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ORIGINAL ARTICLE

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Association of interleukin-6 promoter variant with bone mineral density in pre-menopausal women

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Abstract Interleukin-6 (IL6) has many roles essential to the regulation of the immune response, hematopoiesis, and bone resorption. Three single-nucleotide polymorphisms (SNP) in the *IL6* promoter region were genotyped by the single-base extension method. The frequencies of each SNP were 0.002 (*IL6*−597 G → *A*), 0.27 (*IL6*−572 G → *C*), and 0.002 (*IL6*−174 G → *C*) in a Korean population ($n = 1{,}082$). *IL6*−597 G → *A* and *IL6*−174 G → *C* were totally linked together ($d^2 = 1$) and showed very low allele frequencies (0.002), which are common in Caucasians. On the other hand, the frequency of the *IL6*−572 G → *C***C* allele was much higher (0.27) than that in Caucasian populations (< 0.07). One of the *IL6* promoter SNPs, viz., *IL6*−572 G → *C*, showed significant associations with bone mineral density (BMD), i.e., the C allele was associated with increased BMD ($P = 0.02$, co-dominant model; $P = 0.007$, dominant model). The mean BMD was highest in homozygous C individuals (0.67 ± 0.15), lowest in homozygous G individuals (0.58 ± 0.19), and intermediate in heterozygotes (0.64 ± 0.21). In the present study, we describe a variant in the *IL6* promoter region that shows positive association with higher BMD in a gene-dose-dependent manner in pre-menopausal women.

Keywords Interleukin6 promoter variant · BMD · Osteoporosis · Pre-menopausal women

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Introduction

Allelic variants in the human genome are likely to affect the occurrence of osteoporosis and other bone diseases. Several groups have found genes of which variants are involved in osteoporosis. Polymorphisms of genes encoding the vitamin D receptor (Fontova Garrofe et al. 2000; Ho et al. 1999; Papiha et al. 1999; Sosa Henriquez et al. 1998), the estrogen receptor (Chen et al. 2001a; Sano et al. 1995), collagen type I alpha (Peris et al. 2000; Thiry-Blaise et al. 1995), osteocalcin (Chen et al. 2001b), transforming growth factor-beta (Yamada et al. 1998, 2001), interleukin-1 receptor antagonist, insulin-like growth factor-I, calcitonin receptor (Masi et al. 1998), calcitonin (Masi et al. 1998; Miyao et al. 2000; Taboulet et al. 1998; Tsukamoto and Emi 1998), and interleukin-6 (*IL6*; Ota et al. 2001; Shinohara et al. 2001) have all been implicated as genetic markers for bone mineral density (BMD).

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. Osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in fragility and susceptibility to fracture. The most important predictor of fracture is the BMD, which is influenced by genetic factors and lifestyle. Understanding the genetic factors involved osteoporosis should assist in the diagnosis, prevention, and therapy of osteoporosis.

Interleukin-6 (IL6) 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, IL6 receptor. As IL6 and its receptor stimulate osteoclast development, and thereby, the process of bone resorption, they are possible pathogenic factors in conditions associated with bone loss, especially those triggered by estrogen deficiency (Manolagas et al. 1995). IL6 has also been implicated as a mediator of 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, compared with an enhancement in 50% of postmenopausal controls (Ralston 1994).

In order to discover additional genetic polymorphism(s) implicated in osteoporosis and/or BMD, we have scrutinized the genetic polymorphisms in *IL6* as a potent candidate gene for osteoporosis in a cohort genetic study. We have examined the genetic effects of three polymorphisms in the *IL6* promoter (−597G → A, −572G → C, −174G → C). In the present study, we describe a variant that lies in the *IL6* promoter region and that shows positive association with higher BMD in a gene-dose-dependent manner in pre-menopausal women.

Materials and methods

Patients

Blood DNA samples were obtained from 335 Korean premenopausal women who visited MockDong Hospital of Ewha Woman's University for routine checkup and whose ages ranged from 22 to 49 years (mean ± SD: 37.7 ± 6.7 years). All were volunteers and gave their informed consent prior to this study. No participant had medical complications or was undergoing treatment known to affect bone metabolism.

Measurement of BMD

The BMD of the distal one-third of the radial bone of each participant was measured by dual energy X-ray absorptiometry (DEXA, Lunar Pixi, USA). The device was calibrated twice daily by using a standard BMD phantom provided by the manufacturer. The precision error of the device was 1.2%.

Genomic DNA extraction

Blood samples were obtained, with informed consent, from Korean individuals who visited Ewha Woman's University hospital for routine checkup. Genomic DNA was prepared from each blood sample by using the QIA amp blood kit (QIAGEN, USA). For the exact calculation of allele frequencies in the Korean population, samples from our Korean asthma cohort ($n = 747$) were also included.

PCR procedure

PCR primer sequences are listed in Table 1. PCR was performed in a mixture of 1.25 pmol of each primer, 50 ng genomic DNA, 250 M dNTPs, and 0.15 U *Taq* DNA Polymerase (Applied Biosystems, Foster City, Calif.) in the buffer provided by the manufacturer. Amplification was carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) under touchdown conditions (Don et al. 1991). To clean up the PCR for the primer extension reaction, 1 U SAP (Amersham Life Sciences, Cleveland, Ohio) and 2 U *Exo*I (Amersham Life Sciences) were added to the PCR products. The mixture was incubated at 37°C for 1 h, followed by 15 min at 72°C to inactivate the enzymes.

Primer extension reactions

Primer extension reactions were performed with the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems) as recommended by of the manufacturer. To clean up the primer extension reaction, 1 U SAP was added to the reaction mixture, which was then incubated at 37°C for 1 h, followed by 15 min at 72°C for enzyme inactivation.

Electrophoresis

The DNA samples, plus extension products and Genescan 120 Liz size-standard solution, was added to Hi-Di formamide (Applied Biosystems) as recommended by the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then electrophoresed on an ABI Prism 3100 Genetic Analyzer. The results were analyzed by using the program of the ABI Prism GeneScan and Genotyper (Applied Biosystems).

Statistics

χ^2 tests were used to compare the observed numbers of each genotype with those expected for the population under Hardy-Weinberg equilibrium. Heterozygosity for each locus with allele frequencies p and $q = 1-p$ was given by $H = 1-p^2-q^2 = 2p(1-p)$.

Table 1 Sequences of amplifying and extension primers for *IL6* promoter SNP genotyping by single-base extension method

Locus	Primer	Sequences
IL6−597 G → A	Forward	5'-GCAAAGTCCTCACTGGGAGGA-3'
	Reverse	5'-GGGCTGCGATGGAGTCAGA-3'
	Extension	5'-ATTATAATCAATGATGATTGAAGTAACTGCACGAAATTTGAGG-3'
IL6−572 G → C	Forward	5'-GCAAAGTCCTCACTGGGAGGA-3'
	Reverse	5'-GGGCTGCGATGGAGTCAGA-3'
	Extension	5'-ATCAATGATGATCCAGGCAGTCTACAACAGCC-3'
IL6−174 G → C	Forward	5'-AATGACGACCTAAGCTGCAC-3'
	Reverse	5'-TTGATAAATCTTTGTTGGAGGGTG-3'
	Extension	5'-TCATGATTATAATCAATGATGATTTCCCCCTAGTTGTGTCTTGC-3'

Table 2 Single-nucleotide polymorphisms (SNPs) of the human *IL6* promoter and frequencies in Korean population

Locus	SNP site[a]	Genotype			Frequency[b]	Heterozygosity
IL6−597 G → A	−597	AA	AG	GG	0.002	0.002
		0	5	1,077		
IL6−572 G → C	−572	CC	GC	GG	0.27	0.39
		76	411	576		
IL6−174 G → C	−174	CC	GC	GG	0.002	0.002
		0	5	1,102		

[a]Calculated from the transcriptional start site
[b]Frequencies of rare alleles. For an exact calculation of allele frequencies in Korean population, samples from our Korean asthma cohort ($n = 747$) were also included

Haplotypes were constructed by E-M algorithm (Arlequin, http://anthro.unige.ch/arlequin/). ANOVA and T-tests were performed by using SAS programs (SAS Institute).

Results

Allele and haplotypes frequencies of *IL6* promoter SNPs

Three SNPs in the *IL6* promoter region were genotyped. The frequencies of each SNP were 0.002 (*IL6*−597 G → A), 0.27 (*IL6*−572 G → C), and 0.002 (*IL6*−174 G → C) in our Korean population (Table 2.). Genotype distributions were in Hardy-Weinberg equilibrium ($P > 0.05$). The locations of these SNPs in relation to the genomic structure of the *IL6* gene are shown in Fig. 1. *IL6*−597 G → A and *IL6*−174 G → C were totally linked together and showed very low allele frequencies (0.002), which are common in Caucasians (0.4–0.44). On the other hand, the frequency of the *IL6*−572 G → C*C allele was much higher (0.27) than that of Caucasian populations (0.06; Terry et al. 2000). All of the genotype combinations could be phased without any ambiguity. Only three haplotypes were observed out of eight possible haplotypes. The haplotypes frequencies are shown in Table 3. Two major haplotypes accounted for more than 99% of the haplotype distribution. *IL6*−597 G → A and *IL6*−174 G → C were in absolute LD (D' = 1, $d^2 = 1$), i.e., the A allele at *IL6*−597 G → A occurs only with the C allele at *IL6*−174 G → C, and the G allele at *IL6*−597 G → A occurs only with G allele at *IL6*−174 G → C as indicated by the d^2 value of 1.

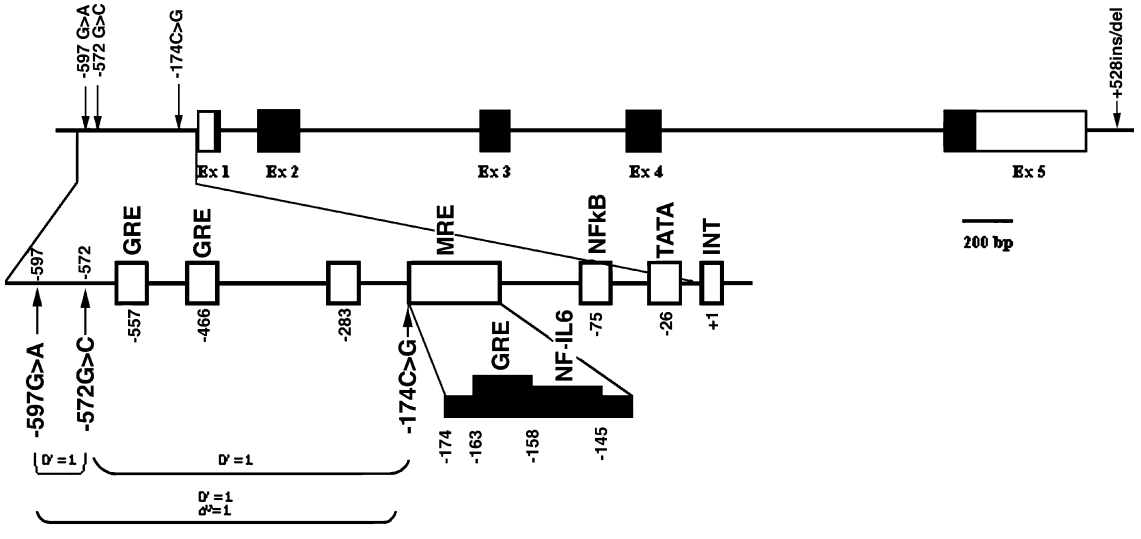

Fig. 1 Map of human *IL6* on chromosome 7p21. The exons, UTR, ins/del, and SNP are shown together with the putative transciptional factor-binding site. *Shaded blocks* Coding exons, *white blocks* 5' and 3' UTRs, *+1* 1st base of the transcriptional start site, *+528ins/del* 528th nucleotide (±40 bp) after the stop codon. The values of D' and d^2 between SNPs are also shown

Association analysis with BMD

The loci *IL6*−597 G → A and *IL6*−174 G → C were not analyzed statistically because the allele frequencies were too low (0.002) in the population. *IL6*−572 G → C associations with BMD were analyzed in pre-menopausal women (37.69 ± 6.67 years old) living in Seoul, Korea. The statistical analyses are summarized in Table 4. Significant associations were detected with *IL6*−572 G → C, i.e., the C allele was associated with increased BMD ($P = 0.02$, co-dominant model; $P = 0.007$, dominant model). The mean BMD was highest in homozygous C individuals (0.67 ± 0.15), lowest in homozygous G individuals (0.58 ± 0.19), and

Table 3 *IL6* promoter haplotypes and their frequencies in Korean population ($n = 1{,}082$) with three *IL6* promoter SNPs (*IL6*−597 G → A, *IL6*−572 G → C, *IL6*−174 G → C)

Haplotype[a]	SNPs in *IL6* promoter			Frequency
	IL6−597 G → A	*IL6*−572 G → C	*IL6*−174 G → C	
ACC	A	C	C	0.002
GCG	G	C	G	0.259
GGG	G	G	G	0.739

[a]Haplotypes were constructed by pair-wise tables. The *IL6* haplotypes could be constructed simply by eye because of absolute LD and/or complete LD. Those haplotypes constructed by eye were identical to those from the E-M algorithm (Arlequin, http://anthro.unige.ch/arlequin/). There were no ambiguous haplotype phases in the population because of complete and/or absolute LDs between SNPs in *IL6*

Table 4 Analysis of association of *IL6* promoter SNP (*IL6*−572 G → C) with distal radius bone mineral density (BMD) in the Korean population

	CC ($n = 23$)	CG ($n = 140$)	GG ($n = 168$)	*P*-value[b]	*P*-value[c]
BMD (mg/cm^2)	0.67 ± 0.15	0.64 ± 0.21	0.58 ± 0.19	0.021	0.007
T-score[a]	3.04 ± 2.64	2.63 ± 3.44	1.69 ± 3.07	0.016	0.005

[a]The T-score is the number of standard deviations from 25-year-old women. The default value (for Asians) of the dual energy X-ray absorptiometer (DEXA, Lunar Pixi, USA) was used for calculating the T-score
[b]P-values for co-dominant models
[c]P-values for dominant models of *IL6*−572 C allele

intermediate in heterozygotes (0.64 ± 0.21). As expected, similar associations were also observed with age-adjusted BMD values (T-score).

Discussion

IL6 is one of the candidate genes of osteopenia and osteoporosis because the gene product stimulates osteoclasts through binding to its cell surface receptor. IL6 protein is a multifunctional cytokine essential to the regulation of bone resorption, and to the immune response and hematopoiesis. It exerts its action through binding to its cell-surface receptor, the IL6 receptor. As IL6 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).

We have observed different genetic background between Koreans and Caucasians with respect to allele frequencies of *IL6* promoter SNPs. The allele frequencies of *IL6*−174 and *IL6*−597 in Caucasians (0.40–0.45; Brull et al. 2001; Burzotta et al. 2001; Cavet et al. 2001; Cox et al. 2001; Fedetz et al. 2001; Helmy et al. 2001; Terry et al. 2000; Zhai et al. 2001) are much higher than those of Koreans (0.002; this study) and the Chinese population (0.002; Zhai et al. 2001). On other hand, the frequency of *IL6*−572 in Caucasians (0.04–0.06) is much lower than that of the Korean population (0.27) and Japanese population (0.184; Ota et al. 2001). Highly frequent population-specific alleles are particularly useful in mapping genes responsible for disease susceptibility and other traits in population (Parra et al. 1998; Stephens et al. 2001). *IL6*−572 might thus be a useful marker for association studies for Asian rather than Caucasian populations.

LD is becoming an important tool in genetic studies because it is applicable to a variety of topics, including disease-gene mapping (Rannala and Reeve 2001; Riley et al. 2000). The SNPs in the *IL6* promoter region are in complete and/or absolute LD so that only three haplotypes are observed out of eight possible haplotypes. Generally, haplotypes are more informative than single SNPs, but, in the case of haplotypes in the *IL6* promoter, haplotypes are not informative in association studies of complex trait diseases such as osteoporosis because of the very low frequencies of *IL6*−597 G → A and *IL6*−174 G → C in the Korean population.

Our findings suggest that individuals who have an *IL6*−572 C-containing genotype may have a beneficial genetic predisposition with respect to BMD. Recently, similar results were published with the same *IL6*−572 C variant in post-menopausal Japanese women (Ota et al. 2001). Here, we report analogous genetic effects of *IL6*−572 G → C in pre-menopausal women.

Many reports can be found in the literature regarding *IL6*−174 in the Caucasian population; C homozygotes are associated with lower bone resorption and a smaller decrease in bone mass in older post-menopausal women (Ferrari et al. 2001). This SNP is also associated with systemic onset juvenile chronic arthritis (Fishman et al. 1998), Alzheimer disease (Bagli et al. 2000; Papassotiropoulos et al. 1999), and increased bone resorption (Ferrari et al. 2001). In pubertal girls, the development of peak bone density in the spine is reported to result from decreased bone resorption (but not increased bone formation), and decreased IL-6 may be in part responsible for the development of maximal peak vertebral bone mass (Manolagas 1998). In a gene reporter assay, the −174 C construct had a lower expression than that of the −174 G construct, but it was nearly impossible to study the genetic effects of this −174 variant because of its very low frequency in the Korean population (0.002). On the other hand, *IL6*−572 G → C variant, which is rare in Caucasian populations (< 0.07), is relatively common in Koreans (0.27; in this study) and Japanese (0.184; Ota et al. 2001).

Although *IL6*−572 G → C is not involved in any known DNA-binding motif (Fig. 1), this site could be part of sequences that bind to unknown gene elements or alter the secondary structure of DNA to affect the access of transcriptional factors. Higher IL6 levels after 6 h following a coronary artery bypass graft (CABG) were detected in carriers of the *IL6*−572 G → C*C allele

than in other genotypes, suggesting that this polymorphism might be functional in IL6 production (Brull et al. 2001). The mechanism of *IL6*−572 G → C in BMD might involve differential expression by the mutant allele (*IL6*−572 G → C*C).

Although the present study is of insufficient power to examine the effects of haplotype, we report the positive association of the *IL6*−572 G → C polymorphism with BMD in pre-menopausal women. Several lines of evidence of *IL6* genetic effects, including those of this study, suggest that variants of *IL6* might have important role(s) in the progress of osteoporosis.

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