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Association of a single-nucleotide polymorphism in low-density lipoprotein receptor-related protein 5 gene with bone mineral density

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Abstract Low-density lipoprotein receptor-related protein 5 (LRP5) is an important regulator of osteoblast growth and differentiation, affecting peak bone mass in vertebrates. Here, we analyzed whether the *LRP5* gene was involved in the etiology of postmenopausal osteoporosis, using association analysis between bone mineral density (BMD) and an *LRP5* gene single-nucleotide polymorphism (SNP). Association of an SNP in the *LRP5* gene at IVS17-1677C > A (intron 17) with BMD was examined in 308 postmenopausal Japanese women (65.2 ± 9.6 years; mean ± SD). The subjects bearing at least one variant A allele (CA + AA; $n = 142$) had significantly lower Z scores for total body and lumbar BMD than the subjects with no A allele (CC; $n = 166$) (total body, 0.08 ± 1.09 versus 0.50 ± 1.03; $P = 0.0022$; lumbar spine, −0.42 ± 1.43 versus −0.02 ± 1.42; $P = 0.013$). These findings suggest that the *LRP5* gene is a candidate for the genetic determinants of BMD in postmenopausal women, and this SNP could be useful as a genetic marker for predicting the risk of osteoporosis.

Key words wnt · LRP5 · osteoporosis · bone mineral density · polymorphism

Introduction

Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors. Twin and sibling studies have revealed that the proportion of variance of bone mineral density (BMD) accounted for by genetic factors is around 50%–90% [1–6]. These studies have suggested that the variation in BMD among individuals is largely caused by genetic factors. Therefore, genetic markers that are correlated with BMD would be useful for predicting future bone loss and for clarifying the mechanism of bone loss in osteoporosis. After an association of BMD with vitamin D receptor (VDR) genotypes was reported [7], polymorphisms in several other genes were investigated [8]. These genes included those implicated in bone formation by the regulation of osteoblast growth and differentiation, such as transforming growth factor beta 1 (TGFβ1) [9], collagen type Ia1 (COLIA1) [10], parathyroid hormone (PTH) [11], and p57Kip2 (CDKN1C) [12]. Considering the polygenetic nature of BMD distribution and the multiplicity of endocrine factors known to regulate bone mass and bone turnover, it is important that the panel of candidate genes could be expanded to elucidate the whole genetic background of osteoporosis.

The Wnt signaling pathway plays a pivotal role in embryonic development and oncogenesis [13,14]. Studies using *Drosophila*, *Xenopus*, and mammalian cells have established a canonical signaling pathway [15–17]. Both genetic and biochemical results have provided solid evidence indicating that FZ proteins function as Wnt receptors. Wnt proteins bind Frizzled (FZ) and prevent glycogen synthase kinase 3 (GSK3)-dependent phosphonylation of β-catenin, leading to the stabilization of β-catenin. Meanwhile, the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) were found to be also required for the Wnt signaling pathway as Wnt co-receptors [18,19]. Recent reports have demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through LRP5 [20–23]. Inactivating mutations in LRP5 decrease bone mass and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome in humans [20] and mice [21]. Conversely, activating mutations in LRP5 are linked to autosomal-dominant high-bone mass traits [22,23]. These data suggest that LRP5, which modulates

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Wnt signaling, controls bone metabolism in vivo in mammals. To examine the possible contribution of the *LRP5* gene to the etiology of involutional osteoporosis, we investigated an association between polymorphism in this gene and BMD in Japanese women.

Subjects and methods

Subjects

Genotypes were analyzed in DNA samples obtained from 308 healthy postmenopausal Japanese women (mean age ± SD; 65.2 ± 9.6 years) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, heparin), or unusual gynecologic history. All women were non-related volunteers and provided informed consent before this study.

Measurement of BMD and biochemical markers

The lumbar spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry, using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin, 1, 25$(OH)_2D_3$, total cholesterol (TC), and triglyceride (TG). We also measured urinary pyridinoline (PD; HPLC method) and deoxypyridinoline (DPD; HPLC method). The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. The Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20000 Japanese women.

SNP Selection

A polymorphic variation of the *LRP5* gene was extracted from the JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html), and was denoted as IVS17-1677C > A according to its localization on the gene.

Genotyping procedure

Genotypes of IVS17-1677C > A were determined using the SNP-dependent (Sd)-polymerase chain reaction (PCR) method, a modified allele-specific PCR of polymorphic sequence as previously described [24,25]. Two allele-specific primers (AS-primers) and one reverse primer were prepared per single-nucleotide polymorphism (SNP). The AS-primers (long and short) have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at the 3′ ends, and an additional artificial mismatch introduced near the 3′ end. Primer sequences used were as follows: IVS17-1677C > A FL-primer: 5′-TTTTTGGGCGGTAAAT ACACGTCTCTCGAG-3′; IVS17-1677C > A FS-primer: 5′-CCGCGGTAAATACACGTCTCTCGAT-3′; and IVS17-1677C > A reverse-primer: 5′-GTTTCC GTCAGAAC GCTGCACTA-3′.

This primer set allowed distinct discrimination of alleles. For the assay, a genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out in a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturalization at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles at 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system, using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, direct re-sequencing was carried out using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the minor A-allele and the group with only the major C-allele encoded at that locus were subjected to analysis. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied Student's *t*-test, using StatView-J4.5 software (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Association of LRP5 *gene polymorphism in intron 17 with BMD*

We analyzed the genotypes for the *LRP5* IVS17-1677C > A polymorphism (rs3781586 in the National Center for Biotechnology Information [NCBI] dbSNP data-

Table 1. Comparison of background and biochemical data between subjects bearing at least one A allele (AA + CA) and subjects with no A allele (CC) at IVS17-1677 (intron 17)

Items	Genotype (mean ± SD)		*P* value
	CC	CA + AA	
No. of subjects	166	142	
Age (years)	65.1 ± 9.6	65.4 ± 9.9	NS
Height (kg)	151.0 ± 6.2	150.3 ± 6.4	NS
Body weight (kg)	50.7 ± 8.4	50.3 ± 8.1	NS
Lumbar spine BMD (g/cm^2)	0.92 ± 0.20	0.87 ± 0.19	0.025
Lumbar spine BMD (Z score)	−0.02 ± 1.42	−0.42 ± 1.43	0.013
Total body BMD (g/cm^2)	1.00 ± 0.11	0.96 ± 0.12	0.015
Total body BMD (Z score)	0.50 ± 1.03	0.08 ± 1.09	0.0022
Ca (mg/dl)	9.2 ± 0.43	9.2 ± 0.45	NS
P (mg/dl)	3.4 ± 0.46	3.4 ± 0.48	NS
ALP (IU/l)	183.7 ± 62.6	195.4 ± 71.0	NS
I-OC (ng/ml)	7.6 ± 4.2	8.3 ± 3.7	NS
PD (pmol/μmol of Cr)	36.1 ± 24.7	34.8 ± 12.0	NS
DPD (pmol/μmol of Cr)	7.6 ± 5.2	7.4 ± 2.4	NS
Intact PTH (pg/ml)	35.1 ± 16.4	35.8 ± 16.6	NS
Calcitonin (pg/ml)	22.8 ± 11.1	23.4 ± 11.7	NS
1,25 $(OH)_2D_3$ (pg/ml)	37.5 ± 12.6	34.3 ± 10.4	NS
TC (mg/dl)	198.7 ± 37.5	195.7 ± 39.2	NS
TG (mg/dl)	141.5 ± 81.4	136.8 ± 71.4	NS
Percent fat	32.1 ± 7.9	31.6 ± 7.4	NS
BMI	22.2 ± 3.2	22.2 ± 2.9	NS

Statistical analysis was performed according to the method described in the text
BMD, bone mineral density; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant

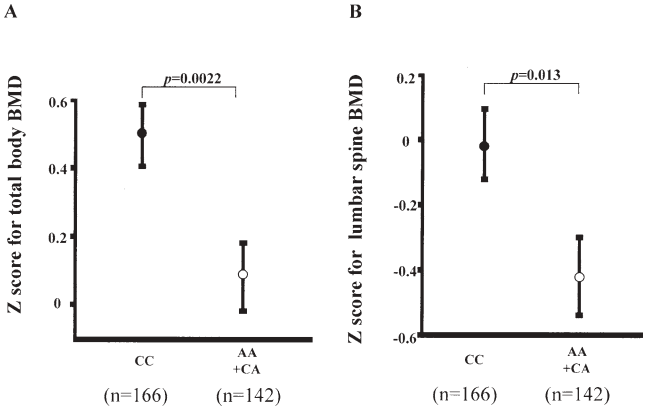

Fig. 1. Z Score values for total body and lumbar bone mineral density (*BMD*) in the groups with each genotype of the *LRP5* gene in intron 17 (IVS17-1677C > A). **A** Z Score values for total body BMD are shown as the *solid circle* for genotype CC at IVS17-1677 and as the *open circle* for genotype AA + CA at IVS17-1677. Values are expressed as means ± SE. Numbers of subjects are shown *in parentheses*. **B** Z Score values for lumbar spine BMD are shown in the same manner as in **A**

base) in 308 subjects, using Sd-PCR methods [25]. Among the 308 postmenopausal volunteers, 24 were AA homozygotes, 118 were CA heterozygotes, and 166 were CC homozygotes. Allelic frequencies were 0.731 for the C allele and 0.269 for the A allele in this population.

We compared Z scores for BMD of total body and lumbar spine between subjects bearing at least one chromosome with the A allele (genotype AA + CA; $n = 142$) and subjects with no A allele (CC; $n = 166$). The former subjects had significantly lower Z scores for total body BMD (0.08 ± 1.09 versus 0.50 ± 1.03; $P = 0.0022$, Fig. 1A) and lumbar BMD (−0.42 ± 1.43 versus −0.02 ± 1.42; $P = 0.013$; Fig. 1B). As shown in Table 1, the background data were not significantly different between these groups.

Discussion

We investigated the influence of a genetic variation of the *LRP5* gene on bone mineral properties. The allelic frequencies of an SNP in intron 17 (0.731 for IVS17-1677C and 0.269 for IVS17-1677A) in Japanese postmenopausal women were in Hardy-Weinberg equilibrium. The allelic frequencies of this SNP in the general Japanese population were reported in the JSNP database (IMS-JST137897). The database reported that the allelic frequencies were 0.726 for IVS17-1677C and 0.274 for IVS17-1677A, indicating that the allelic frequencies in the present study were in line with the JSNP database.

Recently, patients with homozygous *LRP5* gene disruption were reported [20]. There are many types of mutations affecting bone mass accrual during growth, causing the autosomal recessive disorder osteoporosis-pseudoglioma syndrome. Regarding the effect on the bone, these patients showed a marked decrease in their BMD. In addition, Kato et al. [21] created and characterized *LRP5* gene knockout mice. Interestingly, *LRP5* gene knockout mice showed lower bone mass density than wild-type mice because of decreasing osteoblast proliferation. In their report, Kato et al. [21] observed the presence of LRP5 protein in osteoblasts lining the endosteal and trabecular bone surfaces, but not in osteoclasts, by immunohistochemistry in wild-type mice. Recently, a gain-of-function mutation (G171V) in the *LRP5* gene was described in two kindreds with an enhanced bone density [22,23]. In vitro studies showed that the normal inhibition of Wnt signaling by another protein, Dickkopf-1 (Dkk1), was defective in the presence of this mutation, resulting in increased signaling due to unopposed Wnt activity. Thus, LRP5 may be one of the cellular mediators involved in bone formation, by regulating the proliferation and differentiation of osteoblasts.

In the present study, significant correlation was observed between BMD and a polymorphism in intron 17 (IVS17-1677C > A). To our knowledge, this is the first report that a common SNP in the *LRP5* gene affected BMD. However, it is still unclear how BMD is affected by this intronic polymorphism of the *LRP5* gene. For explaining this, three hypotheses could be proposed. (i) This intronic polymorphism may be linked with exon mutations and may contribute to changing LRP5 protein function. (ii) This polymorphism may be linked with mutations of regulatory elements and may affect the levels of expression through transcriptional regulation. (iii) The polymorphism in the *LRP5* gene may be linked with mutation of another unidentified gene adjacent to the *LRP5* gene which causes low BMD directly or indirectly.

In conclusion, our finding suggests that the *LRP5* gene may be a candidate for the genetic determinants of BMD in postmenopausal women. Examining *LRP5* gene variation will, it is hoped, enable us to understand one of the mechanisms of involutional osteoporosis. Wnt and LRP5 signaling have been implicated in other diseases, including cholesterol and glucose metabolism-related diseases [26]. The variant presented here may be involved in the risk of such diseases, as well as osteoporosis.

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