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The *IL6* gene polymorphism −634C>G and *IL17F* gene polymorphism 7488T>C influence bone mineral density in young and elderly Japanese women

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ABSTRACT

Osteoporosis is an important public health problem because of the significant morbidity and mortality associated with its complications, particularly fractures. An important clinical risk factor in the pathogenesis of osteoporosis is the presence of genetic polymorphisms in susceptibility genes.
However, few studies have investigated the relevance of these polymorphisms in premenopausal women. Recent studies have demonstrated interactions between bone and immune cells, and that cytokines produced by immune cells regulate bone turnover. In this study, we examined the associations between bone mineral density (BMD) and polymorphisms in genes encoding interleukin (IL)-6 (−634C>G; rs1800796), tumor necrosis factor (TNF)-α (−308G>A; rs1800629), IL-17F (7488T>C; rs763780), transforming growth factor (TGF)-β (869T>C; rs1800470), osteoprotegerin (OPG; 163A>G; rs3102735) and methylenetetrahydrofolate reductase (MTHFR; 677C>T; rs1801133) in young and elderly Japanese women. Whole-body, lumbar spine (L_1 or L_2–L_4), and femoral neck BMD were measured in 100 young subjects (18–23 years), and 100 elderly subjects (60–83 years). Whole-body, lumbar spine, and femoral neck BMD were 1.13±0.06, 1.14±0.12, and 1.00±0.11 g/cm^2, respectively, in young subjects, and 0.92±0.09, 0.86±0.15, and 0.63±0.10 g/cm^2, respectively, in elderly subjects. The frequencies of the IL-6 CC, CG, and GG genotypes were 48%, 49%, and 3%, respectively. The frequencies of the IL17F TT, TC, and CC genotypes were 79%, 15%, and 6%, respectively, in young subjects. Polymorphisms of the IL-6 and IL17F genes were significantly associated with BMD. To our knowledge, this is the first report to examine these associations in a cohort of 200 Japanese women.

1. Introduction

Osteoporosis is widely recognized as an important public health problem because its complications, including fractures, are associated with significant morbidity and mortality (Diamantopoulos, et al., 2012; Magaziner et al., 1989). Peak bone mass is a major risk factor for osteoporotic fractures. Bone mass attained early in life is perhaps the most important determinant of lifelong skeletal health. Many factors influence the risk of osteoporosis including diet, physical activity, medication use, coexisting disease, aging, and reduced sex steroid production. However, one of the most important clinical risk factors is a positive family history of osteoporosis, emphasizing the importance of genetics in the pathogenesis of osteoporosis.

Genetic factors play critical roles in determining bone mass, and several genes probably regulate these processes. It has become apparent that altered activity of several genes, including those encoding vitamin D receptor (Morrison et al., 1994), estrogen receptor-α (Albagha et al., 2005), IL-6 (Czerny et al., 2010), collagen type Iα1 (Ji et al., 2009), and lipoprotein receptor-related protein 5 (Riancho et al., 2011), play a role in the etiology of osteoporosis. However, most of the studies performed to date have focused on postmenopausal women, and little is known about whether polymorphisms that affect bone metabolism are associated with changes in bone mineral density (BMD) in earlier life.

Bone remodeling involves tight regulation of three proteins, receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB (RANK), and osteoprotegerin (OPG). These proteins are key determinants of osteoclastogenesis and regulate bone resorption (Takayanagi, 2005). Several recent studies have highlighted the interactions between bone and immune cells, and many of products of immune cells, including cytokines, chemokines, and growth

Abbreviations: BMD, bone mineral density; MTHFR, methylenetetrahydrofolate reductase (NAD(P)H); PCR, polymerase chain reaction; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor.

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factors, regulate the activation of cells that control bone turnover. Inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF)-α, can potently upregulate RANKL expression on osteoblasts and accelerate RANKL signaling, and thus direct contribute to bone destruction (Arron and Choi, 2000). Cytokines known to affect bone metabolism include the IL-17-producing helper T (Th) cell subset (Th17 cells), which represent the long-sought-after osteoclastogenic Th cell subset (Sato et al., 2006). The infiltration of Th17 cells into the inflammatory lesion links the abnormal T cell response to bone damage. The production of transforming growth factor (TGF)-β, one of the most abundant cytokines in the bone matrix, is enhanced by estrogen (Oursler et al., 1991).

OPG is secreted as a soluble protein from osteoblasts. It acts by competing with RANK, a surface receptor expressed on osteoclasts and dendritic cells, for binding to RANKL. The major biological role of OPG is to block bone resorption by inhibiting osteoclast differentiation and activity, which has been demonstrated *in vivo* and *in vitro* (Simonet et al., 1997). *MTHFR*, the gene encoding methylenetetrahydrofolate reductase (NAD(P)H), was identified as a candidate gene for osteoporosis (Miyao et al., 2000). A severe deficiency or lack of MTHFR leads to homocysteinuria, including skeletal abnormalities, and early onset osteoporosis.

The aims of the present study were to analysis the association of polymorphisms in genes encoding immune cytokines, such as IL-6, TNFα, IL-17F, TGF-β, OPG, and MTHFR, with the risk of developing osteoporosis, as measured by BMD, in young and elderly Japanese women.

2. Materials and methods

2.1. Subjects

One hundred young women (18–23 years old, mean ± standard deviation 19.3 ± 0.95 years) without evidence of metabolic disorders (assessed by questionnaire) and one hundred elderly women (60–83 years old, mean ± standard deviation 68.1 ± 4.80 years) participated in this study. The young women were enrolled from among students attending Tokyo Metropolitan University (Tokyo, Japan). The elderly subjects were recruited from residents in the city of Hamura, a suburb of Tokyo. The lifestyle of the residents in this area is typical of that in most regions of Japan. A questionnaire was used to assess medical history and lifestyle factors (food intake, behavior, and physical training history). All of the subjects were considered to be healthy, after excluding women with factors that might affect bone metabolism, including chronic diseases/conditions affecting vital organs (e.g., heart, lung, liver, kidney, and brain), women with severe endocrine, metabolic, or nutritional diseases, and women with a known history of metabolic bone disease or hip fracture, and those using medications known to influence bone metabolism. This study was approved by the Research Ethics Committee of Tokyo Metropolitan University and written informed consent was obtained from all subjects.

2.2. Measurement of BMD and general characteristics

The BMD (g/cm^2) of the whole-body, lumbar spine (young; L_1–L_4, elderly; L_2–L_4), and femoral neck were measured by dual-energy X-ray absorptiometry with a Prodigy Advance (GE Healthcare, Piscataway, NJ) for young women and a QDR-A (Hologic Inc., Waltham, MA) for elderly women. For young women, body height (±0.1 cm) and weight (±0.1 kg) were measured using a Tanita body fat analyzer (Model No. BF-220; Tanita Corp., Tokyo, Japan). For elderly women, body height (±0.1 cm) was measured using a Yagami height meter (Model No. ST-2M; Yagami Inc., Nagoya, Japan) and weight (±0.1 kg) was measured using a Tanita body inner scan (Model No. BC-520; Tanita Corp., Tokyo, Japan). BMD of elderly women was recorded as adjusted BMD (adj-BMD) to correct for differences in age, height, and weight using the following formulae: body mass index (BMI) = (body weight, kg)/(body height, m)^2 and adj-BMD = BMD − 0.0052432908 × (73.1716102 − age) + 0.0088382998 × (23.2271299 − BMI) (Kleinbaum et al., 1998).

2.3. Genotyping of polymorphism in target genes

The genotypes of *IL6* −634C>G (rs1800796), *TNFα* −308G>A (rs1800629), *IL17F* 7488T>C (rs763780), *TGFβ* 869T>C (rs1800470), *OPG* 163A>G (rs3102735) and *MTHFR* 677C>T (rs1801133) (Jørgensen et al., 2004; Kawaguchi et al., 2006; Kim et al., 2010; Lario et al., 1999; Miyao et al., 2000; Ota et al., 2001; Seiderer et al., 2008) were analyzed by the polymerase chain reaction (PCR)-RFLP method. Genomic DNA was isolated from the fingernail using a DNA isolation kit (Nippon Gene Co., Ltd, Tokyo, Japan). The concentration of purified DNA was determined using a U-0080D spectrophotometer (Hitachi Ltd., Tokyo, Japan). The extracted genomic DNA was subjected to PCR to amplify *IL6*, *TNFα*, *IL17F*, *TGFβ*, *OPG* and *MTHFR*. PCR was carried out in a 50-μl reaction volume containing 0.5-1 μg of genomic DNA, 1.5 mM or 1 mM magnesium chloride, 0.2 mM deoxy-NTP, 0.4 μM of each primer, 0.5 μl of DNA polymerase (Ampli Taq Gold: Applied Biosystems, Carlsbad, CA or Prime Star HS: Takara Biotechnology Co., Ltd, Shiga, Japan) and each PCR buffer (Applied Biosystems or Takara Biotechnology). *IL17F* and *TGFβ* were amplified by adding 4% DMSO or 3% formamide when we were unable to obtain PCR products. Each primer set was synthesized by Sigma-Aldrich Japan (Tokyo, Japan), and PCR was performed on a thermal cycler (model 2720, Applied Biosystems). The primers and PCR programs for each gene are shown in Table 1. The PCR products were separated by electrophoresis on a 2.5% agarose gel and stained with ethidium bromide. Gels were visualized on a transilluminator under ultraviolet light and photographed. The primers for *IL6*, *TNFα*, *IL17F*, *TGFβ*, *OPG*, and *MTHFR* amplify 180, 107, 412, 500, 253, and 198 bp PCR products. The products were then digested with *Bsr*BI, *Nco*I, *Nla*III, *MSP*A1I, *Mfe*I, and *Hin*fI (New England Biolabs Inc., Hitchin, UK or Promega Corp., Madison, WI), respectively, at 37 °C for 3–16 h, followed by electrophoresis using e-Pagel AE6000 (Model E-T15L: Atto Corp., Tokyo, Japan) and visualization with ethidium bromide staining. Digestions of the *IL6* PCR products with *Bsr*BI generate two fragments of 120 and 60 bp in length. The 60 bp fragment represents the (−634)G allele (Ota et al., 2001). Digestion of the PCR products of *TNFα* with *Nco*I generates two fragments of 87 and 20 bp in length; the (−308)A allele remained uncut with a length of 107 bp (Kim et al., 2010). Digestion of the PCR products of *IL17F* with *Nla*III generated two fragments of 288 and 124 bp in length. The 412 bp product remained undigested for the (7488)C allele (Kawaguchi et al., 2006; Seiderer et al., 2008). Digestion of the *TGFβ* PCR products with *Msp*A1I generated four constant fragments of 67, 67, 41 and 40 bp in length. The (869)C allele was defined by the presence of polymorphic bands of 273 and 12 bp in length, while the (869)T allele was defined by a 285 bp fragment (Lario et al., 1999). Digestion of the *OPG* PCR products by *Mef*I generated two fragments of 232 and 21 bp in length. The (163)A allele remained uncut with a length of 253 bp. The (163)G allele was defined by the presence of 232 and 21 bp fragments (Jørgensen et al., 2004). Digestion of *MTHFR* PCR products by *hin*fI generated two fragments of 175 and 23 bp in length. The (677)C allele remained undigested, and the 175 bp fragment was derived from the (677)T allele (Miyao et al., 2000).

2.4. Statistical analysis

Student's *t*-test and the Mann–Whitney *U*-test were used to examine the effects of each genotype on BMD in young women, and on adj-BMD for elderly women and all women. In all tests, values of $p<0.05$ were considered statistically significant. χ^2 tests were used to determine the Hardy–Weinberg equilibrium for each genotype, and $p<0.05$ was considered statistically significant.