A1330V Variant of the Low-density Lipoprotein Receptor-related Protein 5 (LRP5) Gene Decreases Wnt Signaling and Affects the Total Body Bone Mineral Density in Japanese Women

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Abstract. Wnt signaling is an important regulator of bone homeostasis. The Wnt co-receptor, namely, low-density lipoprotein receptor-related protein 5 (LRP5), initiates Wnt signal transduction. Recently, we and several other groups have shown that there is a single nucleotide polymorphism (SNP) located in the exon 18 of the LRP5 gene that leads to an amino acid change (3989C > T, A1330V), and is associated with lumbar spine, femoral neck, and radial bone mineral density (BMD), and incidence of fracture. These data suggest that the A1330V variation in the LRP5 gene may affect the pathogenesis of osteoporosis. However, the functional basis of the A1330V variation remains unclear. In the present study, we analyzed the effect of the A1330V variation on Wnt activity. We also investigated the association between this LRP5 SNP and total body BMD using 739 postmenopausal women. LRP5 with the A1330V SNP were transiently coexpressed with Wnt3a in 293T cells and their activity was evaluated by the TCF-Lef reporter assay. In vitro, the TCF-Lef activity in presence of Wnt3a in cells expressing LRP5 and carrying the T allele (Valine at 1330 (V1330)) of exon 18 was significantly reduced as compared to the wild-type allele. The association between the A1330V SNP and total body BMD were replicated in 739 postmenopausal Japanese women (AA vs. VV; \( P = 0.0026 \)). These data suggest that the V1330 variant in the LRP5 gene decreases Wnt activity, which in turn decreases the BMD.

Key words: Low-density lipoprotein receptor-related protein 5 (LRP5), Bone mineral density (BMD), Osteoporosis, Single nucleotide polymorphism (SNP)

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in embryonic development and oncogenesis [10, 11]. Studies using *Drosophila*, *Xenopus*, and mammalian cells have established a canonical signaling pathway. Wnt proteins bind Frizzled (FZ) proteins and prevent Glycogen synthase kinase 3 (GSK3)-dependent phosphorylation of β-catenin, leading to the stabilization of β-catenin. Both genetic and biochemical results have provided evidences indicating that FZ proteins function as Wnt receptors. Furthermore, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) were also found to be required for Wnt signaling pathway as Wnt coreceptors. Wnt-β-catenin signaling pathway has been shown to regulate bone density through LRP5 [12-15]. Inactivating mutations in LRP5 decrease the bone mass and cause an autosomal-recessive disorder —osteoporosis-pseudoglioma syndrome— in humans and mice [12, 13]. Meanwhile, activating mutations in LRP5 leads to autosomal-dominant high bone mass traits [14, 15]. These data suggest that LRP5 controls the bone metabolism *in vivo* in mammals. Moreover, we and several groups have shown that a single nucleotide polymorphism (SNP) located in the exon 18 of the LRP5 gene, caused by an amino acid change (3989C>T, A1330V), is associated with the BMD and incidence of fracture [16-19]. These data suggest that the A1330V variation in the LRP5 gene may affect the pathogenesis of osteoporosis through decreasing Wnt activity. However, the functional basis of the A1330V variation remains unclear. In the present study, we analyzed the effect of this A1330V variation on Wnt activity. Moreover, in the previous reports which analyzed the association between the A1330V polymorphism and BMD, BMD was measured at the lumbar spine, the femoral neck and the trochanter but not at the total body. Thus, we here investigate the association of the A1330V polymorphism, with total body BMD using 739 postmenopausal women.

**Materials and Methods**

**In vitro activity of the A1330V SNP in the LRP5 gene**

We used the Lef-TCF sensitize transcription assay to evaluate canonical Wnt3a signaling that was mediated by the wild-type LRP5 or by the V1330 LRP5 variant. HEK293T cells were seeded at a density of 8 × 10⁴ cells/well in 24-well tissue culture plates in 0.5 ml of complete Dulbecco’s Modified Eagle Medium (DMEM) and transfected after 38 h using Fugene HD reagent (Roche, Basel, Switzerland) and LRP5 constructs carrying the SNP in exon 18.

**Subjects**

The subjects in this study were from the central part of Japan. The baseline examination was conducted after obtaining informed consent from 739 unrelated ambulatory postmenopausal volunteers. Genotypes were analyzed in DNA samples obtained from 739 healthy postmenopausal Japanese women (mean age ± SD; 66.2 years ± 7.4). Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications affecting bone metabolism (e.g., corticosteroids, anticonvulsants, heparin sodium), or unusual gynecologic history. None of the women had a history of taking medicines for treatment of osteoporosis, such as active vitamin D3, bisphosphonate, SERM, or calcium. The study was approved by the ethics committees of the University of Tokyo and the Research Institute and Practice for Involutional Diseases.

**Measurement of BMD and biochemical markers**

The total body BMD (g/cm²) of each participant was measured by dual-energy X-ray absorptiometry using the fast-scan mode (DPX-L; Lunar, Madison, WI). We measured the serum concentration of intact-osteocalcin (I-OC) (ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), and calcitonin (CT). We also determined the urinary ratio of urinary deoxypyridinoline (DPD; HPLC method) to creatinine. 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 20,000 Japanese women.

**Determination of a SNP in the LRP5 gene**

We determined the A1330V (c.3989C>T) polymorphism of the LRP5 gene using the TaqMan (Applied Biosystems) polymerase chain reaction (PCR) method [20]. To determine the LRP5 SNP, we used an Assays-on-Demand SNP Genotyping Kit C__25752205_10
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A FunCTIOnAL LRP5 V ARIAnT AFFECTS BMD in 739 postmenopausal Japanese women using the TaqMan methods. Among the 739 postmenopausal women, 355 were AA homozygotes, 318 were AV heterozygotes, and 66 were VV homozygotes. The genotype distribution was found to be in Hardy-Weinberg equilibrium.

The kruskal-Wallis test revealed that there was a significant association between the A1330V genotype and total body BMD (Fig. 2A and Table 1, P = 0.0069). We also compared the Z scores for total body BMD between the subjects bearing at least 1 A allele (AA + AV) and the subjects without the A allele (VV) using an unpaired t-test. Those with the VV genotype had significantly lower Z scores for total body BMD (Z score; AA + AV vs. VV; 0.50 ± 1.07 vs. 0.23 ± 0.98; P = 0.0045) (Fig. 2B, Table 1). The background and biochemical data are shown in Table 1. The Kruskal-Wallis test also revealed a significant association between the A1330V genotype and the serum levels of intact osteocalcin (Table 1, P = 0.029). The other background and biochemical data among the A1330V genotypes were not statistically different.

Functional significance of the A1330V polymorphism in the LRP5 gene

To evaluate the functional significance of LRP5 alanine to valine at codon 1330, we substituted the alanine at codon 1330 in the wild-type LRP5 (LRP5-A1330) to valine (LRP5-V1330). These LRP5 constructs were transfected in HEK293T cells and measured TCF-Lef reporter activity in response to canonical Wnt3a signaling (Fig. 1.). Data are shown as relative values compared with the activity in LRP5-A1330 without Wnt3a. The Wnt3a-induced TCF-Lef activity was significantly reduced in the cells containing the LRP5-V1330 than in those containing the wild-type allele (LRP5-A1330 vs LRP5-V1330, 1.900 ± 0.064 vs 1.597 ± 0.101; mean ± SE). In the case of cells not treated with Wnt3a, TCF-Lef activity of cells with LRP5-V1330 was not significantly changed as that of those with LRP5-A1330 (LRP5-A1330 vs LRP5-V1330, 1 ± 0.056 vs 0.975 ± 0.015; mean ± SE).

Association between the A1330V polymorphism in the LRP5 gene and total body BMD

We also examined the association between the LRP5-A1330V polymorphism and total body BMD in 739 postmenopausal Japanese women using the TaqMan methods. Among the 739 postmenopausal women, 355 were AA homozygotes, 318 were AV heterozygotes, and 66 were VV homozygotes. The genotype distribution was found to be in Hardy-Weinberg equilibrium.

The Kruskal-Wallis test revealed that there was a significant association between the A1330V genotype and total body BMD (Fig. 2A and Table 1, P = 0.0069). We also compared the Z scores for total body BMD between the subjects bearing at least 1 A allele (AA + AV) and the subjects without the A allele (VV) using an unpaired t-test. Those with the VV genotype had significantly lower Z scores for total body BMD (Z score; AA + AV vs. VV; 0.50 ± 1.07 vs. 0.23 ± 0.98; P = 0.0045) (Fig. 2B, Table 1). The background and biochemical data are shown in Table 1. The Kruskal-Wallis test also revealed a significant association between the A1330V genotype and the serum levels of intact osteocalcin (Table 1, P = 0.029). The other background and biochemical data among the A1330V genotypes were not statistically different.
Table 1. Comparison of background, total body BMD and biochemical data among the subjects with AA, AV and VV genotypes in the LRP5 gene (3989C>T, A1330V).

<table>
<thead>
<tr>
<th>Items</th>
<th>Genotype (mean ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>AA 355</td>
<td>AV 318</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.1 ± 7.5</td>
<td>66.0 ± 7.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>150.9 ± 6.1</td>
<td>150.4 ± 5.5</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>51.6 ± 7.9</td>
<td>50.8 ± 7.5</td>
</tr>
<tr>
<td>Total body BMD (g/cm²)</td>
<td>0.979 ± 0.103</td>
<td>0.971 ± 0.102</td>
</tr>
<tr>
<td>Total body BMD (Z score)</td>
<td>0.43 ± 1.02</td>
<td>0.36 ± 1.03</td>
</tr>
<tr>
<td>I-OC (ng/mL)</td>
<td>7.8 ± 3.8</td>
<td>8.3 ± 4.9</td>
</tr>
<tr>
<td>DPD (pmol/μmol/Cr)</td>
<td>7.4 ± 4.0</td>
<td>7.4 ± 2.8</td>
</tr>
<tr>
<td>Intact PTH (pg/mL)</td>
<td>37.3 ± 18.8</td>
<td>37.3 ± 16.2</td>
</tr>
<tr>
<td>Calcitonin (pg/mL)</td>
<td>22.4 ± 9.8</td>
<td>24.6 ± 11.0</td>
</tr>
<tr>
<td>BMI</td>
<td>22.6 ± 3.1</td>
<td>22.5 ± 3.1</td>
</tr>
</tbody>
</table>

BMD, bone mineral density; I-OC, intact-osteocalcin; DPD, deoxypyridinoline; PTH, parathyroid hormone; BMI, body mass index; NS, not significant. Statistical analysis was performed according to the method described in the text. Comparisons of background, BMD and biochemical data in the groups of subjects classified by the LRP5 SNP genotypes were compared by Kruskal-Wallis test.

Fig. 2. Z score values for total body BMD in the groups with each genotype of LRP5 gene in exon 18 (A1330V). (A) Z score values for total body BMD are shown for AA, AV, and VV genotypes. Scores are expressed as mean ± SE. The number of subjects is shown in parentheses. The association of the 3 genotype groups with total body BMD was determined by a Kruskal-Wallis test. (B) Z score values for total body BMD are shown as the solid circle for genotype AA + AV and as the open circle for genotype VV.

Discussion

The discovery of LRP5 as an important regulator in bone metabolism has generated a major interest in the role of LRP5 as a susceptibility gene in the regulation of BMD and/or fracture risk in the general population. Consequently, there have been several recent reports on the association between LRP5 SNPs and different bone phenotypes, including BMD, fracture, and osteophyte formation [16-19, 21-27]. These SNPs included 3 nonsynonymous variations: Q89R, V667M, and A1330V. In Caucasian populations, the V667M polymorphism was found to be significantly associated with BMD [22]. Thus far, no studies on V667M have been reported in Asian populations to our knowledge. This could be due to the low minor allele frequency of this SNP in Asian populations. The Q89R polymorphism has been studied only in Asian popula-
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gand-stimulated TCF-Lef activity. It is possible that variations in LDL repeats may alter the ligand binding and signaling of the LRP5.

Several reports have shown that Wnt and LRP5 pathway activate the bone formation [12-14]. It had been assumed that the effects of Wnt and LRP5 signaling in bone were caused by direct actions in osteoblast precursors, osteoblasts, and osteocytes [13, 29-31]. However, a recent report suggests that a key target is the duodenal enterochromaffin cell in mice [32]. In this report, Wnt signaling transduced by LRP5 was assumed to regulate serotonin synthesis, which acts in an endocrine fashion to regulate bone cell metabolism. Previous reports have shown that LRP5 is also required for normal cholesterol and glucose metabolism in mice model [33, 34]. Moreover, the A1330V polymorphism was found to be associated with the obesity and serum cholesterol levels in human [35-37]. Thus, it is possible that the association between the A1330V polymorphism and BMD is caused by the other metabolism pathways in an indirect manner.

In conclusion, our findings confirm the findings of the previous studies that the A1330V variant in the LRP5 gene may be a genetic determinant of BMD. The A1330V variant also influences Wnt signaling in vitro. These data suggest that the A1330V variant affects Wnt signaling, which in turn changes the bone metabolism and BMD.

Acknowledgments

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