ORIGINAL ARTICLE

Multiple Genetic Loci for Bone Mineral Density and Fractures

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ABSTRACT

BACKGROUND

Bone mineral density influences the risk of osteoporosis later in life and is useful in the evaluation of the risk of fracture. We aimed to identify sequence variants associated with bone mineral density and fracture.

METHODS

We performed a quantitative trait analysis of data from 5861 Icelandic subjects (the discovery set), testing for an association between 301,019 single-nucleotide polymorphisms (SNPs) and bone mineral density of the hip and lumbar spine. We then tested for an association between 74 SNPs (most of which were implicated in the discovery set) at 32 loci in replication sets of Icelandic, Danish, and Australian subjects (4165, 2269, and 1491 subjects, respectively).

RESULTS

Sequence variants in five genomic regions were significantly associated with bone mineral density in the discovery set and were confirmed in the replication sets (combined P values, 1.2×10^{-7} to 2.0×10^{-21}). Three regions are close to or within genes previously shown to be important to the biologic characteristics of bone: the receptor activator of nuclear factor-κB ligand gene (*RANKL*) (chromosomal location, 13q14), the osteoprotegerin gene (*OPG*) (8q24), and the estrogen receptor 1 gene (*ESR1*) (6q25). The two other regions are close to the zinc finger and BTB domain containing 40 gene (*ZBTB40*) (1p36) and the major histocompatibility complex region (6p21). The 1p36, 8q24, and 6p21 loci were also associated with osteoporotic fractures, as were loci at 18q21, close to the receptor activator of the nuclear factor-κB gene (*RANK*), and loci at 2p16 and 11p11.

CONCLUSIONS

We have discovered common sequence variants that are consistently associated with bone mineral density and with low-trauma fractures in three populations of European descent. Although these variants alone are not clinically useful in the prediction of risk to the individual person, they provide insight into the biochemical pathways underlying osteoporosis.

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Osteoporosis confers substantive morbidity and mortality and associated costs and predisposes people to fragility fractures at the hip, spine, forearm, or other skeletal sites.[1] It is a common disease affecting both sexes in populations of various ancestries, although elderly women of European descent are at the highest risk.[2] Bone density is the single best predictor of osteoporotic fractures and is a valuable tool in evaluation of the risk of fractures.[3,4] There is abundant evidence for a genetic contribution to variation in bone mineral density, with heritability estimates between 0.6 and 0.8.[5] Bone mineral density is also influenced by environmental and medical factors. Numerous candidate genes have been tested for their association with bone mineral density and with osteoporotic fractures, yielding varying results.[6,7] Several linkage analyses involving bone mineral density as a quantitative trait have also yielded varying results.[8] A genomewide association study, a test for association between hundreds of thousands of single-nucleotide polymorphisms (SNPs) and a specific phenotype, provides an opportunity to discover genes that contribute to bone mineral density.

METHODS

STUDY POPULATIONS

We studied four populations: a discovery set of 5861 Icelandic persons, 87% of whom are women, and three replication sets — 4165 other Icelandic persons (74% of whom are women), 2269 postmenopausal Danish women,[9,10] and the Australian Dubbo Osteoporosis Epidemiology Study (DOES) cohort[11] of 1491 persons (61% of whom are women). Dual-energy x-ray absorptiometry measurements at the lumbar spine and at the hip (a total hip or femoral neck measurement) were obtained in all patients. Standardized bone mineral density was calculated and corrected for sex, age, and weight (see the Methods section in Supplementary Appendix 1, available with the full text of this article at www.nejm.org). For example, a standardized value of X corresponds to X standard deviations above or below the population average after adjustment for sex, age, and weight. Most low-impact fractures were self-reported by persons in the Icelandic sets; reports of hip fractures in these sets were obtained from hospital records. All fractures in the Danish set were self-reported, and all fractures in the Australian cohort were ascertained through review of radiography reports. Further description of characteristics of the study populations is provided in the Methods section and Table 1 of Supplementary Appendix 1.

All participants provided written informed consent. The study was approved by the Data Protection Commission of Iceland, the National Bioethics Committee of Iceland, the Ethical Committee of Copenhagen County (in Denmark), and the St. Vincent's Ethics Review Committee (in Australia).

The study was funded by deCODE Genetics, which holds the data; the authors in Denmark and Australia also hold the data for those sets. Seven industry authors and one academic author designed the study, six industry authors analyzed the data, and six prepared the manuscript. Six industry authors and two academic authors vouch for the completeness and accuracy of the data.

GENOTYPING

We used either the Infinium HumanHap300 or the HumanCNV370 SNP chip (Illumina) to genotype a total of 317,503 SNPs in DNA samples from 5861 persons; 5858 had measurements of spine bone mineral density and 5715 had measurements of hip bone mineral density. Subsequent analysis was restricted to 301,019 SNPs that were of sufficient quality (see the Methods section in Supplementary Appendix 1). We genotyped SNPs in the replication sets at deCODE Genetics, using the Centaurus platform (Nanogen).[12]

ASSOCIATION ANALYSES

Age- and weight-adjusted standardized values of bone mineral density were computed for each sex and population separately (see the Methods section in Supplementary Appendix 1). For each SNP, a linear regression analysis, with the genotype as an additive covariate and standardized bone mineral density as the response variable, was fitted to test for association. Each SNP was tested separately for its association with bone mineral density of the hip and for its association with bone mineral density of the lumbar spine. The method of genomic control[13] was used to adjust the P values and standard errors for the relatedness of Icelandic subjects in the discovery set. When the discovery set was combined with the Icelandic follow-up set, the effect of relatedness was estimated by simulating the founder gene types through the Icelandic genealogy (see the Methods section in Supplementary Appendix 1). The threshold for genomewide significance was set at a P value of less than 1.7×10^{-7} ($0.05 \div 301{,}019$ SNPs) for each

phenotype and the threshold for replication was set at a P value of less than 0.05 in the replication sets.

To test for association between implicated SNPs and osteoporotic fractures, odds ratios for any low-trauma osteoporotic fracture among case patients as compared with controls were computed. A standard likelihood ratio statistic was used to calculate two-sided P values (see the Methods section in Supplementary Appendix 1). Results from multiple groups of case subjects with any low-impact osteoporotic fracture and controls were combined with the use of a Mantel–Haenszel model.[14] P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

GENOMEWIDE ASSOCIATION ANALYSIS

We observed a substantial excess of SNPs associated with bone mineral density, especially with spine bone mineral density, even after adjustment for relatedness of the subjects and for possible stratification by means of genomic control (adjustment factor for chi-square statistics, $\lambda = 1.18$ for the hip and $\lambda = 1.22$ for the spine) (Fig. 1). A total of 77 SNPs (which capture at least 48 independent association signals) were significantly associated with spine bone mineral density, with adjusted P values of less than 0.0001 (adjusted chi-square statistics >15.14); only 30.1 SNPs were expected to satisfy this criterion on the basis of chance alone. These results indicate that a substantial fraction of the most strongly associated SNPs could have true associations with bone mineral density. The 50 SNPs most strongly associated with each phenotype, ranked according to P value, are listed in Table 2 in Supplementary Appendix 1; the rankings of all SNPs tested are provided in Supplementary Appendixes 2 and 3.

Three SNPs — rs9594759 (located on 13q14) and rs2504063 and rs851982 (on 6q25) — achieved genomewide significance ($P<1.7\times10^{-7}$) for spine bone mineral density, whereas no SNP reached genomewide significance for hip bone mineral density (Table 2 in Supplementary Appendix 1). We then genotyped these SNPs in the three replication sets, in addition to SNPs with P values less than 3.0×10^{-5} for either skeletal site and a few SNPs with weaker associations that clustered around interesting candidate genes. In all, we genotyped 74 SNPs representing 32 chromosomal loci (Table 3 in Supplementary Appendix 1).

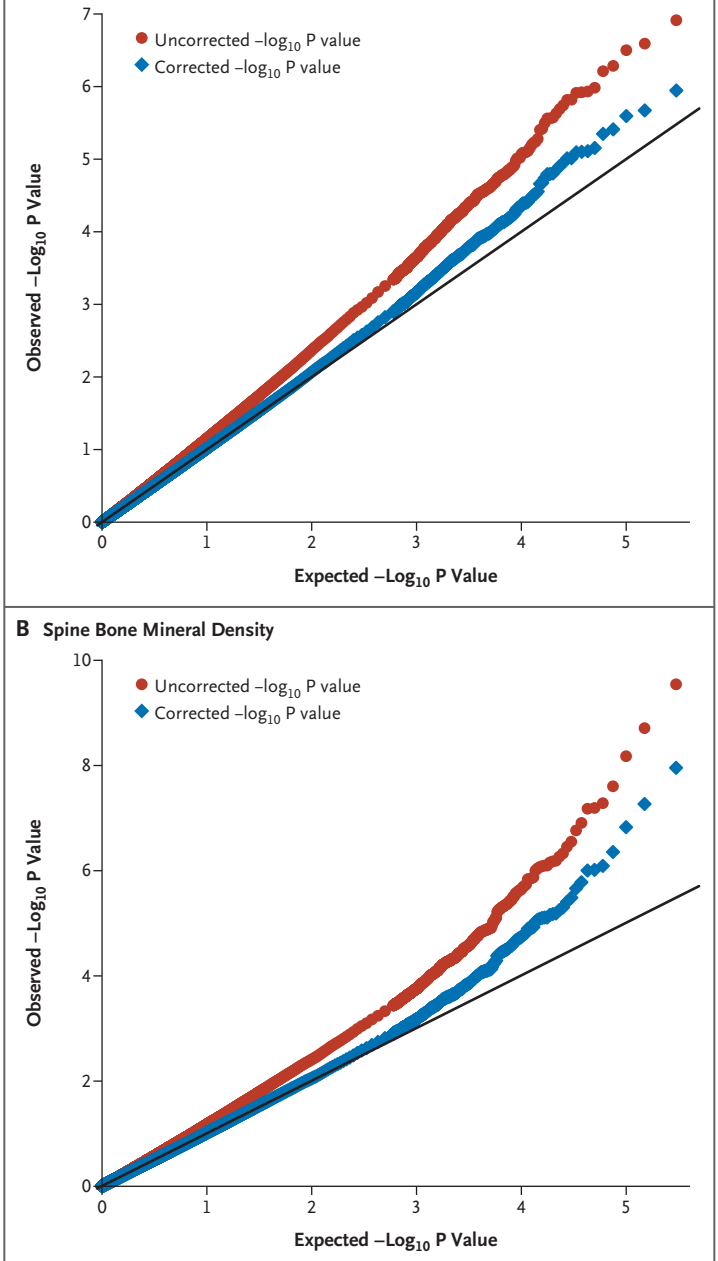

Figure 1. Quantile–Quantile Plots of Test Statistics (P Values) for the Associations of Single-Nucleotide Polymorphisms with Bone Mineral Density.

Panel A shows data for hip bone mineral density; Panel B shows data for spine bone mineral density. The P values were corrected by means of the genomic control method (adjustment factor for chi-square statistics, $\lambda = 1.18$ for hip bone mineral density and $\lambda = 1.22$ for spine bone mineral density). Under the null hypothesis of no association at any locus, the data points would be expected to follow the black line.

Twelve SNPs were both nominally significant (P<0.05) in the Danish and Australian sets combined and had genomewide significance

Table 1. Estimated Effects on Bone Mineral Density of the Spine of Single-Nucleotide Polymorphisms (SNPs) at the Five Regions with Genomewide Significance for Either the Spine or the Hip.*

Allele and SNP	SNP Frequency in Icelandic Discovery Set	Icelandic Discovery Set (N=5858)	P Value	Icelandic Replication Set (N=4158)	P Value	P Value for both Icelandic Sets (N=10,023)
		effect (95% CI)		*effect (95% CI)*		
1p36						
A, rs7524102	0.817	−0.08 (−0.13 to −0.02)	0.009	−0.19 (−0.27 to −0.11)	1.8×10^{-6}	1.8×10^{-7}
G, rs6696981	0.858	−0.11 (−0.17 to −0.04)	0.001	−0.18 (−0.27 to −0.10)	4.0×10^{-5}	2.9×10^{-7}
6p21 (MHC)						
T, rs3130340	0.787	−0.10 (−0.16 to −0.05)	2.2×10^{-4}	−0.11 (−0.18 to −0.03)	0.006	1.4×10^{-5}
6q25 (*ESR1*)						
C, rs9479055	0.348	−0.08 (−0.13 to −0.03)	5.3×10^{-4}	−0.08 (−0.15 to −0.02)	0.01	2.3×10^{-5}
T, rs4870044	0.281	−0.11 (−0.16 to −0.07)	4.1×10^{-6}	−0.12 (−0.19 to −0.05)	4.1×10^{-4}	8.1×10^{-9}
G, rs1038304	0.469	−0.10 (−0.14 to −0.06)	8.3×10^{-6}	−0.15 (−0.21 to −0.09)	2.0×10^{-6}	6.7×10^{-11}
A, rs6929137	0.298	−0.10 (−0.15 to −0.05)	3.0×10^{-5}	−0.14 (−0.20 to −0.07)	6.3×10^{-5}	6.5×10^{-9}
C, rs1999805	0.437	−0.11 (−0.15 to −0.06)	2.3×10^{-6}	−0.08 (−0.14 to −0.02)	0.01	3.8×10^{-7}
8q24 (*OPG*)						
C, rs6993813	0.496	−0.10 (−0.14 to −0.06)	8.5×10^{-6}	−0.18 (−0.24 to −0.12)	7.8×10^{-9}	1.7×10^{-13}
A, rs6469804	0.510	−0.09 (−0.14 to −0.05)	3.6×10^{-5}	−0.20 (−0.26 to −0.13)	4.1×10^{-10}	3.2×10^{-13}
13q14 (*RANKL*)						
T, rs9594738	0.562	−0.15 (−0.22 to −0.08)	1.4×10^{-5}	−0.19 (−0.25 to −0.13)	5.5×10^{-10}	1.5×10^{-14}
T, rs9594759	0.624	−0.13 (−0.18 to −0.09)	1.2×10^{-8}	−0.12 (−0.19 to −0.06)	1.5×10^{-4}	2.2×10^{-12}

* The estimated effects are expressed as standardized values (standard deviations above or below the population average) per copy of the SNP allele. All P values are two-sided and were corrected for the relatedness within the Icelandic populations. The SNP frequency is that in the Icelandic discovery set, except for rs9594738, for which the frequency is given for the Icelandic replication set. Similar allele frequencies were observed in all populations. *ESR1* denotes the estrogen receptor 1 gene, MHC the major-histocompatibility-complex region, *OPG* the osteoprotegerin gene, and *RANKL* the receptor activator of nuclear factor-κB ligand gene.

($P<1.7\times10^{-7}$) for at least one phenotype when all sets (including the Icelandic discovery set) were combined. Eleven SNPs from five chromosomal regions — 1p36, 6p21, 6q25, 8q24 and 13q14 — were associated with spine bone mineral density, meeting the criteria of nominal significance in the Danish and Australian sets combined and genomewide significance in all sets combined (Table 1). On the basis of these same criteria, six SNPs from three chromosomal regions (1p36, 6q25, and 8q24) were associated with hip bone mineral density (Table 2). These three loci were also associated with spine bone mineral density.

We estimated that the alleles associated with low bone mineral density resulted in a decrease of 0.05 to 0.17 standardized unit. We did not observe sex-specific associations at any of the five loci, apart from 8q24, where a significantly stronger effect was observed in men than in women ($P=0.03$ to 0.050) (Table 4 in Supplementary Appendix 1).

THE 13q14 (*RANKL*), 8q24 (*OPG*), AND 18q21 (*RANK*) REGIONS

The SNP most strongly associated with spine bone mineral density in the discovery set, rs9594759 on 13q14, is located 113 kb upstream of the receptor activator of nuclear factor-κB ligand gene (*RANKL*) and in a linkage-disequilibrium block adjacent to that gene (Fig. 1A in Supplementary Appendix 1). The association between rs9594759 and spine bone mineral density was consistent in each replication set. Another SNP, rs9594738, selected on the basis of HapMap data, showed the strongest association with spine bone mineral density when genotyped in the replication sets (combined $P=2.0\times10^{-21}$) (Table 1). We estimate that the T allele of rs9594738 lowers spine bone mineral density by 0.17 standardized value per

Danish Replication Set (N=2260)	Australian Replication Set (N=1490)	P Value for Danish and Australian Sets	All Sets Combined	P Value
effect (95% CI)			*effect (95% CI)*	
–0.12 (–0.21 to –0.02)	–0.04 (–0.14 to 0.07)	0.01	–0.11 (–0.15 to –0.07)	9.2×10^{-9}
–0.11 (–0.22 to 0.00)	–0.01 (–0.14 to 0.12)	0.08	–0.12 (–0.16 to –0.07)	1.7×10^{-7}
–0.12 (–0.20 to –0.04)	–0.04 (–0.14 to 0.05)	0.002	–0.10 (–0.13 to –0.06)	1.2×10^{-7}
–0.06 (–0.13 to 0.01)	–0.07 (–0.16 to 0.01)	0.009	–0.08 (–0.11 to –0.05)	6.2×10^{-7}
–0.11 (–0.19 to –0.03)	–0.07 (–0.16 to 0.02)	4.8×10^{-4}	–0.11 (–0.14 to –0.08)	1.6×10^{-11}
–0.05 (–0.12 to 0.02)	–0.06 (–0.14 to 0.03)	0.04	–0.10 (–0.13 to –0.07)	4.0×10^{-11}
–0.08 (–0.15 to 0.00)	–0.06 (–0.14 to 0.03)	0.007	–0.10 (–0.13 to –0.07)	2.5×10^{-10}
–0.06 (–0.13 to 0.01)	–0.07 (–0.16 to 0.02)	0.02	–0.09 (–0.12 to –0.06)	2.2×10^{-8}
–0.08 (–0.14 to –0.01)	–0.05 (–0.14 to 0.03)	0.006	–0.12 (–0.15 to –0.09)	1.8×10^{-14}
–0.07 (–0.14 to 0.00)	–0.08 (–0.16 to 0.00)	0.002	–0.12 (–0.15 to –0.09)	7.4×10^{-15}
–0.18 (–0.25 to –0.11)	–0.09 (–0.17 to 0.00)	1.8×10^{-8}	–0.17 (–0.21 to –0.14)	2.0×10^{-21}
–0.14 (–0.21 to –0.07)	–0.07 (–0.15 to 0.01)	9.7×10^{-6}	–0.13 (–0.16 to –0.10)	1.1×10^{-16}

allele. With respect to its association with hip bone mineral density, this SNP reached genomewide significance in the combined Icelandic samples (Table 2) and had a nominally significant association in the Danish set but not in the Australian set.

Two correlated SNPs on 8q24, rs6469804 and rs6993813, reached genomewide significance for both spine and hip bone mineral density and were significantly associated in the replication sets (Tables 1 and 2). These two SNPs are in strong linkage disequilibrium (pairwise coefficient of determination [r^2]=0.94) and are in a linkage-disequilibrium block including the osteoprotegerin gene (*OPG*) (Fig. 1B in Supplementary Appendix 1). This gene has been tested for association with bone mineral density and osteoporosis, with inconsistent results.[15-25] The two most studied SNPs in this gene, G1181→C (rs2073618) and T950→C (rs2073617), are in linkage disequilibrium with both rs6469804 (r^2=0.57 and r^2=0.77, respectively) and rs6993813 (r^2=0.44 and r^2=0.59, respectively) in the HapMap–CEU data set (consisting of Centre d'Etude du Polymorphisme Humain [CEPH] subjects who are Utah residents of northern and western European ancestry). Our results are consistent with those from previous studies reporting associations with bone mineral density.[16,18,22-24]

RANKL and *OPG* encode known regulators of bone remodeling which, together with the receptor activator of the nuclear factor-κB protein RANK, are central to osteoclastogenesis and activation of bone resorption. A SNP (rs3018362) located 27 kb downstream of *RANK* and within the same linkage disequilibrium block, on 18q21 (Fig. 1C in Supplementary Appendix 1), showed a consistent association with hip bone mineral density, although the association did not meet genomewide significance (Table 3). We did not find significant interaction between the associated SNPs at these three genes, despite their close involvement at the protein level (see the Methods section in Supplementary Appendix 1).

THE 6q25 *ESR1* REGION

The genomewide association study uncovered a complex pattern of association in the 6q25 region. SNPs in this region showed an association with bone mineral density of both the hip and spine (Tables 1 and 2, and Table 3 in Supplementary Ap-

Table 2. Estimated Effects on Bone Mineral Density of the Hip of Single-Nucleotide Polymorphisms (SNPs) at the Five Regions with Genomewide Significance for Either the Spine or the Hip.*

Allele and SNP	SNP Frequency in Icelandic Discovery Set	Icelandic Discovery Set (N=5715)	P Value	Icelandic Replication Set (N=4165)	P Value	P Value for both Icelandic Sets (N=9880)
		effect (95% CI)		*effect (95% CI)*		
1p36						
A, rs7524102	0.817	−0.13 (−0.18 to −0.08)	2.4×10^{-6}	−0.18 (−0.25 to −0.11)	6.1×10^{-7}	7.5×10^{-12}
G, rs6696981	0.858	−0.15 (−0.21 to −0.09)	1.3×10^{-6}	−0.17 (−0.25 to −0.09)	2.6×10^{-5}	3.0×10^{-10}
6p21 (MHC)						
T, rs3130340	0.787	−0.04 (−0.09 to 0.01)	0.13	−0.09 (−0.15 to −0.02)	0.02	0.01
6q25 (*ESR1*)						
C, rs9479055	0.348	−0.07 (−0.12 to −0.03)	7.0×10^{-4}	−0.10 (−0.16 to −0.04)	7.1×10^{-4}	1.7×10^{-6}
T, rs4870044	0.281	−0.09 (−0.14 to −0.05)	9.9×10^{-5}	−0.07 (−0.14 to −0.01)	0.02	1.4×10^{-5}
G, rs1038304	0.469	−0.09 (−0.13 to −0.05)	1.8×10^{-5}	−0.13 (−0.18 to −0.07)	1.2×10^{-5}	6.7×10^{-10}
A, rs6929137	0.298	−0.10 (−0.14 to −0.05)	1.4×10^{-5}	−0.11 (−0.17 to −0.05)	0.0004	3.1×10^{-8}
C, rs1999805	0.437	−0.07 (−0.11 to −0.02)	0.002	−0.08 (−0.14 to −0.02)	0.005	4.8×10^{-5}
8q24 (*OPG*)						
C, rs6993813	0.496	−0.04 (−0.09 to 0.00)	0.03	−0.15 (−0.21 to −0.09)	1.6×10^{-7}	2.1×10^{-8}
A, rs6469804	0.510	−0.04 (−0.08 to 0.00)	0.04	−0.17 (−0.23 to −0.11)	3.2×10^{-9}	2.0×10^{-7}
13q14 (*RANKL*)						
T, rs9594738	0.562	−0.08 (−0.13 to −0.04)	2.7×10^{-4}	−0.14 (−0.19 to −0.08)	2.4×10^{-6}	8.2×10^{-9}
T, rs9594759	0.624	−0.08 (−0.12 to −0.03)	4.5×10^{-4}	−0.08 (−0.14 to −0.02)	0.005	1.6×10^{-6}

* The estimated effects are expressed as standardized values (standard deviations above or below the population average) per copy of the SNP allele. All P values are two-sided and were corrected for the relatedness within the Icelandic populations. The SNP frequency is that in the Icelandic discovery set, except for rs9594738, for which the frequency is given for the Icelandic replication set. Similar allele frequencies were observed in all populations. *ESR1* denotes the estrogen receptor 1 gene, MHC the major histocompatibility-complex-region, *OPG* the osteoprotegerin gene, and *RANKL* the receptor activator of nuclear factor-κB ligand gene.

pendix 1), and many had modest or minimal linkage disequilibrium (with respect to one another). None of these SNPs could fully account for the associations. The SNPs spanned 273 kb and were distributed over several linkage-disequilibrium blocks (Fig. 1D in Supplementary Appendix 1). Neither of the two significantly associated SNPs identified in the genomewide association study, rs2504063 and rs851982, showed associations with bone mineral density in the non-Icelandic replication sets. In total, associations between eight SNPs and spine bone mineral density were consistently replicated (Table 3A in Supplementary Appendix 1), with four (rs4870044, rs1038304, rs6929137, and rs1999805) reaching genomewide significance in the combined analysis (Table 1). Five of the eight SNPs were also associated with hip bone mineral density; two of these five, rs9479055 and rs4870044, reached nominal significance in the Danish and Australian replication sets and genomewide significance in the combined analysis (Table 2). The five SNPs have pairwise r^2 values ranging from 0.001 to 0.51 and are dispersed across three linkage-disequilibrium blocks (Fig. 1B in Supplementary Appendix 1).

No single SNP could fully explain the association with 6q25; at least three SNPs (e.g., rs4870044, rs1038304, and rs1999805) seemed to be required to account for the overall association (see the Methods section in Supplementary Appendix 1). The SNP rs1999805 is within an intron of the U68068 splice variant of the estrogen receptor 1 gene (*ESR1*). The SNPs rs4870044 and rs1038304 are located 44 kb to 75 kb upstream of the gene, possibly affecting its regulation of transcription. However, these two SNPs lie within *C6orf97*, the chromosome 6 open reading frame 97 gene of unknown function, now a candidate

Danish Replication Set (N=2260)	Australian Replication Set (N=1490)	P Value for Danish and Australian Sets	All Sets Combined	P Value
effect (95% CI)			*effect (95% CI)*	
–0.14 (–0.23 to –0.04)	–0.15 (–0.25 to –0.04)	1.4×10^{-5}	–0.15 (–0.19 to –0.11)	5.0×10^{-16}
–0.12 (–0.23 to –0.02)	–0.10 (–0.23 to 0.02)	0.002	–0.14 (–0.19 to –0.10)	3.8×10^{-12}
–0.06 (–0.14 to 0.02)	0.01 (–0.09 to 0.11)	0.30	–0.05 (–0.08 to –0.01)	0.007
–0.06 (–0.13 to 0.01)	–0.08 (–0.17 to 0.00)	0.005	–0.08 (–0.11 to –0.05)	3.1×10^{-8}
–0.11 (–0.19 to –0.04)	–0.03 (–0.11 to 0.06)	0.003	–0.08 (–0.11 to –0.05)	1.6×10^{-7}
–0.04 (–0.11 to 0.02)	–0.01 (–0.09 to 0.07)	0.22	–0.08 (–0.11 to –0.06)	5.3×10^{-9}
–0.04 (–0.11 to 0.03)	–0.02 (–0.11 to 0.06)	0.20	–0.08 (–0.11 to –0.05)	1.0×10^{-7}
–0.01 (–0.08 to 0.06)	–0.03 (–0.12 to 0.06)	0.45	–0.06 (–0.09 to –0.03)	1.2×10^{-4}
–0.09 (–0.15 to –0.02)	–0.08 (–0.17 to 0.00)	3.9×10^{-4}	–0.09 (–0.12 to –0.07)	3.3×10^{-11}
–0.06 (–0.12 to 0.00)	–0.08 (–0.16 to 0.00)	0.003	–0.08 (–0.11 to –0.06)	2.5×10^{-9}
–0.08 (–0.15 to –0.01)	0.00 (–0.10 to 0.11)	0.06	–0.10 (–0.13 to –0.06)	1.9×10^{-8}
–0.07 (–0.14 to –0.01)	0.03 (–0.05 to 0.11)	0.18	–0.07 (–0.10 to –0.04)	2.1×10^{-6}

gene for osteoporosis (Fig. 1D in Supplementary Appendix 1).

THE 1p36 REGION

Two SNPs in linkage disequilibrium on chromosome 1p36, rs7524102 and rs6696981 ($r^2=0.73$), showed an association with both hip bone mineral density and spine bone mineral density (Tables 1 and 2). The marker showing the stronger association, rs7524102, was significant in all study populations, with similar effects on hip bone mineral density among all populations. These SNPs are located in a linkage-disequilibrium block that does not contain any known gene (Fig. 1E in Supplementary Appendix 1). The closest gene is the zinc finger and BTB domain containing 40 gene (*ZBTB40*), located in an adjacent linkage-disequilibrium block, 80 kb downstream from the signal. This gene, of unknown function, is expressed in bone (UniGene accession number, Hs.418966), indicating a potentially unidentified role in the biologic characteristics of bone. Farther from the signal, 250 kb in the opposite direction, lies the wingless-type MMTV integration site family member 4 gene (*WNT4*).

THE 6p21 REGION

One SNP, rs3130340, in the major histocompatibility complex region was associated with spine bone mineral density in the replication sets and in the combined overall analysis (Table 1). This SNP is located downstream of the uncharacterized chromosome 6 open reading frame 10 gene *C6orf10* (Fig. 1F in Supplementary Appendix 1). This association is weaker than those of SNPs at the other loci.

RISK OF OSTEOPOROTIC FRACTURE

We tested for associations between low-trauma fracture and the SNPs associated with bone mineral density (Table 4). The 1p36 locus was the most strongly associated with fractures. It was significantly associated with not only the broadly defined group of fractures of any kind but also individually with forearm fractures, fractures of the hip, and vertebral fractures (Table 4, and Table 5 in Supplementary Appendix 1). The 8q24 locus also was associated with all categories of fracture, albeit not as strongly as 1p36. The 6p21 MHC locus was significantly associated with the broadly defined group of fractures as well as with

Table 3. Estimated Effects on Bone Mineral Density of the Spine or the Hip of SNPs at Three Regions with Suggested Associations with Bone Mineral Density and Fracture.*

Region and Allele and SNP	Frequency	Icelandic Discovery and Replication Sets (N = 10,026)	P Value	Danish Replication Set (N = 2260)	Australian Replication Set (N = 1490)	P Value for Danish and Australian Sets	All Sets Combined	P Value
		effect (95% CI)		*effect (95% CI)*			*effect (95% CI)*	
Spine bone mineral density								
2p16								
G, rs11898505	0.674	–0.10 (–0.14 to –0.06)	2.0×10^{-7}	–0.02 (–0.09 to 0.06)	–0.04 (–0.13 to 0.04)	0.28	–0.08 (–0.11 to –0.05)	8.4×10^{-7}
Hip bone mineral density								
18q21 (*RANK*)								
A, rs3018362	0.354	–0.08 (–0.11 to –0.04)	2.4×10^{-5}	–0.08 (–0.15 to –0.01)	–0.03 (–0.12 to 0.05)	0.01	–0.07 (–0.10 to –0.04)	9.9×10^{-7}
11p11 (*LRP4*)								
G, rs2306033	0.854	–0.10 (–0.15 to –0.05)	9.2×10^{-5}	–0.04 (–0.13 to 0.06)	–0.09 (–0.21 to 0.03)	0.11	–0.08 (–0.12 to –0.04)	3.4×10^{-5}
G, rs7935346	0.769	–0.06 (–0.10 to –0.02)	0.003	–0.06 (–0.14 to 0.02)	–0.06 (–0.16 to 0.04)	0.04	–0.06 (–0.10 to –0.03)	2.6×10^{-4}

* The estimated effects are expressed as standardized values (standard deviations above or below the population average) per copy of the SNP allele. All P values are two-sided and were corrected for the relatedness within the Icelandic populations. The allelic SNP frequency is that in the Icelandic discovery set. *LRP4* denotes the gene encoding the low-density lipoprotein receptor–related protein 4, and *RANK* the receptor activator of the nuclear factor-κB gene.

hip and forearm fractures individually (Table 5 in Supplementary Appendix 1). The 18q21 *RANK* locus was modestly associated with fractures; the 6q25 *ESR1* and 13q14 *RANKL* loci were not.

Some loci that did not reach genomewide significance in our combined analysis of association with bone mineral density did show evidence of an association with osteoporotic fractures (Tables 3 and 4). These are rs11898505, in the spectrin β nonerythrocytic 1 gene (*SPTBN1*) on chromosome 2p16, which was associated with fractures in all sample sets, most strongly with vertebral fractures (Table 5 and Fig. 1G in Supplementary Appendix 1), and SNPs within and close to the gene encoding the low-density lipoprotein receptor–related protein 4 (*LRP4*) on chromosome 11p11 (Tables 3 and 5 and Fig. 1H in Supplementary Appendix 1).

OSTEOPOROSIS CANDIDATE GENES

Numerous candidate genes have been studied for association with bone mineral density and with osteoporotic fractures.[26,27] We looked at the ranking, in our genomewide association study data set, of 77 candidate genes previously reported to be associated with bone mineral density or fractures and their neighboring regions (Table 6 in Supplementary Appendix 1). Apart from *ESR1*, *RANKL*, and *RANK*, only two of these genes had two or more markers in the list of the 500 SNPs most significantly associated with either hip or spine bone mineral density: the sclerosteosis gene (*SOST*) and the nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) gene (*NR3C1*). SNPs in and around the more intensively studied vitamin D (1,25-dihydroxyvitamin D3) receptor gene *VDR* and the low-density lipoprotein receptor–related protein 5 gene *LRP5* were among the 1000 SNPs most significantly associated with bone mineral density, whereas SNPs in the collagen type Iα1 gene *COL1A1* (which was also intensively studied) did not even feature in the top 5000 SNPs.

DISCUSSION

We report here the results from our large genomewide association study of bone mineral density: associations with five genomic regions were replicated consistently in three replication sets of subjects of European descent (Danish, Australian, and Icelandic subjects). The four loci 1p36 (*ZBTB40*–

Table 4. Association of Single-Nucleotide Polymorphisms (SNPs) with Any Low-Trauma Osteoporotic Fracture.*

Allele and SNP	Iceland				Danish Replication Set				Australian Replication Set				All Sets Combined	
	Frequency among Case Subjects (N=2986)	Frequency among Controls (N=35,400)	Odds Ratio	P Value	Frequency among Case Subjects (N=870)	Frequency among Controls (N=1040)	Odds Ratio	P Value	Frequency among Case Subjects (N=550)	Frequency among Controls (N=345)	Odds Ratio	P Value	Odds Ratio (95% CI)	P Value
1p36														
A, rs7524102	0.829	0.817	1.09	0.03	0.861	0.834	1.23	0.02	0.846	0.811	1.29	0.059	1.12 (1.05–1.20)	8.4×10^{-4}
G, rs6696981	0.872	0.858	1.13	0.004	0.902	0.879	1.27	0.02	0.897	0.880	1.18	0.28	1.15 (1.07–1.25)	2.4×10^{-4}
6p21 (MHC)														
T, rs3130340	0.800	0.786	1.09	0.02	0.782	0.761	1.13	0.12	0.775	0.777	0.98	0.89	1.09 (1.02–1.16)	0.008
6q25 (*ESR1*)														
C, rs9479055	0.355	0.348	1.03	0.29	0.390	0.356	1.16	0.03	0.413	0.406	1.03	0.77	1.05 (1.00–1.11)	0.06
T, rs4870044	0.281	0.281	1.00	1.00	0.277	0.252	1.14	0.08	0.302	0.300	1.01	0.95	1.02 (0.97–1.09)	0.14
G, rs1038304	0.472	0.469	1.01	0.72	0.511	0.476	1.15	0.03	0.524	0.485	1.17	0.12	1.04 (0.99–1.10)	0.11
A, rs6929137	0.299	0.298	1.00	0.90	0.323	0.288	1.18	0.02	0.361	0.317	1.22	0.054	1.05 (0.99–1.10)	0.12
C, rs1999805	0.440	0.437	1.01	0.72	0.413	0.401	1.05	0.47	0.471	0.434	1.16	0.17	1.03 (0.97–1.08)	0.35
8q24 (*OPG*)														
C, rs6993813	0.508	0.496	1.05	0.09	0.532	0.517	1.06	0.36	0.539	0.519	1.08	0.44	1.06 (1.00–1.11)	0.04
A, rs6469804	0.521	0.510	1.05	0.13	0.551	0.540	1.04	0.51	0.574	0.540	1.15	0.17	1.05 (1.00–1.11)	0.052
13q14 (*RANKL*)														
T, rs9594738	0.575	0.559	1.07	0.09	0.488	0.486	1.01	0.90	0.466	0.486	0.92	0.42	1.04 (0.98–1.11)	0.23
T, rs9594759	0.631	0.624	1.03	0.33	0.551	0.559	0.97	0.65	0.531	0.533	0.99	0.94	1.02 (0.97–1.07)	0.52
2p16														
G, rs11898505	0.693	0.673	1.10	0.004	0.663	0.635	1.13	0.08	0.663	0.616	1.22	0.054	1.11 (1.05–1.17)	1.8×10^{-4}
18q21 (*RANK*)														
A, rs3018362	0.365	0.353	1.06	0.08	0.382	0.342	1.18	0.01	0.349	0.324	1.12	0.29	1.08 (1.02–1.14)	0.005
11p11 (*LRP4*)														
G, rs2306033	0.865	0.854	1.10	0.03	0.874	0.860	1.13	0.21	0.877	0.859	1.17	0.31	1.11 (1.03–1.19)	0.007
G, rs7935346	0.776	0.768	1.05	0.21	0.783	0.756	1.16	0.053	0.792	0.750	1.27	0.055	1.08 (1.01–1.14)	0.02

* The odds ratios are for any low-trauma osteoporotic fracture among case patients as compared with controls. All P values are two-sided and were obtained with the use of the multiplicative model. For the Icelandic case–control group, the P values and odds ratios were adjusted for relatedness. For the combined group, we calculated odds ratios and P values using a Mantel–Haenszel model.[14] Frequencies are the allelic SNP frequencies. *ESR1* denotes the estrogen receptor 1 gene, *LRP4* the gene encoding the low-density lipoprotein receptor–related protein 4, *OPG* the osteoprotegerin gene, MHC the major histocompatibility complex region, *RANK* the receptor activator of the nuclear factor-κB gene, and *RANKL* the receptor activator of nuclear factor-κB ligand gene.

WNT4), 6q25 (*ESR1–C6orf97*), 8q24 (*OPG*), and 13q14 (*RANKL*) influence bone mineral density at both the spine and the hip, whereas the 6p21 locus is associated with spine bone mineral density only. We identified loci that warrant further investigation in additional samples. The 1p36, 6p21, and 8q24 loci had a significant association with low-trauma fractures. Three other loci for which genomewide significance for bone mineral density was not reached but that had an association with low-trauma fractures were 2p16 (the location of the *SPTBN1* gene), 11p11 (*LRP4*), and 18q21 (*RANK*). That some loci were found to show association with bone mineral density but not fracture may reflect differences in pathologic characteristics. However, other issues such as the power to detect an association with a specific type of fracture could also affect these results.

None of the loci we describe here were reported to have an association that reached genomewide significance with bone mineral density in the Framingham Heart Study, which is, to our knowledge, the only previous publication describing a genomewide association study of bone-related phenotypes.[28] Genes at three of the bone mineral density loci have been previously tested for association with bone mineral density or osteoporosis. Of these, *ESR1* is one of the most intensively studied candidate genes for osteoporosis.[29] A recent meta-analysis of more than 18,000 subjects indicated that the most intensively studied polymorphisms in *ESR1* had no effect on bone mineral density but were associated with a risk of fracture.[30]

The SNPs at the *ESR1* locus that we describe here have not been previously reported to be associated with bone mineral density, nor are they in substantive linkage disequilibrium with two of the most widely studied polymorphisms in *ESR1*: rs2234693 (T397→C) and rs9340799 (C351→G) (r^2, 0.001 to 0.09).[29,31] Moreover, the SNPs we have identified in and around *ESR1* are generally not in substantive linkage disequilibrium with one another, indicating the existence of more than one associated signal in this region and demonstrating a complex pattern of association, perhaps analogous to that observed between prostate cancer and variants in the 8q24 region.[32] Furthermore, some of the associated SNPs are not only within and upstream of *ESR1* but are within another uncharacterized gene, *C6orf97*. However, given the complexity of *ESR1* transcripts and tissue-specific regulation of its different messenger RNA variants,[33] it is possible that all associated SNPs in this region affect bone mineral density by modulating *ESR1* expression levels or function.

The *RANKL*, *RANK*, and *OPG* gene pathway has been extensively studied since the discovery of OPG[34,35] and the subsequent discovery that it interacts with RANKL[36-38] and RANK.[39] The critical involvement of this pathway in the cellular regulation of bone remodeling is evident. Furthermore, there are ongoing phase 3 clinical trials investigating the effect of a human anti-RANKL antibody (denosumab) on bone loss in patients with osteoporosis and other bone-related diseases.[40] Studies testing for association between markers at these genes and osteoporosis are few, and most of them are underpowered.[15-25] Hitherto, there has been no report of consistent association between markers in these genes and bone mineral density across different populations.

The variants listed in Tables 1 and 2 are estimated to account for approximately 3% of the total variation in hip and spine bone mineral density (calculated from the replication sets). This reinforces our belief that there are many more sequence variants relevant to osteoporosis to be identified. The variants that we have identified provide insights into the biologic pathways that influence osteoporosis. Some of them are common in the population and therefore have a greater influence on population attributable risk than would otherwise be the case. (Population attributable risk is the fraction of cases that would not occur in the population if the adverse effect of the at-risk variants could be eliminated.) For example, the odds ratio of any low-trauma fracture associated with the A allele of rs7524102 in the 1p36 region is modest (1.12) and by itself not clinically useful for purposes of prediction. Since it has a frequency of over 80% in the population, however, its population attributable risk is 17%. Follow-up studies performed with multiple and large sample sets are needed before the effect of these variants can be fully and accurately evaluated.

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