn tract around −447 in the promoter region and the G/A substitu-

Table 1. Sequences of the forward and reverse primers used to generate each PCR product for sequence analysis of the *IL-6* gene

Primer names	Nucleotide sequence of primers (forward/reverse)
IL-6 Promoter A	5′-CTCAATGACGACCTAAGCTG-3′/5′-GCCTCAGACATCTCCAGTC-3′
IL-6 Promoter B	5′-ACTCTTTGTCAAGACATGCC-3′/5′-TGGGGCTGATTGGAAACCTT-3′
IL-6 Promoter C	5′-TCAGTTCAGAACATCTTTGGTT-3′/5′-ATGTCTTGACAAAGAGT-3′
IL-6 Promoter D	5′-CTTCTTCATAATCCAGGCTTG-3′/5′-AAAGCTGAAGTCATGCACGAAG-3′
IL-6 Promoter E	5′-GAGACGCCTTGAAGTAACTG-3′/5′-AACCAAAGATGTTCTGAACTGA-3′
IL-6 Ex. 1	5′-TATTAGAGTCTCAACCCCCAA-3′/5′-CTCGACCGGCTGGCAGTT-3′
IL-6 Int. 1	5′-GCTATGAACTCCTTCTCCAC-3′/5′-AGAAGGCAACTGGACCGAAG-3′
IL-6 Ex. 2A	5′-GTGCTGTCAGCTCACCCC-3′/5′-CGTCGAGGATGTACCGAATT-3′
IL-6 Ex. 2B	5′-CCCAGGAGAAGATTCCAAAG-3′/5′-AAAGACCTCCTAATGCAGGC-3′
IL-6 Ex. 3A	5′-TGGTGTTTGTTTTAGGGACAC-3′/5′-GGAGAAGTTTTGCCTAAGGAA-3′
IL-6 Ex. 4A	5′-GGCGATAACCAATTTTCCCAC-3′/5′-CTGTCTTTGAGCCTGTCTTC-3′
IL-6 Ex. 5A	5′-ATTTATTCAACATTTAAACAATCCT-3′/5′-CCGAAGAGCCCTCAGGCT-3′
IL-6 Ex. 5B	5′-CTGCGCAGCTTTAAGGAGTT-3′/5′-TAGTGTCCTAACGCTCATACT-3′
IL-6 Ex. 5C	5′-GGCACAGAACTTATGTTGTTC-3′/5′-CTGCATAGCCACTTTCCATTA-3′

PCR, Polymerase chain reaction; IL, interleukin

tion at 4391 in the 3′ noncoding region of exon 5 of the *IL6* gene were genotyped by the PCR-SSCP method, as previously described (Hirayama et al. 1998). To analyze the *Bsr*BI restriction fragment length polymorphism (RFLP) in the promoter region of the *IL-6* gene, PCR amplifications were carried out as described previously (Tsukamoto et al. 1998), using the primers IL6PAF (5′-GAGACGCCTTGA AGTAACTG-3′) and IL6PAR (5′-AACCAAAGATGTT CTGAACTGA-3′). After the PCR product was digested with *Bsr*BI endonuclease, the restriction digest was separated in a 2% agarose gel, generating a 120-bp fragment and a 60-bp fragment. The 60-bp fragment represents the "G" allele.

Construction of haplotypes and their frequencies; statistical analysis of linkage disequilibrium, and association test

Haplotype frequencies among the 940 alleles investigated were calculated with Arlequin software (Geneva, Switzerland). Linkage disequilibrium was investigated for all possible two-way comparisons of the three polymorphisms, using Thompson's method (D, D′, and r^2; Thompson et al. 1988; Miller et al. 2000). That is, for loci L_A and L_B, each with two alleles A (B) and a (b), let p_A be the frequency of allele A, let p_{AB} be the frequency of the AB haplotype, and (in general) p_A be the frequency of allele i, and p_{ij} the frequency of the ij haplotype. The coefficient of disequilibrium, D, is the difference between the observed haplotype frequency and the frequency expected under statistical independence: $D = p_{AB}\ p_{ab} - p_{Ab}\ p_{aB}$. The normalized disequilibrium coefficient is obtained by dividing D by its maximum possible value ($D' = D/|D|_{max}$); $|D|_{max} = \min(p_A\ p_B, P_a\ p_b)$ if $D < 0$, and $|D|_{max} = \min(p_A\ p_b, P_a\ p_B)$ if $D > 0$. The correlation coefficient is $r^2 = D^2/(A\ B\ a\ b)$. Significance levels were determined by χ^2 statistics for the corresponding 2×2 table. ADJBMD values were compared between three genotypic categories using nonparametric (Student-Newman-Keuls; StatView J 4.5; Abacus Concepts, Cany, NC, USA, 1992) analysis. Differences in means were considered significant for *P* values of less than 0.05.

Results

Allelic association studies and sib-pair linkage analyses that we carried out earlier in the Japanese population suggested that variations at the IL6 locus were involved in the pathogenesis of osteoporosis (Ota et al. 1999; Tsukamoto et al. 1999). In the work reported here, we followed up these results by screening DNA from a large panel of unrelated, postmenopausal Japanese women for sequence variations in essential parts of the *IL6* gene. PCR-SSCP analysis of all five exons, intron 1, and the 5′ and 3′ flanking regions of the gene identified three sequence variations: a G/C substitution at nt −634 in the 5′ regulatory region, a G/A substitution at nt 4391 in the 3′ noncoding portion of exon 5, and a variation in the composition of the AnTn tract around nt −447 (Fig. 1). An A9T11 allele was previously described in the Japanese population (Yasukawa et al. 1987). Two alleles, one consisting of A10T10 and the other of A10T11, were observed in Japanese women in the present study, but another published polymorphism, a G/C substitution at position −237 (Fishman et al. 1998) was not. The G/C variation at −634 created a restriction site for *Bsr*BI. The nucleotide numbering adopted here corresponds to that published by Yamasaki et al. (1988).

Genotypes for each of the three variant sites observed in our panel were determined, and were correlated with BMD in this cohort of 470 postmenopausal women living in northern Japan. The genotypic data are summarized in Table 2. A significant correlation with BMD was identified only with the G/C substitution at nt −634 in the regulatory region of the *IL-6* gene; i.e., the G allele was associated with decreased BMD in the statistical analysis of variance. When

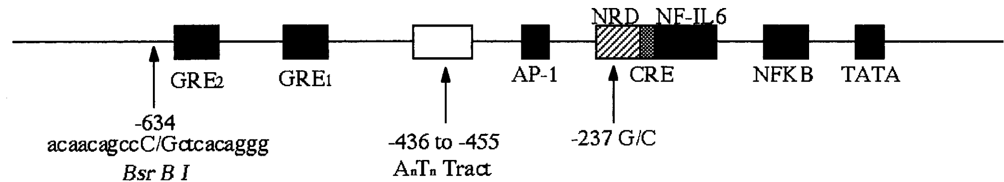

Fig. 1. Schematic representation of the 5′-flanking region of the interleukin-6 (*IL-6*) gene. The TATA box is at −90 to −87; other domains are NFKB, −136 to −127; NF-IL6 (C/EBPβ), −221 to −208; CRE (cyclic adenoside monophosphate [cAMP] responsive element), −226 to −221; NRD (negative regulatory domain), −288 to −227; AP-1, −346 to −340; an AnTn tract, −455 to −436; and GRE_1 and GRE_2 (glucocorticoid responsive elements 1 and 2), −529 to −524 and −620 to −615, respectively (Tanabe et al. 1988). The numbering corresponds to the published sequence of Yamasaki et al. (1988)

Table 2. Genotypic classification of three *IL-6* polymorphisms, and age-adjusted bone mineral density (ADJBMD) values in the test population

Position	Nucleotide substitution (x/X)	Allele frequency		ADJBMD (mean ± SD g/cm^2)		
		X	x	XX	Xx	xx
−634[a]	G/C	0.814	0.184	0.314 ± 0.059 (n = 316)	0.303 ± 0.066 (n = 133)	0.284 ± 0.062 (n = 21)
AnTn around −447	A10T11/A10T10	0.869	0.131	0.310 ± 0.061 (n = 397)	0.300 ± 0.060 (n = 55)	0.293 ± 0.060 (n = 5)
+4391 (exon 5)	A/G	0.900	0.100	0.310 ± 0.063 (n = 417)	0.307 ± 0.050 (n = 46)	(n = 0)

Total number of the test population was 470. Not all subjects had genotypes unambiguously determined for each polymorphisms and, therefore, the total number of amendable subjects presented in the table varied among polymorphisms

[a] When BMD values were compared among the three genotypic categories at −634 (G/G, G/C, C/C), BMD was lowest among G/G homozygotes, highest in C/C homozygotes ($P < 0.05$), and intermediate among heterozygotes

Table 3. Clinical characteristics of 470 postmenopausal Japanese women, classified by genotypes at the *IL6* −634 G/C site

	CC (n = 316)	GC (n = 133)	GG (n = 21)
Age (years)	73.054 ± 5.842	73.634 ± 5.735	72.511 ± 5.115
Weight (kg)	49.5 ± 8.6	49.4 ± 8.0	49.9 ± 8.9
Height (cm)	145.1 ± 6.0	144.5 ± 5.8	143.8 ± 7.9
ADJBMD (g/cm^2)	0.314 ± 0.059	0.303 ± 0.066	0.284 ± 0.062

Values are means ± SD

Table 4. Frequencies of haplotypes constructed from three SNPs, including AnTn tract variation

No.	Frequency	−634 (G/C)	AnTn	4391 (A/G)
1	0.74909	G	A	T11
2	0.12674	C	A	T11
3	0.06062	C	A	T10
4	0.05100	G	G	T11
5	0.01255	G	A	T10

SNP, Single-nucleotide polymorphism

BMD values were compared among the three genotypic categories (G/G, G/C, C/C), BMD was lowest among G/G homozygotes (mean ± SD, 0.284 ± 0.062 g/cm^2), highest in C/C homozygotes (0.314 ± 0.059 g/cm^2), and intermediate among heterozygotes (0.303 ± 0.066 g/cm^2; $P < 0.05$) (Table 2). Deviation of genotype frequencies from Hardy-Weinberg equilibrium was tested. No deviation from the equilibrium was observed in any of the three polymorphisms in the present population.

Construction of haplotypes and calculation of their frequencies were carried out using the Arlequin algorithm; the results are summarized in Table 4. We identified five distinct haplotypes among the 470 subjects, Two of the five haplotypes were more frequent than 10%, one accounting for 75% of these haplotypes (Table 4). We analyzed linkage disequilibrium for all possible two-way comparisons of the three polymorphisms, using Thompson's method (D, D′, and r^2). The results are summarized in Table 5, i.e., the polymorphism at position −634 was in linkage disequilibrium with the polymorphism at position 4391 (D′ = −1), and in weak linkage disequilibrium with the variation in the AnTn tract (D′ = 0.79; χ^2 = 192). Given the several lines of evidence provided by diverse genetic studies, we suggest that *IL-6* is one of the genes affecting bone metabolism that may harbor variants leading to osteoporosis.

Table 5. Analysis of linkage disequilibrium for all possible two-way comparisons among two SNPs and the AnTn tract variation

	AnTn	4391 (A/G)
−634 (G/C)	D′ = 0.7889 r^2 = 0.2131 É‘2 = 192.2 $P = 1.03 * 10^{-43}$	D′ = −1.0 r^2 = 0.0124 É‘2 = 11.176 P = 0.0083
AnTn		D′ = −1.0 r^2 = 0.042 É‘2 = 3.827 P = 0.0504

See text for explanation of analysis method (according to Thompson et al. 1998; Miller et al. 2000)

Discussion

In the study reported here, we screened all five exons and exon/intron junctions of the *IL-6* gene, as well as 1 kb of the promoter region, by PCR-SSCP analysis of DNAs from 40 Japanese women, and by analysis of genotypes in DNA from a total population sample of 470 women. Three different molecular variants were identified, but significant association with BMD was detected only in the case of a G/C substitution at position −634 in the regulatory region, where BMD values were lowest in G/G homozygotes, intermediate in G/C heterozygotes, and highest in C/C homozygotes. The data implied that variation in the regulatory region of the *IL6* gene may affect bone metabolism and, eventually, lead to variations in BMD. Lowered BMD in elderly women could be a result of abnormally rapid bone loss and/or lower peak bone mass that had occurred when they were young adults. Although the variation at nt −634 does not affect any known DNA-binding motifs (Tanabe et al. 1988), this polymorphic site could be part of a sequence that binds to unknown gene elements or alters the secondary structure of DNA to affect the access of *cis*-acting transcription factors to the promoter/enhancer regions, or it could be a marker in linkage disequilibrium with an undiscovered causative variation. Therefore, studies must be carried out to define the function of the −634 G/C variation, as well as to examine its effect on BMD in different populations.

Recently, another allelic variant in the *IL6* regulatory region was correlated with the occurrence of systemic lupus erythematosus and with elevated levels of IL6 expression (Linker-Israeli et al. 1999). Moreover, a separate G/C polymorphism in the IL6 promoter was shown to affect transcription of the *IL6* gene; the C/C genotype at that site was found less frequently in patients with systemic-onset juvenile chronic arthritis (Olomolaiye et al. 1998). In this connection, it is worth noting that Murray et al. (1997) have described a positive association between *IL-6* polymorphism and BMD among women living in northeastern Scotland. These investigators measured BMD in the lumbar spine, but we measured BMD at the radius, following the Guidelines for Osteoporosis Screening in the health checkup program conducted by the Ministry of Health and Welfare of Japan. The latter method is recommended for BMD measurement in elderly women because they often suffer concurrent osteoarthritis of the spine. Nevertheless, the similar results in different populations support the conclusion that *IL6* gene variation can affect BMD. Takacs et al. (2000) tested for linkage and association between *IL6* gene variable-number tandem repeats (VNTR) polymorphism in the 3′ untrans lated region (UTR) region and peak bone mass in a large population of healthy sib-pairs, and found no linkage/association. Their subjects were young premenoposal adults; in addition, BMD was measured in the spine/hip, unlike the radial BMD measured in the present study. Discrepancies between their findings and ours may be related to these fundamental differences in each of the study designs, which may reflect different physiological features.

We had considered the possibility that *IL6* could be a candidate gene for osteoporosis because of its potential involvement in osteoclast differentiation and function (Roodman 1992). Our observations of linkage between osteoporosis and the *IL6* locus suggested that molecular variants in this gene may, indeed, contribute to this disease. There are several well documented instances of nucleotide polymorphisms occurring within regulatory regions of cytokine genes; some of them are associated with an altered rate of gene expression and/or with a specific disease. For instance, polymorphism in the promoter region of the *IL1 alpha* gene is associated with pauci-articular juvenile rheumatoid arthritis (McDowell et al. 1995), and a strong association has been noted between a polymorphism in the TNF-alpha promoter and susceptibility to cerebral malaria and mucocutaneous leishmaniasis (McGuire et al. 1994). In the latter case the associated allele induced stronger expression of a reporter gene than did the common allele. Moreover, nucleotide substitutions in regulatory regions of the angiotensinogen gene (Inoue et al. 1997) and the apolipoprotein A-I gene (Angotti et al. 1994) affect the basal transcription rates of these genes.

The present study has yielded some genetic as well as biological insights into a possible mechanism of genetic predisposition to osteoporosis; that is, *IL-6* alleles carrying guanine at position −634 may be expressed more efficiently than C alleles, and this heightened expression may represent the direct cause of lower BMD levels in individuals carrying the G allele.

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