Table 1. Comparison of background, BMD and biochemical data between subjects bearing at least one A allele (AA + GA) and subjects with no A allele (GG) in the SXR gene (IVS1-579A/G).

Items	Genotype (mean ± SD)		*P* value
	AA+AG	GG	
Number of subjects	247	47	
Age (years)	65.2 ± 9.0	66.7 ± 8.7	NS
Height (cm)	150.8 ± 6.5	151.6 ± 5.4	NS
Body weight (kg)	50.5 ± 8.1	51.5 ± 7.7	NS
Lumber spine BMD (Z score)	-0.224 ± 1.475	0.083 ± 1.547	NS
Total body BMD (Z score)	0.268 ± 1.061	0.635 ± 1.031	0.0298
ALP (IU/L)	190.9 ± 62.7	177.3 ± 57.8	NS
I-OC (ng/mL)	8.3 ± 4.2	7.5 ± 3.1	NS
DPD (pmol/μmol Cr)	7.8 ± 4.4	6.8 ± 2.5	NS
Intact PTH (pg/mL)	34.7 ± 16.8	33.7 ± 8.6	NS
Calcitonin (pg/mL)	22.4 ± 10.4	20.7 ± 14.7	NS
1,25 $(OH)_2D_3$ (pg/ mL)	35.1 ± 11.3	34.5 ± 10.3	NS
% fat	31.6 ± 8.1	32.6 ± 6.2	NS
BMI	22.1 ± 3.2	22.4 ± 3.1	NS

BMD, bone mineral density; ALP, alkaline phosphatase; 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.

Fig. 1.

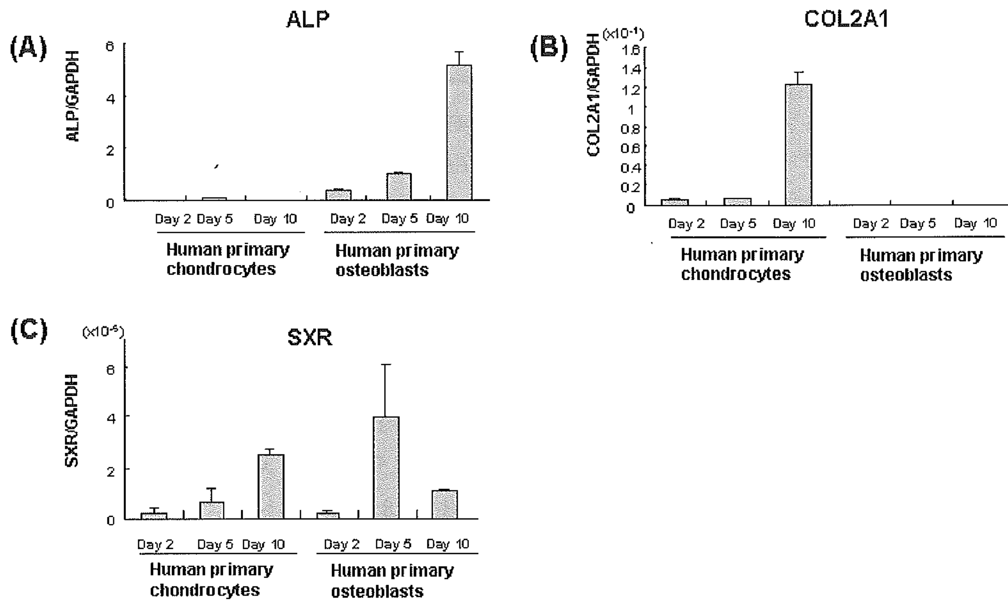
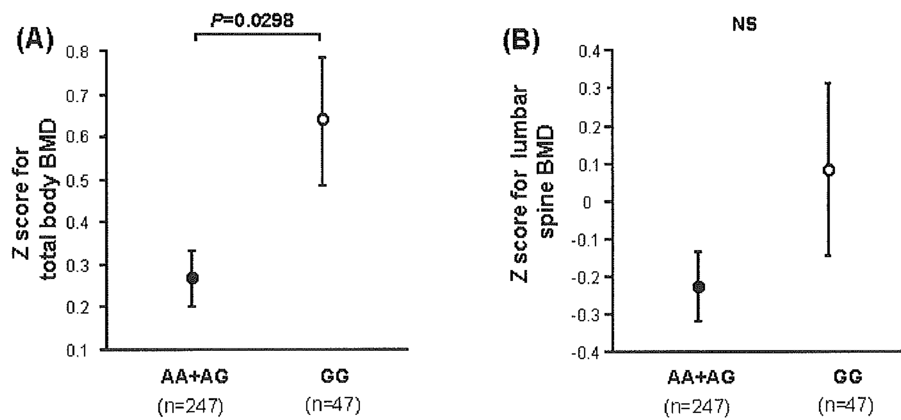


Fig. 2.



Bone (in press)

Association of a Single-Nucleotide Variation (A1330V) in the Low-Density Lipoprotein Receptor-related Protein 5 Gene (*LRP5*) with Bone Mineral Density in Adult Japanese Women

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Abstract

Low-density lipoprotein receptor-related protein 5 (LRP5), a co-receptor of Wnt signaling, is an important regulator of bone development and maintenance. Recently we identified correlation between an intronic single-nucleotide polymorphism (SNP) in the *LRP5* gene and vertebral bone mineral density (BMD), indicating that a genetic ground exists at this locus for determination of BMD. In the study reported here, we searched for nucleotide variation(s) that might confer susceptibility to osteoporosis among an extended panel of 387 healthy subjects recruited from the same hospital (Group-A), as well as among 384 subjects from the general population in eastern Japan (Group-B). We basically focused on two potentially functional variations, Q89R (c.266A>G) and A1330V (c.3989C>T), whose functional effects by the amino-acid changes were estimated by the SIFT software program; it predicted the 1330V allele as deleterious ("intolerant") although the minor allele of Q89R was questionable. By analyzing associations between the variant alleles and the BMD, reproducible association of the minor variant of A1330V to lower adjusted BMD levels was detected; i.e., In Group-A subjects 1330-V significantly associated with the spinal BMD Z-score (p=0.034), and in Group-B it associated with low radial BMD (p = 0.019). From haplotype and linkage disequilibrium (LD) analysis for 29 SNPs, we detected two separate LD blocks within the entire 137-kb *LRP5* locus, basically consistent with a previous report on Caucasians. One of the second block haplotype significantly associated with adjusted BMD (r=0.15, p=0.004). Possible combined effect of Q89R and A1330V belonging to different LD blocks was denied by multiple regression analyses. Our results indicate that genetic variations in *LRP5* are important factors affecting BMD in adult women and that 1330V may contribute to osteoporosis susceptibility, at least in Japanese.

Key words

Single-nucleotide polymorphism, LRP5, low-density lipoprotein receptor-related protein, bone mineral density, association study, quantitative trait

Introduction

Osteoporosis is a common, multi-factorial disease characterized by reduced bone mass, microarchitectual deterioration of bone tissue, and increased risk of fragility fractures. Achievement of high peak bone mass before maturation, as well as avoidance of postmenopausal bone loss, is important for prevention of osteoporosis (1). Since complicated processes during periods of development, maturation, and aging are regulated through multiple endocrine and local systems, many aspects of the mechanisms affecting control of bone mass remain to be clarified.

Wnt signaling is likely to be one of the most important systems involved in regulating developmental and homeostatic control of the skeletal system (2-4). Low-density lipoprotein receptor-related protein 5 (LRP5) is a co-receptor for Wnt (5,6), and by studies on osteoporosis pseudoglioma syndrome (OPGS) family, causative mutations were identified in the *LRP5* gene (7,8). On the other hand, certain mutations in *LRP5* cause an inherited trait of high bone mass in some families (9,10), and autosomal dominant osteopetrosis in others (11). These observations strongly suggest an important general role of LRP5 in acquisition and/or maintenance bone mass.

Genes responsible for monogenic inherited diseases sometimes also play roles in the phenotypic manifestations of common diseases (12-14), and thus are first-choice candidates for testing associations. Previously we suggested a possible contribution of LRP5 in determination of BMD, after detecting an intronic nucleotide variation of the *LRP5* gene (IVS17-1677C>A) associated with age-adjusted values of spinal BMD (Z-score) in a set of adult Japanese women. Although no definitive responsible variation(s) were established at that time (15), three other groups subsequently reported association of different missense nucleotide variations in *LRP5* with bone mineral density among Asian populations (Korean and Japanese; Q89R and A1330V: Refs. 16,17) and in Caucasians (V667M; Ref. 18). Although the significance levels and the suggested responsible variations were different, the reports were consistent in implicating the *LRP5* locus.

To examine in more detail the possibly responsible variation(s) of that gene in terms of BMD, we investigated multiple genetic variations within the *LRP5* locus in two independently recruited subject groups, comprising a total of 771 adult women in Japan. We constructed haplotypes, analyzed linkage disequilibrium (LD), and searched for mutations in these subjects.

Materials and Methods

Subjects

The 308 subjects recruited for the previous report (15) were from an outpatient clinic in Nagano prefecture (Research Institute and Practice for Involutional Diseases). Although BMD levels distributed in a wide range without skewness, these subjects were not from a population-based panel, and thus adjustment by multiple regression was not applicable. However instead, quantitative association analysis was possible using spinal BMD Z-scores, and we detected significant association with an intronic variant of *LRP5*. To verify that association, extended panel of 387 adult female was recruited from the same clinic (Group-A); these are basically healthy individuals who visited the same clinic up to December 2003. Mean ages and body-mass indices (BMI) with standard deviations (SD) were 64.6 ± 10.8 (range 25-89) years, and 22.2 ± 2.9 (range 14.3-32.9) kg/m^2, respectively. The BMD of lumbar vertebral bodies (from L1 to L4; expressed in

g/cm^2) was measured in each participant by DXA using DPX-L (GE Medical Systems Lunar Corporation, Madison WI).Coefficients of variation (CV) for the antero-posterior view of the lumbar BMD was 0.5% as described (40). Z-scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20,000 Japanese women (19). Eight of the subjects had remarkably high BMD (Z-scores >3.0), and four had remarkably low BMD (Z-scores <-3.0). For distribution analysis, we selected 91 subjects with Z-scores >+1.0 and 139 with Z-scores < -1.0. Among those women, the Z-score was greater than 2.0 in 33 and smaller than –2.0 in 34 of them. Biochemical markers of bone turnover including serum intact osteocalcin, urinary pyridinoline, and deoxypyridinoline were measured in most of the 387 subjects ascertained from the Institute; each had given informed consent prior to the study.

DNA samples for a population-based analysis were obtained from peripheral blood of 384 adult Japanese women (20,21). In this group (Group-B), mean ages and body mass indices (BMI) with standard deviations (SD) were 58.4 ± 8.6 (range 32-69) years, and 23.7 ± 3.61 (range 14.7-38.5) kg/m^2 respectively. The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry (DXA) using DTX-200 (Osteometer Meditech Inc., Hawthorne, CA, USA), and was normalized for differences in age, height, and weight by multiple linear-regression analysis (21). Forearm BMD in the distal radius was measured according to the Guidelines for Osteoporosis Screening in a health check-up program in Japan (22), and the coefficients of variation (CV) for the applied instrument was 0.8%. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism (21). All were non-related volunteers, and written informed consent was obtained from each of them. The ethical committee of the Institutional Review Board approved the entire project.

Mutation Search, SNP Selection, and Genotyping,

Mutation analysis of all 23 exons and flanking regions was carried out in DNA from the 12 subjects with the highest (>3.0, n=8) and lowest (<-3.0, n=4) Z-scores by direct sequencing of PCR products in the ABI Prism 377 DNA system (Applied Biosystems).

As potentially functional SNPs, two previously reported missense coding-SNPs, A1330V (c.3989C>T) and Q89R (c.266A>G), were selected for testing association of BMD levels, but a third, V667M, was eliminated because it was undetectable among our subjects (Table 1). Another intronic variation previously reported to associate with low BMD also was tested for association. For LD analysis, we first tested more than 40 variations within the *LRP5* locus, including all 38 polymorphic variations archived in the JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html), dbSNP and from the literature (11, 23). However, since some of the variations showed minor-allele frequencies of < 0.05 among our Group-B 384 subjects, we chose to use only 29 SNPs for LD analysis.

Genotypes for these 29 selected SNPs were determined either by the Sd-PCR method (20), Invader assay (Third Wave Technologies, Madison, WI) (24), or TaqMan Assay (Applied Biosystems). The Sd-PCR method was used for 16 SNPs, according to a protocol described previously (20). In brief, the Sd-PCR reaction was carried out using two allele-specific primers (AS-primers) and one reverse primer in a standard reaction mixture containing fluorescence-labeled dCTP. Discrimination of alleles, on the basis of by five-base differences between the AS-primers, was achieved using an ABI Prism 377 DNA system (21). The Invader assay was applied for 10 other SNPs, according to the manufacturer's protocol. In brief, 1ul of the diluted PCR

product (1:333 in distilled water) of the region flanking each SNP was used as template in a 6-ul reaction mixture in 384-well plates, and fluorescent signals for FAM and Redmond Red were detected by a plate-reader after standard 1-hour incubation (25). The other three SNPs were genotyped by TaqMan Assay according to the manufacturer's protocol.

Haplotyping, LD Analysis and Statistical Analysis

Maximum-likelihood haplotype frequencies among the 582 chromosomes of 291 subjects from Group-B were estimated by an EM algorithm using SNPAlyze v3.1 (DYNA-COM, Chiba, Japan). The LD for all possible two-way combinations of SNPs was tested with D, D', and r^2 (26, 27). After defining LD blocks and tag-SNPs in each block, each individual diplotype was estimated by SNPAlyze v3.1 software.

Quantitative associations between genotypes and adjusted BMD values (g/cm^2) were examined by analysis of variance (ANOVA), with regression analysis as a post-hoc test using Instat 3 software (GraphPad Software, San Diego, CA). The three genotypic categories of each SNP were converted into incremental values (0, 1, and 2), which represent the number of chromosomes carrying the major allele. Significant association was defined when the given *p*-value of the ANOVA F-test was less than 5% ($p<0.05$). Similarly, quantitative association was tested for major 5- and 4-haplotypes (frequency > 5%) defined for each LD block in the *LRP5* gene locus. Chi-square tests were used to ascertain Hardy-Weinberg equilibrium among genotypes ($p>0.05$). Multiple linear-regression analysis was applied for examining potential combined effects of Q89R and A1330V alleles, using Instat 3 software. Distribution analysis of the genotype frequencies among phenotypically divided groups, i.e. BMD Z-scores >1.0 (n=91) and Z-scores <-1.0 (n=139) was conducted by Chi-square tests as in our previous experiments (21). Predictive analysis of protein function for the two missense coding SNPs was conducted using the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html) (28). When the given score was less than 0.05, the alteration was deemed to be intolerant (i.e., deleterious).

Results

To identify the genetic ground involved in a previously detected association of an intronic *LRP5* polymorphism with low BMD in Group-A subjects (15), we first searched for a causative mutation anywhere among the 23 exons of the gene. However, among 12 subjects who had extremely high or low BMD Z-scores (> 3.0 or <-3.0), no mutations were detected apart from two already known missense variations (Q89R and A1330V). We analyzed the effect of these amino acid alterations, using predictive computer program SIFT (28); this program estimated a deleterious effect of 1330-V (score =0.03) and possible mild effect of 89-R (score =0.19). Although the lowest score was predicted for another missense variation V667M (score = 0.01), this variant was not detected in our test population. Based on the notion that strong candidate for the causative polymorphism would be missense variation, we investigated association of these SNPs among the entire Group-A subjects.

To analyze the effects on BMD, all the extended 387 subjects were genotyped for these two SNPs as well as previously tested IVS17-1677C>A (Table 2). No association of the Q89R genotypes was detected for BMD Z-score (r = 0.03, NS). However, we detected significant correlation of the A1330V genotype with spinal BMD Z-score (r = 0.11, $p = 0.034$); homozygous

carriers of the minor T-allele (V/V) had the lowest BMD Z-scores (-0.47 ± 1.47, n = 35), heterozygous individuals (A/V) were intermediate (-0.35 ± 1.38; n = 174), and homozygous carriers of the C-allele (A/A) had the highest BMD Z-scores (-0.03 ± 1.62; n = 178) (Table 2 and 3). The result was consistent with previously detected results for the intron SNP IVS17-1677C>A (15), as indicated (Table 2). We noticed that these SNPs were in strong linkage disequilibrium (see Fig 1). Consistent results were achieved in a distribution analysis of phenotypically divided subjects (high BMD Z-scores: n =91 and low BMD Z-scores: n = 139), using 2x3 Chi-square tests for detecting trends (p = 0.046) (Table 4). A similar result was obtained by analyzing subjects with more prominent phenotypes (Z-scores >+2.0: n = 33, and Z-scores < -2.0: n = 34; p = 0.025) (Table 4).

No significant differences were detected in physical characteristics (body weight, height, or BMI) between groups genotypically classified according to A1330V alleles. Although we detected a significant correlation of A1330V with levels of intact osteocalcin in serum (p=0.004), this might be affected by a correlation detected for age distribution (p=0.01) (Table 2).

To examine the reproducibility of that correlation, 384 adult Japanese women recruited from general Japanese population were analyzed (Group-B) on adjusted values of radial BMD by ANOVA with linear regression. As a result, significant correlation of the A1330V genotype with adjusted BMD was replicated (Table 2 and 5). Homozygous carriers of the minor T-allele (V/V) had the lowest adjusted BMDs (0.385 ± 0.041 g/cm^2, n = 34); heterozygous individuals (A/V) were intermediate (0.395 ± 0.059 g/cm^2; n = 155); and homozygous C-allele carriers (A/A) had the highest adjusted BMDs (0.405 ± 0.053 g/cm^2; n = 195), indicating a possible co-dominant effect of the minor T-allele (corresponding to the second nucleotide for a valine codon, g<u>T</u>g) on lowering adjusted BMD levels (Fig. 2A; r=0.12, p = 0.019; n = 384). Interestingly in these subjects, we observed a tendency for association of Q89R minor G-allele (corresponds to arginine; c<u>G</u>g) to low adjusted BMD, although this tendency did not quite reach statistical significance (Fig. 2B; r=0.10, p=0.052). We also examined if any patient characteristics correlated with the genotype; however, no significant differences were detected except in body weight (r = 0.11, p=0.03) or BMI (r = 0.12, p=0.02). A contribution of A1330V to control of body mass appeared to be a possibility (Table 5). For analysis of possible combined effects of Q89R and A1330V on BMD determination, multivariate linear regression analysis estimated a fitting equation explained about 2% of BMD variances (r^2 = 0.023, p = 0.01). However only the A1330V genotype contributed significantly to the equation (p=0.004).

We then analyzed LD in the LRP5 locus, by genotyping these subjects for informative 29 SNPs (minor allele frequencies > 0.05) (Table 1). Average distance between neighboring SNPs was 4.6kb (median, 3.0kb; range, 33-21,779 bp). Genotype, allelic frequency, and heterozygosity were clarified successfully for each SNP, and no deviation was detected from Hardy-Weinberg equilibrium. Indices of pair-wise LD (D' and r^2) were calculated by estimating the maximum-likelihood haplotypes and their frequencies from 293 Group G subjects genotyped for all 29 SNPs. These procedures detected two LD blocks separated by a region of about 11kb, from intron 5 to intron 7 (Fig. 1). In the second block, the indices of pair-wise LD between the A1330V and IVS17-1677C>A variants were remarkably high (D'=0.99, r^2=0.98). More important, the two missense SNPs, Q89R (c.266A>G) and A1330V (c.3989C>T), appeared to localize in different LD blocks, although the indices of pair-wise LD between the two SNPs were slightly higher than the

neighboring intronic SNPs (D' = 0.64, r^2 = 0.07). By analyzing haplotype frequency estimated by all the belonging SNPs for each LD block (11 and 18 respectively), representative 4 and 5 SNPs were selected (Table 6 and 7). By estimating diplotype in each individual, association was examined for 9 haplotypes (5 and 4 haplotypes for each block). Significant association was represented by haplotype-#2-3 (C-G-G-V-C) for the second LD block (r=0.15, p=0.0041); homozygous and heterozygous haplotype#3 carriers had lower adjusted BMD (0.384± 0.049, n=6 and 0.379±0.057, n=57) compared to non-carriers (0.402±0.053, n=299).

Discussion

In the study reported here, we investigated a possible contribution of *LRP5* polymorphisms to determination of bone-mineral density in Japanese women. A search for mutations, an LD analysis, and a study for association between two candidate SNPs and adjusted BMD values in two independently collected groups revealed reproducible association of the *LRP5* variations with BMD levels. During the preparation and submitting this manuscript, five independent groups reported similar results. One reported consistent correlation of A1330V with radial BMD in adult Japanese women (16), and the other reported a similar tendency of Q89R to be associated with hip BMD in young Korean males (17). Although another study, involving Caucasians, reported a c.2047G>A variation (V667M: nucleotide numbering is from the cap site of mRNA in that report; ref. 18), that variant appeared to be so rare in our test subjects that we excluded it from our investigations. Recent two articles further supported consistent association of A1330V, although both reports emphasized association of other intron SNPs in strong LD with A1330V (29, 30). We propose that *LRP5* variations may be important determinants of BMD in the general population, and believe that the A1330V polymorphism is the most likely LRP5 determinant of bone mass as well, at least in adult Japanese women.

Although the molecular basis for the function of LRP5 in determining BMD is not fully clarified, evidence is accumulating. Several mutations in the *LRP5* gene have been identified as causing inherited diseases with bone phenotypes, including OPGS and autosomal-dominant types of osteopetrosis (8-11). Functional molecular analyses, including gene targeting experiments and analysis of mutated LRP5 products, have revealed a mechanistic basis for bi-directional phenotypic expression, where a key is the Wnt pathway and binding of its antagonists to the YWTD motif in the extracellular domains of this co-receptor (4,10, 31). So far, however, the cellular and *in vivo* mechanisms causing reduction of bone mass are not well understood. Therefore functional differences focusing on the alanine residue at 1330 and the LDLR class-A domain should be investigated, and binding affinity for Wnt ligands or other co-binding regulators like dickkopfs (Dkks) (6) should be examined. In our experiments, a predictive informatics program (SIFT, ref. 28) for functional alterations caused by the two missense variations in *LRP5* estimated an intolerable alteration of alanine to valine at A1330V (score = 0.03) but not for glutamine to arginine in Q89R (score = 0.19). Those estimates were consistent with our assumption from association analysis that the effect of Q89R might be weaker than that of A1330V. However, a cross-sectional study conducted elsewhere had indicated a selective influence of a V667M variant on vertebral bone mass among Caucasians where the SIFT program predicted an intolerant effect of 667M (score = 0.01). Longitudinal human studies, or an *in vivo* animal study, may clarify the

developmental period(s) during which *LRP5* variants affect BMD most strongly.

The feasibility of a candidate-gene association study generally depends on control of confounding factors resulting from population bias, unreliability of diagnosis or phenotypic evaluation, interaction with environmental factors, or deficiencies in statistical power (32-34). In our study a reliable measurement of BMD, a quantitative trait with significant heredity, was evaluated with adjustment for age and BMI among unbiased population with no evidence of the stratification. Moreover, the reproducibly demonstrated association of the 1330V variant with low BMD among independent groups of subjects, in other studies and ours (16, 17), seems to provide comprehensive evidence for a functional contribution of this variant, even though in all of the studies cited, the significance levels were moderate without adjustment for multiple testing (16-18). Supportive data from linkage analysis includes a quantitative trait locus (QTL) for spinal BMD at 11q12-13 that has been identified in general populations (35). A possible combined effect of the multiple coding SNP(s) tested in our multiple regression analysis should be re-evaluated in a larger cohort study.

The existence of overlapping mechanisms for lipid metabolism, body-mass regulation, and bone metabolism is a classically discussed issue (36-38). These processes are under systemic control of leptin and neuro-endocrinological systems (37-39), but are influenced by apolipoprotein E polymorphisms (40), subject to developmental controls of mesenchymal-cell differentiation by transcription factors like PPAR-gamma (41), and affected by statins (42). In addition, the Wnt-LRP system may affect regulation of both bone and body mass (43). Our data support that notion because body-weight and BMI, as well as adjusted BMD, correlated with A1330V genotypes among 384 adult women from the general Japanese population. Other effector molecules in the Wnt signaling system might contribute as well.

In summary, our data suggest a functional effect of polymorphic variants in a candidate osteoporosis-susceptibility gene, *LRP5*, whose common polymorphisms significantly correlated with bone mineral density of women recruited from two independent populations. Because osteoporosis is a multi-factorial disease, other genes, especially genes acting through the Wnt pathway, may have to be examined for potential contributions to disease susceptibility. That information will help to clarify the complex mechanism of BMD determination *in vivo*, and may explain, at least in part, the pathogenesis of postmenopausal osteoporosis. Such studies should provide a novel viewpoint for establishing suitable treatment designs and preventive strategies for the disease.

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Figure Legends

Figure 1

Linkage disequilibrium analysis among 29 SNPs within the *LRP5* locus. **A.** Indices of linkage disequilibrium D' (upper half) and r^2 (lower half), calculated from the predicted haplotype frequencies and presented as separate tables. D' values greater than 0.5 and r^2 values greater than 0.1 are highlighted by gray-to-black gradients. Arrows indicate a possible hot spot for recombination. **B.** Schematic diagram of the *LRP5* gene showing locations of the 23 exons. Locations of the 29 tested SNPs are indicated by downward arrows.

Figure 2

Reproducible association of two missense *LRP5* variations (Q89R and A1330V) with adjusted BMD. **A.** Plots of adjusted BMD values for three genotypically classified subgroups by A1330V among 384 subjects from the general population. **B.** Plots of adjusted BMD values for three genotypically classified subgroups by Q89R. Open circles indicate mean values and error bars indicate standard deviations. Correlations between the number of minor allele possessed and the adjusted BMD were tested by linear regression analysis.

1. Summary of LRP5 polymorphisms analyzed among 384 adult women in the general Japanese population

SNP Name	nt.	cSNP Characteristics[†]	dbSNP-ID[‡]	Allele Frequency (%-Heterozygosity)	n[§]	Distance (bp)[*]	Genotyping Method
IVS1+4689C>G	G/C		rs312014	0.53:0.47 (45%)	378	9469	TaqMan
IVS1+14158G>A	G/A		rs312024	0.44:0.56 (46%)	383	310	Invader
IVS1+14468T>C	T/C		rs634008	0.26:0.74 (41%)	367	7259	Sd-PCR
IVS1-13315A>G	G/A		rs606989	0.91:0.09 (16%)	378	9089	TaqMan
IVS1-4226T>C	T/C		rs74744	0.09:0.91 (18%)	384	3832	Invader
IVS1-394A>G	A/G		rs312782	0.09:0.91 (17%)	369	567	Sd-PCR
Q89R	A/G	(c.266A>G)	-	0.93:0.07 (13%)	383	3075	Invader
IVS2+2852T>C	T/C		rs312783	0.09:0.91 (18%)	381	197	Sd-PCR
IVS2+3049T>C	T/C		rs312784	0.11:0.89 (21%)	375	3535	Sd-PCR
IVS2-2823T>G	T/G		rs312788	0.09:0.91 (18%)	384	9317	Invader
IVS4+201G>A	A/G		rs178352	0.91:0.09 (18%)	377	21779	TaqMan
IVS5-393C>T	C/T		rs3781592	0.06:0.94 (11%)	381	5761	Sd-PCR
IVS7+1632G>A	G/A		rs3781590	0.09:0.91 (16%)	374	11224	Invader
IVS7-575T>C	T/C		rs685095	0.30:0.70 (40%)	382	7134	Sd-PCR
c.2220C>T	C/T	silent	rs2306862	0.26:0.74 (36%)	372	218	Sd-PCR
IVS10+120T>C	T/C		rs667126	0.30:0.70 (40%)	384	907	Invader
IVS10-269C>T	C/T		rs583545	0.33:0.67 (45%)	370	33	Sd-PCR
IVS10-236A>G	A/G		rs23691	0.34:0.66 (42%)	381	498	Invader
IVS11+78G>A	G/A		rs689179	0.30:0.70 (41%)	378	13524	Sd-PCR
c.3357A>G	A/G	silent	rs556442	0.42:0.58 (51%)	373	6662	Sd-PCR
IVS17-1718G/del	G/del		rs3837372	0.39:0.61 (46%)	383	41	Invader
IVS17-1677C>A	C/A		rs3781586	0.28:0.72 (41%)	375	941	Sd-PCR
IVS17-736G>A	G/A		rs3781585	0.05:0.95 (10%)	379	110	Sd-PCR
IVS17-626G>A	G/A		rs3781584	0.05:0.95 (10%)	383	101	Invader
IVS17-525C>T	C/T		rs3781583	0.05:0.95 (10%)	384	750	Invader
A1330V	C/T	(c.3989C>T)	rs3736228	0.29:0.71 (40%)	384	1285	Invader
IVS18+1274A>G	A/G		rs638076	0.39:0.61 (44%)	377	2998	Sd-PCR
IVS19-336T>C	T/C		rs901823	0.36:0.64 (44%)	370	4140	Sd-PCR
IVS21+2334T>C	T/C		rs3781579	0.17:0.83 (28%)	375	-	Sd-PCR

cleotide positions in the coding sequence are indicated for missense cSNPs

s are from dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/); §: number of genotyped subjects, *: Number of otides to the next SNP (bp)

Table 2. Association analysis of adjusted BMD in Group-A and -B

SNP	Genotype	Group-A(n=387) (Spinal BMD Z-score)				p-value	Group-B(n=384) (Radial adjusted BMD)				p-value
		means		SD	n		means		SD	n	
Q89R	QQ	-0.15	±	1.50	332	0.85	0.401	±	0.055	333	0.052
	QR	-0.14	±	1.54	35		0.387	±	0.049	48	
	RR	-0.57	±	1.10	3		0.360	±	0.059	2	
A1330V	AA	-0.04	±	1.61	178	0.034*	0.405	±	0.053	195	0.019*
	AV	-0.35	±	1.38	174		0.395	±	0.059	155	
	VV	-0.47	±	1.47	35		0.385	±	0.038	34	
IVS17-1677C>A (before expansion)	CC	-0.01	±	1.42	166	0.005**	0.405	±	0.053	194	0.055
	CA	-0.35	±	1.44	118		0.395	±	0.059	152	
	AA	-0.78	±	1.36	24		0.390	±	0.040	29	

+:p-values were calculated for linear regression.
±:Data was from the previous study before subject expansion.
*:p<0.05, **:p<0.01

Table 3. Physical and clinical characteristics of the subjects in Group-A (Healthy subjects from a clinic)

Group-A Subjects (n-387)	A1330V AA	A1330V AV	A1330V VV	p-value*
Number	178	174	35	-
Age(Year)	65.6±10.1 (41-86)	64.1±10.4 (33-89)	59.3±15.0 (25-87)	0.012
Weight(Kg)	51.1±7.9 (34-74)	50.0±7.9 (33-76)	50.2±8.1 (34-70)	NS
BMI(Kg/m^2)	22.5±3.0 (16.4-32.9)	21.9±2.7 (14.3-31.8)	21.9±3.2 (15.3-29.5)	NS
Height(cm)	150.8±6.2 (135-167)	150.8±6.7 (134-172)	151.1±5.4 (140-162)	NS
Spine BMD(g/cm^2)	0.914±0.215 (0.387-1.732)	0.878±0.188 (0.400-1.506)	0.898±0.196 (0.518-1.310)	NS
BMD Z-Score	-0.03±1.62 (-3.1 - 7.1)	-0.35±1.38 (-3.5 - 5.3)	0.45±1.47 (-2.9 - 3.1)	0.034
Intact-OC†(ng/ml)	7.18±3.16 (n=117)	8.40±3.82 (n=118)	8.87±3.81 (n=18)	0.004
Pyridinolin (pmol/μmol crea)	34.2±11.1 (n=138)	33.6±11.2 (n=133)	34.5±11.4 (n=20)	NS
Deoxypyriginoline (pmol/μmol crea)	7.20±2.50 (n=139)	7.29±2.42 (n=133)	7.85±3.01 (n=20)	NS

*:p-values were calculated for regression analysis with ANOVA F-test.
†: Serum intact osteocalcin level.

Table 4. Physical and clinical characteristics of the subjects in Group-B (Cohort subjects)

Group-B Subjects	A1330V			p-value*
(n-384)	AA	AV	VV	
Number	195	155	34	-
Age(Years)	58.4±8.72	58.3±8.14	58.9±9.93	NS
	(34-69)	(32-69)	(32-69)	
Weight(Kg)	53.2±9.01	55.1±8.73	55.9±8.7	0.027
	(34.8-84.8)	(28.8-89.0)	(44.6-80.0)	
BMI(Kg/m^2)	23.3±3.63	23.9±3.61	24.6±3.29	0.018
	(16.2-37.7)	(14.7-38.5)	(17.5-31.3)	
Height(cm)	151.2±6.04	151.7±5.41	150.6±6.65	NS
	(130.5-169.0)	(139.0-166.0)	(140.0-163.4)	
Radial BMD(g/cm^2)	0.402±0.082	0.398±0.084	0.390±0.079	NS
	(0.387-1.732)	(0.178-0.613)	(0.270-0.566)	
Adj-BMD(g/cm^2)	0.405±0.053	0.395±0.059	0.385±0.041	0.019
	(0.275-0.551)	(0.225-0.554)	(0.298-0.448)	

*:p-values are calculated for regression analysis with ANOVA F-test.

Table 5. Contingency table analysis on Group-A subjects

Group-A	n	A130V			p-value#
		AA	AV	VV	
Z-score > 3.0	8	5	2	1	NA
Z-score < -3.0	4	1	3	0	
Z-score > 2.0	33	22	8	3	0.025*
Z-score > -2.0	34	13	14	7	
Z-score > 1.0	91	52	33	6	0.046*
Z-score > -1.0	139	61	63	15	

#: p-values are calculated in chi-square test for trend.
*: $p<0.05$, NA: not applicable.

able 6. Representative haplotypes for LD block-1

Hap-No	IVS1+4689C>G	IVS1+14158G>A	IVS 1+14468T>C	IVS2+2852T>C	Frequency	SUM
#1-1	0†	0	0	0	37.6%	
#1-2	1†	1	0	0	26.4%	
#1-3	1	1	1	0	15.0%	
#1-4	1	1	1	1	6.5%	
#1-5	0	1	0	0	6.3%	
#1-6	1	0	1	0	2.1%	
#1-7	1	0	0	0	2.1%	
#1-8	0	0	1	0	1.0%	
#1-9	0	0	0	1	0.9%	98.0%

0 and 1 represent major and minor alleles respectively.

able 7. Representative haplotypes for LD block-2

Hap-No	IVS7-575T>C	c.3357A>G	IVS17-626G>A	A1330V	IVS21+2334T>C	Frequency	SUM
#2-1	0†	0	0	0	0	53.0%	
#2-2	1†	1	0	1	0	13.9%	
#2-3	1	1	0	1	1	9.2%	
#2-4	0	1	0	0	0	3.8%	
#2-5	1	1	0	0	0	3.5%	
#2-6	0	1	0	1	0	3.4%	
#2-7	1	0	0	0	0	3.2%	
#2-8	0	1	1	0	1	2.9%	
#2-9	0	1	0	1	1	1.8%	
#2-10	0	1	1	0	0	1.6%	
#2-11	0	1	0	0	1	1.4%	97.6%

0 and 1 represent major and minor alleles respectively.

Figure 1

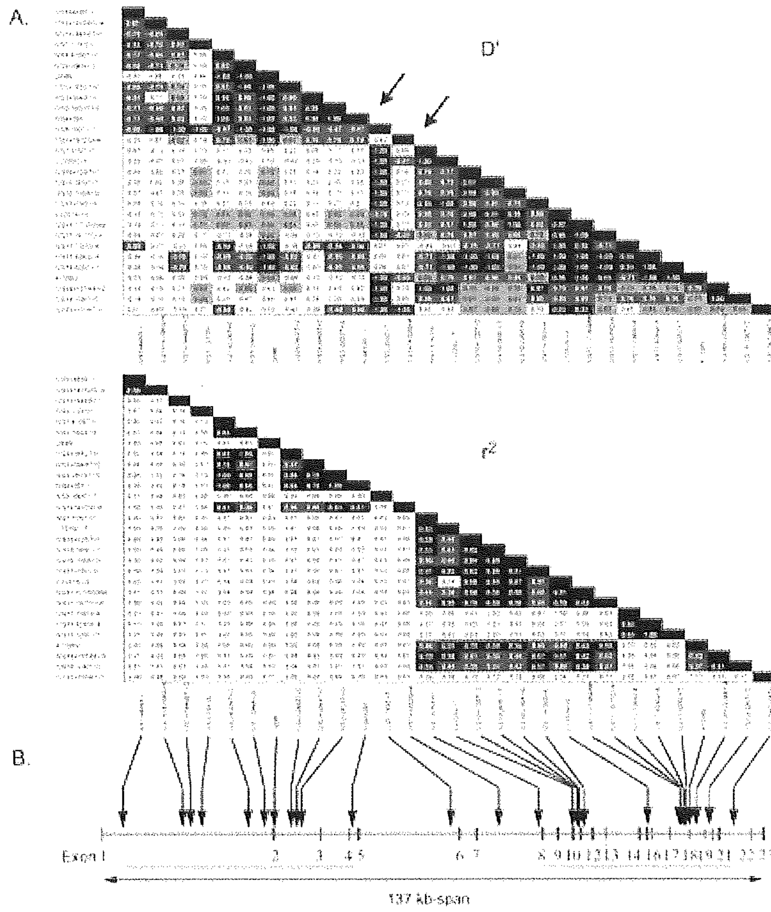
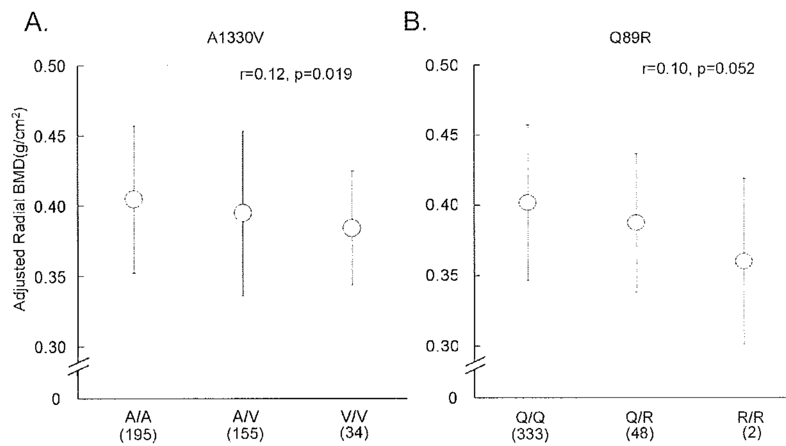


Figure 2



Spine (in press)

Q89R polymorphism in the LDL receptor-related protein 5 gene is associated with spinal osteoarthritis in postmenopausal Japanese women

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