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A Haplotype-Based Analysis of the *LRP5* Gene in Relation to Osteoporosis Phenotypes in Spanish Postmenopausal Women

Lídia Agueda,[1,2,3] Mariona Bustamante,[1,2,3] Susana Jurado,[4] Natalia Garcia-Giralt,[4] Manel Ciria,[5] Guillem Saló,[6] Ramon Carreras,[7] Xavier Nogués,[4] Leonardo Mellibovsky,[4] Adolfo Díez-Pérez,[4] Daniel Grinberg,[1,2,3] and Susana Balcells[1,2,3]

ABSTRACT: *LRP5* encodes the low-density lipoprotein receptor-related protein 5, a transmembrane protein involved in Wnt signaling. LRP5 is an important regulator of osteoblast growth and differentiation, affecting bone mass in vertebrates. Whether common variations in *LRP5* are associated with normal BMD variation or osteoporotic phenotypes is of great relevance. We used a haplotype-based approach to search for common disease-associated variants in *LRP5* in a cohort of 964 Spanish postmenopausal women. Twenty-four SNPs were selected, covering the *LRP5* region, including the missense changes p.V667M and p.A1330V. The SNPs were genotyped and evaluated for association with BMD at the lumbar spine (LS) or femoral neck (FN) and with osteoporotic fracture, at single SNP and haplotype levels, by regression methods. Association with LS BMD was found for SNP 1, rs312009, located in the 5′-flanking region ($p = 0.011$, recessive model). SNP 6, rs2508836, in intron 1, was also associated with BMD, both at LS ($p = 0.025$, additive model) and FN ($p = 0.031$, recessive model). Two polymorphisms were associated with fracture: SNP 11, rs729635, in intron 1, and SNP 15, rs643892, in intron 5 ($p = 0.007$ additive model and $p = 0.019$ recessive model, respectively). Haplotype analyses did not provide additional information, except for haplotype "GC" of the block located at the 3′end of the gene. This haplotype spans intron 22 and the 3′ untranslated region and was associated with FN BMD ($p = 0.029$, one copy of the haplotype versus none). In silico analyses showed that SNP 1 (rs312009) lies in a putative RUNX2 binding site. Electro-mobility shift assays confirmed RUNX2 binding to this site.
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Key words: LRP5, osteoporosis, association, haplotype-tagging SNP, BMD, fracture, RUNX2

INTRODUCTION

Osteoporosis is a disease characterized by decreased bone strength and increased susceptibility to fracture. It constitutes a major health problem worldwide as it incurs significant costs, morbidity, and mortality.[1] BMD is a primary predictor of osteoporotic fractures. Each SD reduction in BMD is associated with a 1.5- to 2.5-fold increase in fracture risk.[2] Both fracture risk and BMD are strongly regulated by genetic factors and constitute complex traits, as shown by twin and family studies, which indicate that heritability accounts for 50–80% of the variance in BMD.[3,4] Environmental factors have also been shown to affect BMD and to be associated with osteoporosis risk.

Chromosome 11q12–13 was initially shown to contain a key quantitative trait locus (QTL) that contributes to variations in BMD.[5–8] Within this region, the *LRP5* gene was identified as the causal gene for various bone phenotypes. Thus, in the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG), very low bone mass and blindness have been linked to loss-of-function mutations in the *LRP5* gene.[9] Concurrently, increased BMD phenotypes in several extended pedigrees have been associated with mutations in the *LRP5* gene.[10–12] In particular, the high bone mass phenotype, caused by the gain of function mutation p.G171V, is characterized by greater amounts of both cortical and trabecular bone and increased strength.[6] In mice, ablation of the *LRP5* gene mimics the skeletal phenotypes reported in humans with OPPG.[13] Conversely, transgenic mice containing the high bone mass mutation have greater bone mass, density, and strength than nontransgenic littermates.[14]

The *LRP5* gene encodes the low-density lipoprotein receptor-related protein 5 (LRP5), a transmembrane protein involved in Wnt signaling. The Wnt pathway has been shown to be related to bone mass and metabolism.[9,11] In osteoblasts, LRP5 can transduce canonical signals to promote renewal of stem cells, stimulation of pre-osteoblast

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[1]Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; [2]Institut de Biomedicina de la Universitat de Barcelona (IBUB), Barcelona, Spain; [3]CIBER de Enfermedades Raras (CIBERER), Barcelona, Spain; [4]Internal Medicine, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, Spain; [5]Department of Rheumathology, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, Spain; [6]Department of Traumathology and Orthopaedic Surgery, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, Spain; [7]Department of Gynecology and Obstetrics, URFOA, IMIM, Hospital del Mar, Autonomous University of Barcelona, Barcelona, Spain.

replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis by increasing the levels of β-catenin and altering gene expression through the Lef/Tcf transcription factors.[15]

Rare mutations in *LRP5* are associated with severe phenotypic changes, whereas more common variants may result in normal BMD variation within normal or osteoporotic ranges.[16–18]

Haplotype-based association studies have been proposed as a powerful approach to identifying the causal genetic variation underlying complex diseases.[19,20] Recently, studies have shown that the human genome is comprised of genomic segments (blocks) that display little evidence of historical recombination and low haplotype diversity.[19–21] Because of the high degree of LD observed between SNPs within these blocks, ancestral disease variants may be shown by evaluating the underlying haplotypes. This methodology does not require the causal variant to be identified and tested directly. Instead, it has the potential to highlight physical regions that harbor putative disease-associated variants.

In this study, we used a haplotype approach to comprehensively examine the contribution of common variations in the *LRP5* genomic region to osteoporosis risk among 964 Spanish postmenopausal women. We evaluated 24 SNPs individually and at the haplotype level, in relation to lumbar spine (LS) BMD, femoral neck (FN) BMD, and osteoporotic fracture.

MATERIALS AND METHODS

Study population

Nine hundred sixty-four postmenopausal Spanish women, all with Spanish ancestry, were recruited from the Menopausal Unit of the Hospital del Mar, Barcelona, Spain. All of the participants were consecutive, unselected, postmenopausal women who attended the outpatient clinic for a baseline visit because of menopause. Women were prospectively recruited and included in the study regardless of their BMD values. Subjects with a history of bone disease, metabolic or endocrine diseases, hormone-replacement therapy, and a history of drugs that could affect bone mass were excluded. Age at menarche, age at menopause, years since menopause, age, weight, and height were recorded in the final cohort, named BARCOS.[22,23] The characteristics of the study population are shown in Table 1. Blood samples and written informed consent were obtained in accordance with the regulations of the Hospital del Mar Human Investigation Review Committee for Genetic Procedures.

BMD and fractures

Areal BMD was measured (g/cm^2) in the LS (L_2–L_4) and in the nondominant FN using DXA (Hologic QDR 4500 SL). In our center, this technique has an in vivo CV of 1.0% for LS measurements and 1.65% for FN measurements.

Prevalent fractures were estimated by obtaining a fracture history for each subject including age at the time of fracture, the site of fracture, and how the fracture was sustained. Only fractures caused by minimal trauma were included. Fractures of the face, skull, fingers, and toes were excluded. A questionnaire was used to take the history of peripheral fractures. Vertebral fractures were confirmed by spine X-ray reports.

TABLE 1. CHARACTERISTICS OF THE BARCOS COHORT

Variable	*Mean ± SD*	*n*
Age (yr) LS*	55.5 ± 8.7	944
Weight (kg)	64.5 ± 10.0	944
Height (cm)	156.3 ± 6.3	944
Years since menopause LS*	7.7 ± 8.6	944
FN BMD (g/cm^2)	0.683 ± 0.109	513
LS BMD (g/cm^2)	0.853 ± 0.153	944
Breast-feeding (mo)	8.3 ± 13.2	883
Age at menarche (yr)	12.9 ± 1.6	927
Age at menopause (yr)	47.7 ± 4.5	936
Fracture		143 (18.5%)
No fracture		630 (81.5%)

* For femoral neck BMD data, the mean age was 57.6 ± 8.2 yr and the years since menopause was 9.8 ± 8.2 (n = 513).

SNP selection

Twenty-four SNPs in the *LRP5* genomic region were selected on the basis of the following criteria: (1) haplotype tagging; (2) putative functional changes; and/or (3) previous association with osteoporotic phenotypes.

(1) The selection of haplotype-tagging SNPs (htSNPs) was based on the HapMap Project white reference panel genotype data (CEU).[24] Available data from the chromosomal region Chr11: 67829880–67980145 bp covering 136.6 kb of LRP5 and 6.8 kb of the neighboring regions (HapMap Data Release 20/PhaseII Jan 06 on NCBI assembly dbSNPb125) were downloaded. Seventy of the 149 SNPs genotyped in this region were polymorphic, resulting in an average SNP density of 1 SNP/2 kb. Haplotypes were constructed using Haploview Software 3.32[25] and by applying both the Gabriel (or CIs) method[20] and the Four Gamete Rule (FGR),[26] with the default parameters. Briefly, in the Gabriel method, the haplotype block is defined as a region over which <5% of comparisons among informative SNP pairs show strong evidence of historical recombination. In FGR, blocks are formed by consecutive markers where, for each pair, only three of the four possible gametes are observed. For each resulting block, the minimal combination of SNPs that identified ≥95% of the haplotypes was selected. The LD select program[27] was used to identify highly correlated SNPs among this htSNP subset in terms of linkage disequilibrium (r^2). When a pair of SNPs was correlated with $r^2 \geq 0.80$, only one of the SNPs was selected for genotyping, because the other was considered redundant. Twenty-two htSNPs were finally selected according to this criterion (all but 16 and 19 in Table 2).

(2) Four putatively functional missense changes have been described in the *LRP5* gene. The Val667Met change (rs4988321) was included in the panel as SNP 19. The

TABLE 2. CHARACTERISTICS OF THE 24 SNPS USED IN THE LRP5 ANALYSIS

SNP	rs	Position in Chr11*	Location	Base change	MAF in BARCOS	MAF in CEU-HAPMAP†	HWE p value
1	rs312009	67833814	5′ UTR	C>T	0.150	0.225	0.9811
2	rs312016	67838979	Intron 1	G>A	0.277	0.325	0.5083
3	rs7111296	67839486	Intron 1	C>T	0.001	0.017	0.9988
4	rs11228202	67840804	Intron 1	C>T	0.142	0.092	0.8716
5	rs4988300	67845407	Intron 1	T>G	0.439	0.442	0.2374
6	rs2508836	67847841	Intron 1	C>T	0.323	0.255	0.8730
7	rs7924398	67849769	Intron 1	C>T	0.083	0.037	0.6193
8	rs3781600	67849913	Intron 1	G>C	0.113	0.085	0.7524
9	rs606989	67858576	Intron 1	C>T	0.088	0.050	0.9796
10	rs314756	67868248	Intron 1	A>G	0.071	0.108	0.9462
11	rs729635	67868336	Intron 1	A>C	0.147	0.068	0.7450
12	rs312788	67878871	Intron 2	T>G	0.480	0.350	0.2828
13	rs638051	67897990	Intron 5	A>G	0.464	0.308	0.0262
14	rs11826287	67903237	Intron 5	T>C	0.215	0.167	0.0913
15	rs643892	67905029	Intron 5	T>A	0.439	0.258	0.4475
16‡	rs627174	67920090	Intron 7	T>C	0.226	0.152	0.1170
17	rs545382	67927589	Exon 8 F549F	G>A	0.091	0.076	0.6491
18	rs2277268	67930698	Exon 9 E644E	C>T	0.109	0.042	0.4216
19§	rs4988321	67930765	Exon 9 V667M	G>A	0.069	0.039	0.6218
20	rs583545	67935211	Intron 10	G>A	0.291	0.208	0.6242
21	rs531163	67951072	Intron 16	T>C	0.397	0.242	0.4838
22§	rs3736228	67957871	Exon 18 A1330V	C>T	0.174	0.117	0.1787
23	rs2242340	67970695	Intron 22	G>A	0.173	0.068	0.8514
24	rs676318	67973996	3′ UTR	T>C	0.067	0.050	0.6689

* Build 126 NCBI dbSNP.
† MAF from the HapMap white reference panel, except for SNPs 16 and 19, where MAF was from Perlegen and from Applera, respectively.
‡ Selected on the basis of previously published associations.[28]
§ Missense change, putatively functional.

Ala1330Val change (rs3736228) was already selected as htSNP 22. The Val1204Leu change (rs11607268) appears as a nonsynonymous SNP in public genome databases but was not included in the panel because it was not polymorphic in 40 Spanish control chromosomes tested. Finally, the Ala1525Val SNP (rs11574422) was not included in the panel because its minimum allele frequency in Europeans was <1% (minor allele frequency [MAF] 0.9%).

(3) Most of the SNPs that have previously been associated with osteoporosis lay within the blocks described above. Only SNP 16 (rs627174), associated with FN and LS BMD,[28] fell in a gap between Gabriel blocks 4 and 5 (FGR blocks 5 and 6). Thus, it was added to the collection.

SNP genotyping

Genomic DNA was extracted from buffy coats (centrifuged whole blood) by a salting out procedure.[29] DNA concentration was assessed by spectrophotometry (NanoDrop ND-1000 Spectrophotometer; NanoDrop Technologies). Some of the samples that had failed a first round of genotyping because of impurities were repurified with the Puregene TM DNA isolation kit (Gentra Systems), according to the manufacturer's instructions.

Genotyping of the 24 selected SNPs was performed simultaneously by SNPlex (Applied Biosystems) at the genotyping platform of the Centro Nacional de Genotipado (CEGEN, Barcelona, Spain). SNPlex system probe sets were designed using an automated assay design pipeline and included in a 48-plex. A previous accurate quantification of the DNA samples was performed by Picogreen (Invitrogen).

Twenty-one of the SNPs were successfully genotyped (pass rate 87.5%), with an average of a 95.6% call rate over 964 samples. SNPlex performance at the CEGEN platform was evaluated by testing one 41-SNP pool in genomic DNA samples from 367 individuals. SNPlex reproducibility was >99% (99.93% in 14,136 counts), in concordance with reported standards from AB.[30] Because two of the *LRP5* SNPs (19 and 22) were also analyzed by Taqman (Applied Biosystems) for a different study, the comparison of the two series of genotypes constituted an additional genotyping quality control. Results showed a 99.75% average concordance between the two techniques.

To complete the genotyping, the three SNPs failed by SNPlex (9, 13, and 23) were typed by Taqman (Applied Biosystems). Primer and probe sequences are available on request. Amplification and detection were performed under standard manufacturer's protocol in an ABI PRISM 7900HT Sequence Detection System and analyzed with AB SDS 2.2 Software. For these SNPs, the genotype call rate was slightly lower: 86% on average. Quality control was assessed by regenotyping 6% of the samples (selected at random) by PCR-restriction fragment length polymorphism (RFLP). This gave a >95% reproducibility.

LD analysis and haplotype reconstruction

To determine whether individual variants at each locus were in Hardy-Weinberg equilibrium (HWE) in the population, χ^2 tests were applied. MAFs and HWE p values for all the SNP were calculated using the SNPATOR web tool developed by CEGEN (http://bioinformatica.cegen.upf.es/public/principal/dataMngA.php).

In the BARCOS cohort, Lewontin's D′ and the linkage disequilibrium (LD) coefficient r^2 were examined between all pairs of SNPs using HAPLOVIEW.[25] Haplotype frequencies in the BARCOS cohort were also estimated using this software.

The MAFs of all the SNPs, D′, and r^2 values between BARCOS and the HapMap white reference panel were compared by standard lineal regression.

For each individual, haplotypes were inferred using the PHASE v2.1.1 program.[31] Only haplotypes with >0.90 posterior probability were used in further analyses.

Statistical analyses

Initially, all genotyped SNPs were tested for single-marker allelic association. The quantitative traits LS BMD and FN BMD were adjusted for age, weight, and height and analyzed by ANCOVA under a general model. Alternative inheritance models were evaluated when the general model showed a trend of significance. Logistic regression models were used for the phenotype "fracture." Calculations were performed with SPSS v11.5 (SPSS, Chicago, IL, USA).

For haplotype analyses, a global test on all the haplotypes in each block with a frequency >1% was performed by logistic regression with a stepwise selection procedure using the expected number of copies. Logistic regression analyses were also adjusted by age, weight, and height (SPSS). The significance level was set at $p < 0.05$.

The statistical power was calculated with Quanto 1.1 software.[32] For FN BMD (mean = 0.683 and SD = 0.109) at a significance level of 0.05, an MAF of 0.10 in 450 individuals provides a minimal power of 90% to detect differences in BMD of −0.04 g/cm^2 under an additive model. The same assumptions for an MAF of 0.40 allow differences of 0.03 g/cm^2 to be detected. For LS BMD (mean = 0.853 and SD = 0.153) at a significance level of 0.05, an MAF of 0.10 and 950 individuals provides a 90% power to detect differences in BMD of −0.04 g/cm^2 under an additive model. The same assumptions for a MAF of 0.40 allow differences of 0.03 g/cm^2 to be detected. For fractures (150 cases, 4.4 controls per case, and a 20% population risk in our cohort) under an additive model for MAFs of 0.10 and 0.40, there would be enough power (80%) to detect ORs of 1.75 and 1.45, respectively.

The presence of population stratification was investigated by the program Structure 2.2,[33] using 12 independent loci that had been genotyped in the cohort. The highest probability was obtained under the assumption of one population (k = 1) and was close to 1. All other assumptions (k = 2 to k = 5) had probabilities $<10^{-58}$.

EMSAs

SAOS-2 human osteosarcoma cells (ATCC number HBT-85™) were grown in DMEM (Gibco), supplemented with 10% FBS (Gibco). Nuclear extracts were prepared according to Schreiber et al.[34] using a modified buffer C (10% glycerol and 1.5 mM of $MgCl_2$). Protein concentrations were determined by the Bradford method, and nuclear extracts were stored at −80°C until use.

Single-stranded DNA oligonucleotides were automatically synthesized (Sigma Aldrich) for each allele of SNP 1, both forward and reverse strands. Forward oligonucleotides were as follows: *C* allele, 5′-CCTTTGTTC*CTGTGGC***C**-GGA- 3′; *T* allele, 5′-CCTTTGTTC*CTGTGGCT***T**GGA-3′ (bases indicated in bold correspond to the polymorphic site and those in italics to the predicted RUNX2 binding site). Other oligonucleotides used were those containing binding sites for RUNX2 (OSE), the glucocorticoid receptor (GRE), and Sp1. Double-stranded probes were obtained by annealing complementary oligonucleotides and end-labeling with [γ-^{32}P] ATP (GE Healthcare), using the OptiKinase (USB) standard protocol. The unincorporated nucleotides were removed using a quick-spin G-25 Sephadex column (ROCHE Molecular Biochemicals). Binding reactions typically contained 10 μg of nuclear extract, 0.5 μg of double-stranded poly (dI-dC; Amersham Pharmacia Biotech), 0.5 μg of double-stranded poly (dA-dT; ROCHE), 6 μg of acetylated BSA (Promega), and 100,000 cpm of radiolabeled probe. The reaction mixtures were incubated for 30 min at room temperature in a buffer containing 20 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol in a total volume of 20 μl. Protein–DNA complexes were resolved from the free probes in nondenaturing 7% polyacrylamide (29:1) gels containing 2.5% glycerol. Electrophoresis was performed at 4°C in 1× TBE buffer at 20 mA for ~3 h. Gels were vacuum-dried and exposed to Storage Phosphor screens (Kodak) at room temperature for 3–12 h. In competition assays, the binding reactions were performed in the presence of an excess of unlabeled competitor oligonucleotide, as indicated in each case. For supershift experiments, 2 μg of the RUNX2 polyclonal antibody (Calbiochem), 0.5 μg of NFATc1 antibody (Santa Cruz Biotechnology), or 2 μg PPCA antibody (Rockland Immunochemicals) was preincubated with nuclear proteins in the binding buffer on ice for 15 min before adding the probe.

RESULTS

LD block definition, SNP selection, genotyping, and block reconstruction

The CEU-HapMap population data were used to construct LD blocks across the LRP5 genomic region by the Four Gamete rule and the Gabriel methods. This resulted in six and eight distinct haplotypic blocks, with two to four tag SNPs per block, respectively (Fig. 1A). The 22 SNPs that best tagged the resulting LD blocks, together with two other relevant SNPs (SNP 16 and 19; see Materials and Methods section), are depicted in Fig. 1 and listed in Table 2. This 24-plex was genotyped in the BARCOS cohort. All SNPs but one (SNP 13) were found to be in HWE in this cohort (Table 2). SNP 3 was practically nonpolymorphic and was not used in further analyses. MAFs and pairwise

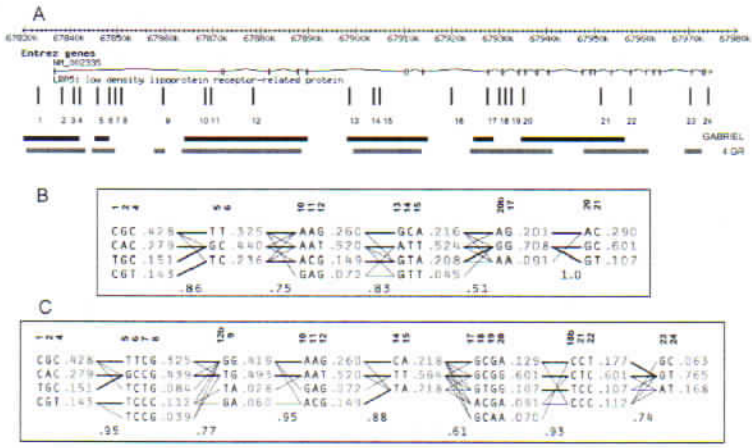

FIG. 1. LRP5 genomic structure, the approximate position of genotyped SNPs, and the resulting block structure for the two haplotype block definitions (A). Reconstruction of Haplotype Blocks by the Gabriel Method (B) and the Four Gamete Rule (C). SNPs 12b, 18b, and 20b correspond to rs7944040, rs7111370, and rs491347 and were not genotyped. Instead, they were substituted by SNPs 12, 18, and 20, which have r^2 values of 0.804, 1, and 1, respectively.

LD values (r^2) in BARCOS were comparable to those of the CEU-HapMap (Figs. 2A and 2B). The genotypes obtained were used to reconstruct in the BARCOS cohort the LD blocks dictated by the CEU-HapMap data. Major haplotypes and their frequencies are shown in Figs. 1B and 1C. These results are also comparable to the CEU-HapMap data (Figs. 2C and 2D).

Single SNP and haplotype level association analysis

The 23 SNPs were individually tested for association under a general model with the three phenotypes (LS BMD, FN BMD, and osteoporotic fracture), adjusting by age, height, and weight. Table 3 lists the analysis of covariance (ANCOVA) p values for the BMDs and the logistic regression p values for fracture. SNPs with $p < 0.05$ (nominal significance) or <0.1 (indicative of a trend) were given further attention. p values corresponding to alternative models (additive, recessive, or dominant) are provided in parentheses.

SNP 1 (rs312009) was associated with LS BMD under a recessive model ($p = 0.011$). SNP 6 (rs2508836) was associated with LS (additive, $p = 0.025$; recessive, $p = 0.030$) and FN BMD (recessive, $p = 0.031$). The effects of these SNPs on BMD are shown in Table 4. For SNP 1, an increase of 0.07 (0.02–0.13) g/cm^2 was observed for the minor allele homozygote (TT). In the case of SNP 6, the minor allele homozygote (TT) showed risk effects for both LS and FN BMD (–0.018 [–0.03 to 0.00] g/cm^2 of LS BMD per copy of T; –0.03 [–0.06 to 0.00] g/cm^2 of FN BMD for TT versus CC/CT). SNPs 11 and 15 were associated with fracture under alternative models ($p = 0.007$ additive, and $p = 0.019$ recessive, respectively; Table 3). Their effects are indicated in Table 4. For both models, minor homozygotes had a higher risk of osteoporotic fracture (OR, 1.70 [1.15–2.51] per copy of C allele; OR, 1.79 [1.10–2.92] AA versus TT/TA, respectively).

The association between the six blocks generated by the Gabriel Method, the eight blocks generated by the FGR, and the three bone phenotypes was also tested under a general model (Table 5). No nominally significant results were obtained. Block 4 of Gabriel and block 8 of FGR showed a trend with FN BMD ($p = 0.056$ and $p = 0.051$, respectively). For these two blocks, individual haplotypes were tested for association under an additive model. Only haplotype "G-C" of FGR block 8 (frequency of 0.063) showed a nominally significant association with FN BMD ($p = 0.029$) and an effect of 0.021 (0.040–0.002) g/cm^2 for one copy of the "G-C" haplotype versus none.

Functional analysis: EMSA

Two of the four SNPs that showed association with bone phenotypes were located in the 5′-flanking region or within the 5′ half of intron 1 of the gene. To test the possible functionality of these two SNPs, we assessed whether they lay in putative binding sites for transcription factors. According to MATINSPECTOR and ElDorado TFBS prediction tools (GENOMATIX), SNP 1 (rs312009) lies within a conditional site for RUNX2, a *runt* domain transcription factor essential to osteoblast differentiation. For SNP 6, no site was identified.

To test if RUNX2 was able to bind to this site, a labeled double-stranded 20-mer bearing the rs312009 T allele was incubated with nuclear extracts from the osteosarcoma cell line SAOS2 in a gel shift assay (EMSA). As shown in Fig. 3A, a band shift pattern was produced that was competed by increasing amounts of the cold oligonucleotide but not by other unrelated DNA molecules such as GRE or Sp1. Both oligonucleotides, the one containing a T and the one containing a C at the SNP, seemed to exert the same level of competition. Clear competition by the oligonucleotide containing an OSE, the canonical RUNX2 binding site in the osteocalcin promoter, was also observed. Additionally, a supershift was produced when the incubation was performed in the presence of RUNX2 antibodies, as shown in Fig. 3B. This supershift was not produced when the unrelated antibodies used were those against NFAT or PPCA. An experiment using the C allele probe produced a similar result (data not shown).

DISCUSSION

This study used a haplotype-based approach to scan the *LRP5* gene for common genetic variants related to bone

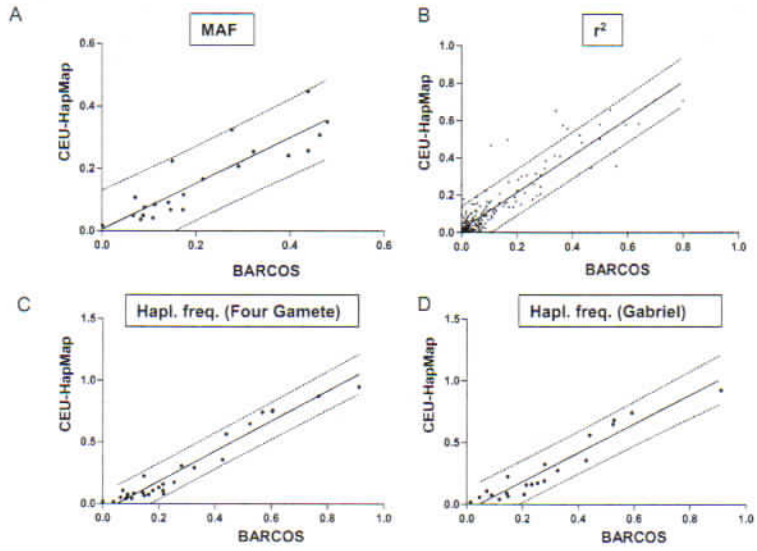

FIG. 2. Comparisons between the CEU-HapMap population and the BARCOS cohort. (A) Minor allele frequencies (Pearson correlation $R = 0.801$, $p < 0.0001$). (B) Pairwise LD values (r^2; $R = 0.828$, $p < 0.0001$). (C) Frequencies of the haplotypes constructed with the Four Gamete Rule ($R = 0.946$, $p < 0.0001$). (D) Frequencies of the haplotypes constructed with the Gabriel Method ($R = 0.917$, $p < 0.0001$).

TABLE 3. ASSOCIATION STUDIES OF INDIVIDUAL SNPS AND OSTEOPOROTIC PHENOTYPES ADJUSTED BY AGE, WEIGHT, AND HEIGHT

SNP	*LS BMD*	*FN BMD*	*Osteoporotic fracture*
1	0.026 (r: 0.011)	0.693	0.612
6	0.053 (a: 0.025, r: 0.030)	0.062 (r: 0.031)	0.187
11	0.299	0.437	0.028 (a: 0.007)
15	0.425	0.365	0.062 (r: 0.019)
17	0.691	0.078 (d: 0.057)	0.533

Values are p value for the general model (p value for the alternative model).

a, additive; d, dominant; r, recessive.

phenotypes. Four SNPs were found to be associated either with BMD or fracture risk. One of these SNPs was located in the 5′-flanking region within a binding site for RUNX2.

Because LRP5 was found to be an important gene for bone biology,[35] several studies have been performed to study association between SNPs in this gene and BMD or fracture risk. Most of these studies have been limited to only one or a few polymorphisms. Several studies focused on the two nonsynonymous SNPs V667M and A1330V. Results varied among the different studies. This could be because of differences in sex, age, ethnicity, or other features of the cohorts. Overall, it seems that these variants mainly affect peak bone mass in men.[16] However, when the effects of the SNPs were very small, any discrepancies could be caused by a lack of power of the individual studies. Only appropriate meta-analyses can resolve this issue. In this respect, the recent publication of van Meurs et al.[36] on the full GENOMOS consortium (>37,000 whites) indicated that these two variants have a modest but highly significant effect on BMD and fracture risk. Our results, which were included in the GENOMOS study, showed a trend in the same direction. However, they did not reach significance.

One limitation of previous studies is that they have only focused on particular missense SNPs. They did not address variability across the gene. Moreover, although these SNPs are missense, their functionality has not been experimentally proven. A new approach, based on whole gene variation, has been adopted in recent studies.[17,28,37–41] Some such studies analyzed the haplotypic block structure, allowing the use of a limited set of tag-SNPs to evaluate all the variability within the blocks, as done here. In general, the block structure was quite similar among studies. In all cases, a gap where LD is lost was detected in intron 7, where rs627174 (SNP 16 in this study) is located. This is in agreement with the existence of a recombination hotspot described by Twells et al.,[42] which divides the gene into a 5′ and a 3′ region. The 3′ region is characterized by one block of strong LD, which in some of the studies may be split into minor blocks. The LD across the 5′ side of the gap is split into three or more blocks in the white population.

The two SNPs associated with BMD in this study (SNP 1: rs312009 and SNP 6: rs2508836) have not been tested by other researchers. Both of them are in the 5′ end of the gene. SNP 1 is in the 5′-flanking region and is ~2900 bp upstream of the transcription start site. It lies within a large LD block that spans from nearly 50 kb upstream of the gene to the 5′ region of intron 1.[42] No SNPs upstream of SNP 1 have been included in other *LRP5* gene-wide analyses. However, some groups have analyzed SNPs within the same block. They found that rs4988330 and rs4988331,[37] as well as rs682429,[38] were associated with LS BMD. In addition, rs682429 was associated with hip BMD.[17,43] This would be consistent with variants within this block being involved in determining bone phenotypes. The fact that we found a stronger association for SNP 1 than for its haplotype block and that SNP 1 lies within a RUNX2 binding site, suggests that it could be a functional SNP. Further replication and functional analyses are required to confirm this hypothesis. SNP 6 is in a small block within intron 1. A previous study[28] analyzed a very close marker (rs514529)

TABLE 4. EFFECTS ON BMD (G/CM^2) OR FRACTURE (OR) OF SIGNIFICANTLY ASSOCIATED SNPS

SNPs associated with BMD

SNP	*rs*	*Phenotype*	*Genotype*	*Adjusted BMD [mean (SE)]*	*n**	*Difference between means (g/cm^2) for the alternative model (95% CI)*
1	rs312009	LS BMD	CC	0.848 (0.005)	654	TT vs. CC/CT 0.07 (0.02–0.13)
			CT	0.857 (0.009)	231	
			TT	0.925 (0.029)	21	
6	rs2508836	LS BMD	CC	0.861 (0.007)	408	Per copy of the *T* allele: −0.02 (−0.03 to 0.00)
			CT	0.851 (0.007)	396	
			TT	0.825 (0.014)	95	
		FN BMD	CC	0.672 (0.006)	209	TT vs. CC/CT −0.03 (−0.06 to 0.00)
			CT	0.681 (0.006)	226	
			TT	0.646 (0.013)	49	

SNPs associated with fracture

SNP	*rs*	*Genotype*	*No fracture**	*Fracture**	*OR (95% CI)*
11	rs729635	AA	431 (83.5%)	85 (16.5%)	per copy of the *C* allele: 1.70 (1.15–2.51)
		AC	125 (76.2%)	39 (23.8%)	
		CC	11 (64.7%)	6 (35.3%)	
15	rs643892	TT	179 (81.4%)	41 (18.6%)	AA vs. TA/TT: 1.79 (1.10–2.92)
		TA	293 (84.4%)	54 (15.6%)	
		AA	107 (74.8%)	36 (25.2%)	

* Discrepancies in the total number of cases among the different SNPs and in relation to numbers in Table 1 are because of missing genotypes.

TABLE 5. ASSOCIATIONS OF LRP5 HAPLOTYPES WITH BMD (G/CM^2) OR FRACTURE (OR)

		p for the general model		
Block identification	*Tag SNPs*	*LS*	*FN*	*Fracture*
1G, 1FGR	1, 2, 4	0.120	0.639	0.772
2G	5, 6	0.260	0.366	0.638
3G, 4 FGR	10, 11, 12	0.556	0.630	0.497
4G	13, 14, 15	0.283	0.056	0.769
5G	20b, 17	0.407	0.365	0.903
6G	20, 21	0.585	0.411	0.755
2 FGR	5, 6, 7, 8	0.529	0.622	0.819
3 FGR	12b, 9	0.850	0.975	0.694
5 FGR	14, 15	0.250	0.191	0.303
6 FGR	17, 18, 19, 20	0.364	0.073	0.952
7 FGR	18b, 21, 22	0.621	0.153	0.957
8 FGR	23, 24	0.152	0.051	0.303

within the same block and found that it was associated with LS BMD in white premenopausal women.

We found two SNPs associated with fracture risk: SNP 11 (rs729635) in intron 1 and SNP 15 (rs643892) in intron 5. SNP 11 has recently been analyzed by Sims et al.,[41] who found that it was associated with FN BMD. SNP 15 was also analyzed by Xiong et al.,[17] who found that it was associated with FN BMD in a Chinese population.

In general, although comparisons are difficult because of the disparity in SNP selection, ethnicity, age, sex, and design, a global picture emerges from the various gene-wide analyses mentioned above. Three regions of the gene seem to be relevant for determining bone phenotypes: the 5′-flanking-intron 1 region, which includes the SNPs found in this study; a region in intron 7, which coincides with the previously mentioned gap; and the 3′ end of the gene. However, at individual SNP level, several discrepancies exist. For example, SNP 16 (rs627174), which was selected to replicate a previous positive finding by Koller et al.,[28] gave a negative result both in our study and in studies by Xiong et al.[17,38]

The availability of HapMap data on white polymorphisms and LD structure allowed us to use them for designing an extensive coverage of the variation in the *LRP5* gene. However this coverage is still incomplete. For instance, gaps between defined blocks could harbor unassayed variants. We carefully screened those regions for functionality, using available predictive tools. However, these are also of limited performance.

As in any association study, multiple testing and power considerations were taken into account. Our sample size and study design enables the moderate effects of common variants to be detected. Effects under this threshold or functional rare SNPs will not have been detected. In addition, our current sample size does not allow for an accurate analysis of gene–environment interactions. Such interactions may be relevant for osteoporosis, because the disease is known to be a polygenic condition with a strong environmental component.[44]

Given the number of tests performed, multiple testing has to be considered because some of the significant results may be false positives (type I error). Classical multiple testing corrections such as Bonferroni are considered to be too conservative and not applicable to these kinds of studies, basically because the selected 23 SNPs are not totally independent. In addition, the independence of the three bone phenotypes is still under discussion. A more recent approach proposed by Nyholt[45] takes into account the non-independence of the SNPs, considering the LD between the studied SNPs. This method can be applied to obtain an

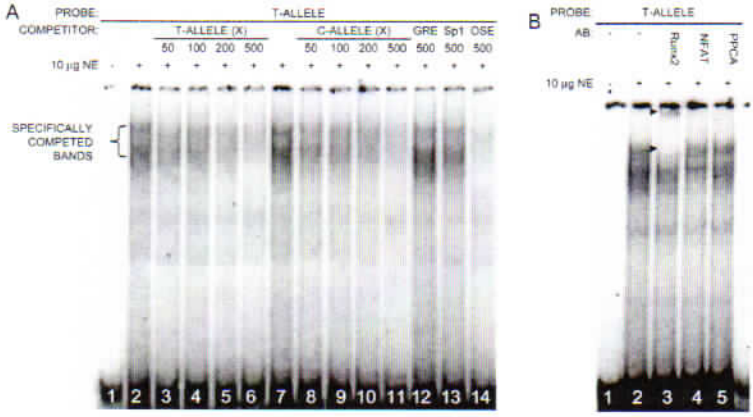

FIG. 3. The DNA sequence containing SNP1 binds a transcription factor present in osteoblast nuclear extracts. DNA binding was analyzed by a gel retardation assay. (A) Labeled double-stranded oligonucleotide containing allele *T* was incubated with 10 μg of nuclear extract. Competition experiments were performed with cold allele *T* or allele *C* oligonucleotide; with the GRE or Sp1 binding sites as nonspecific competitors; and with an oligonucleotide containing the OSE consensus sequence. (B) Addition of an anti-Runx2 antibody shifted the top retarded band (arrowheads), whereas addition of either anti-NFAT or anti-PPCA antibodies had no effect on the retarded bands.

effective number of independent SNPs (Meff). The application of SNPSpD to our 23 SNPs sample set gave a Meff of 17 SNPs, which leads to a significance threshold of 0.05/17 = 0.003. None of our significant results stand this correction. Alternatively, a false discovery rate (FDR) of 12% was estimated with the r-based QVALUE software.[46] However, FDR has some limitations, such as penalizing for a small list of *p* values. Moreover, it does not take into account the dependence between the SNPs and the phenotypes studied. To confirm these results, they should be replicated in other independent cohorts.

Further support for a preliminary positive result may be obtained by functional testing of the associated SNP. For SNP1, the identification of the RUNX2 binding site makes the association more relevant. Similar studies are currently being performed for other SNPs. Among other strengths of our study, it should be mentioned that special effort was made to obtain a highly homogeneous cohort. The sample only included postmenopausal women of confirmed Spanish origin from a limited geographic region, with no illnesses or treatments that affect bone. In addition, careful phenotypic characterization was performed, especially for osteoporotic fractures. Smoking and estrogen replacement therapy, considered the most relevant environmental factors, were filtered in our study. Additionally, the SNPs selected, most of which validated HapMap SNPs, should enable comparison with other studies and facilitate potential meta-analyses.

In summary, we showed that variation along the *LRP5* gene region is associated with bone phenotypes, with moderate effects. Preliminary functional analyses suggest a regulatory role for one of the SNPs in the 5′-flanking region. Genome-wide association analyses, such as the one published recently by Kiel et al.,[18] will help to identify new candidate loci for osteoporosis and to replicate previously reported candidate genes such as *LRP5*.

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REFERENCES

1. Cummings SR, Melton LJ 2002 Epidemiology and outcomes of osteoporotic fractures. Lancet **359:**1761–1767.
2. Center JR, Nguyen TV, Schneider D, Sambrook PN, Eisman JA 1999 Mortality after all major types of osteoporotic fracture in men and women: An observational study. Lancet **353:**878–882.
3. Komori T 2003 Requisite roles of Runx2 and Cbfb in skeletal development. J Bone Miner Metab **21:**193–197.
4. Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S 1987 Genetic determinants of bone mass in adults. A twin study. J Clin Invest **80:**706–710.
5. Koller DL, Rodriguez LA, Christian JC, Slemenda CW, Econs MJ, Hui SL, Morin P, Conneally PM, Joslyn G, Curran ME, Peacock M, Johnston CC, Foroud T 1998 Linkage of a QTL contributing to normal variation in bone mineral density to chromosome 11q12-13. J Bone Miner Res **13:**1903–1908.
6. Johnson ML, Gong G, Kimberling W, Recker SM, Kimmel DB, Recker RB 1997 Linkage of a gene causing high bone mass to human chromosome 11 (11q12-13). Am J Hum Genet **60:**1326–1332.
7. Heaney C, Shalev H, Elbedour K, Carmi R, Staack JB, Sheffield VC, Beier DR 1998 Human autosomal recessive osteopetrosis maps to 11q13, a position predicted by comparative mapping of the murine osteosclerosis (oc) mutation. Hum Mol Genet **7:**1407–1410.
8. Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, Peltonen L, Somer H, Hirose T, Dallapiccola B, De Paepe A, Swoboda W, Zabel B, Superti-Furga A, Steinmann B, Brunner HG, Jans A, Boles RG, Adkins W, van den Boogaard MJ, Olsen BR, Warman ML 1996 Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. Am J Hum Genet **59:**146–151.
9. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege

B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML 2001 LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell **107:**513–523.

10. Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP 2002 High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med **346:**1513–1521.
11. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR, Johnson ML 2002 A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet **70:**11–19.
12. Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Benichou O, Scopelliti D, Key L, Renton T, Bartels C, Gong Y, Warman ML, De Vernejoul MC, Bollerslev J, Van Hul W 2003 Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. Am J Hum Genet **72:**763–771.
13. Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass DA II, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G, Chan L 2002 Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol **157:**303–314.
14. Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Bouxsein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA, Bex F 2003 High bone mass in mice expressing a mutant LRP5 gene. J Bone Miner Res **18:**960–974.
15. Krishnan V, Bryant HU, Macdougald OA 2006 Regulation of bone mass by Wnt signaling. J Clin Invest **116:**1202–1209.
16. Balemans W, Van Hul W 2007 The genetics of low-density lipoprotein receptor-related protein 5 in bone: A story of extremes. Endocrinology **148:**2622–2629.
17. Xiong DH, Lei SF, Yang F, Wang L, Peng YM, Wang W, Recker RR, Deng HW 2007 Low-density lipoprotein receptor-related protein 5 (LRP5) gene polymorphisms are associated with bone mass in both Chinese and whites. J Bone Miner Res **22:**385–393.
18. Kiel DP, Demissie S, Dupuis J, Lunetta KL, Murabito JM, Karasik D 2007 Genome-wide association with bone mass and geometry in the Framingham Heart Study. BMC Med Genet **8**(Suppl 1)**:**S14.
19. Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES 2001 High-resolution haplotype structure in the human genome. Nat Genet **29:**229–232.
20. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D 2002 The structure of haplotype blocks in the human genome. Science **296:**2225–2229.
21. Patil N, Berno AJ, Hinds DA, Barrett WA, Doshi JM, Hacker CR, Kautzer CR, Lee DH, Marjoribanks C, McDonough DP, Nguyen BT, Norris MC, Sheehan JB, Shen N, Stern D, Stokowski RP, Thomas DJ, Trulson MO, Vyas KR, Frazer KA, Fodor SP, Cox DR 2001 Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. Science **294:**1719–1723.
22. Bustamante M, Nogues X, Agueda L, Jurado S, Wesselius A, Caceres E, Carreras R, Ciria M, Mellibovsky L, Balcells S, Diez-Perez A, Grinberg D 2007 Promoter 2 -1025 T/C polymorphism in the RUNX2 gene is associated with femoral neck bmd in Spanish postmenopausal women. Calcif Tissue Int **81:**327–332.
23. Bustamante M, Nogues X, Mellibovsky L, Agueda L, Jurado S, Caceres E, Blanch J, Carreras R, Diez-Perez A, Grinberg D, Balcells S 2007 Polymorphisms in the interleukin-6 receptor gene are associated with bone mineral density and body mass index in Spanish postmenopausal women. Eur J Endocrinol **157:**677–684.
24. The International HapMap Consortium 2003 The International HapMap Project. Nature **426:**789–796.
25. Barrett JC, Fry B, Maller J, Daly MJ 2005 Haploview: Analysis and visualization of LD and haplotype maps. Bioinformatics **21:**263–265.
26. Wang N, Akey JM, Zhang K, Chakraborty R, Jin L 2002 Distribution of recombination crossovers and the origin of haplotype blocks: The interplay of population history, recombination, and mutation. Am J Hum Genet **71:**1227–1234.
27. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA 2004 Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am J Hum Genet **74:**106–120.
28. Koller DL, Ichikawa S, Johnson ML, Lai D, Xuei X, Edenberg HJ, Conneally PM, Hui SL, Johnston CC, Peacock M, Foroud T, Econs MJ 2005 Contribution of the LRP5 gene to normal variation in peak BMD in women. J Bone Miner Res **20:**75–80.
29. Miller SA, Dykes DD, Polesky HF 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res **16:**1215.
30. Tobler AR, Short S, Andersen MR, Paner TM, Briggs JC, Lambert SM, Wu PP, Wang Y, Spoonde AY, Koehler RT, Peyret N, Chen C, Broomer AJ, Ridzon DA, Zhou H, Hoo BS, Hayashibara KC, Leong LN, Ma CN, Rosenblum BB, Day JP, Ziegle JS, De La Vega FM, Rhodes MD, Hennessy KM, Wenz HM 2005 The SNPlex genotyping system: A flexible and scalable platform for SNP genotyping. J Biomol Tech **16:**398–406.
31. Stephens M, Smith NJ, Donnelly P 2001 A new statistical method for haplotype reconstruction from population data. Am J Hum Genet **68:**978–989.
32. Gauderman W, Morrison J 2006 QUANTO 1.1: A Computer Program for Power and Sample Size Calculations for Genetic-Epidemiology Studies. Available at http://hydra.usc.edu/gxe. Accessed October 2006.
33. Pritchard JK, Stephens M, Donnelly P 2000 Inference of population structure using multilocus genotype data. Genetics **155:**945–959.
34. Schreiber E, Matthias P, Muller MM, Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res **17:**6419.
35. Baron R, Rawadi G 2007 Wnt signaling and the regulation of bone mass. Curr Osteoporos Rep **5:**73–80.
36. van Meurs JBJ, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, Kiel DP, Langdahl BL, Lips P, Ljunggren O, Lorene R, Obermayer-Pietsch B, Ohlsson C, Pettersson U, Reid DM, Rousseau F, Scollen S, van Hul W, Agueda L, Åkesson K, Ferrari SL, Hallmans G, Hofman A, Husted LB, Kruk M, Kaptoge S, Karasik D, Karlsson MK, Lorentzon M, Masi L, McGuigan FEA, Mellström D, Mosekilde L, Nogues X, Pols HAP, Reeve J, Renner W, Rivadeneira F, van Schoor NM, Ioannidis JPA, Uitterlinden AG 2008 Large-scale analysis of association between polymorphisms in the LRP-5 and -6 genes and osteoporosis: The GENOMOS Study. JAMA **299:**1277–1290.
37. Kiel DP, Ferrari SL, Cupples LA, Karasik D, Manen D, Imamovic A, Herbert AG, Dupuis J 2007 Genetic variation at the low-density lipoprotein receptor-related protein 5 (LRP5) locus modulates Wnt signaling and the relationship of physical activity with bone mineral density in men. Bone **40:**587–596.
38. Xiong DH, Shen H, Zhao LJ, Xiao P, Yang TL, Guo Y, Wang W, Guo YF, Liu YJ, Recker RR, Deng HW 2006 Robust and comprehensive analysis of 20 osteoporosis candidate genes by very high-density single-nucleotide polymorphism screen

among 405 white nuclear families identified significant association and gene-gene interaction. J Bone Miner Res **21**:1678–1695.
39. Ezura Y, Nakajima T, Urano T, Sudo Y, Kajita M, Yoshida H, Suzuki T, Hosoi T, Inoue S, Shiraki M, Emi M 2007 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. Bone **40**:997–1005.
40. Cheung CL, Huang QY, Chan V, Kung AW 2007 Association of Low-Density Lipoprotein Receptor-Related Protein 5 (LRP5) Promoter SNP with Peak Bone Mineral Density in Chinese Women. Hum Hered **65**:232–239.
41. Sims AM, Shephard N, Carter K, Doan T, Dowling A, Duncan EL, Eisman J, Jones G, Nicholson G, Prince R, Seeman E, Thomas G, Wass JA, Brown MA 2007 Genetic Analyses in a Sample of Individuals With High or Low Bone Density Demonstrates Association With Multiple Wnt Pathway Genes. J Bone Miner Res **23**:499–506.
42. Twells RC, Mein CA, Phillips MS, Hess JF, Veijola R, Gilbey M, Bright M, Metzker M, Lie BA, Kingsnorth A, Gregory E, Nakagawa Y, Snook H, Wang WY, Masters J, Johnson G, Eaves I, Howson JM, Clayton D, Cordell HJ, Nutland S, Rance H, Carr P, Todd JA 2003 Haplotype structure, LD blocks, and uneven recombination within the LRP5 gene. Genome Res **13**:845–855.
43. Cheung CL, Huang QY, Chan V, Kung AW 2008 Association of low-density lipoprotein receptor-related protein 5 (LRP5) promoter SNP with peak bone mineral density in Chinese women. Hum Hered **65**:232–239.
44. Clark AG, Boerwinkle E, Hixson J, Sing CF 2005 Determinants of the success of whole-genome association testing. Genome Res **15**:1463–1467.
45. Nyholt DR 2004 A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet **74**:765–769.
46. Storey JD, Tibshirani R 2003 Statistical significance for genomewide studies. Proc Natl Acad Sci USA **100**:9440–9445.

Address reprint requests to:
Susana Balcells, PhD
Departament de Genètica
Universitat de Barcelona
Av. Diagonal 645
E-08028 Barcelona, Spain
E-mail: sbalcells@ub.edu

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