

## High-Density Association Study of 383 Candidate Genes for Volumetric BMD at the Femoral Neck and Lumbar Spine Among Older Men

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**ABSTRACT:** Genetics is a well-established but poorly understood determinant of BMD. Whereas some genetic variants may influence BMD throughout the body, others may be skeletal site specific. We initially screened for associations between 4608 tagging and potentially functional single nucleotide polymorphisms (SNPs) in 383 candidate genes and femoral neck and lumbar spine volumetric BMD (vBMD) measured from QCT scans among 862 community-dwelling white men  $\geq 65$  yr of age in the Osteoporotic Fractures in Men Study (MrOS). The most promising SNP associations ( $p < 0.01$ ) were validated by genotyping an additional 1156 white men from MrOS. This analysis identified 8 SNPs in 6 genes (*APC*, *DMPI*, *FGFR2*, *FLT1*, *HOXA*, and *PTN*) that were associated with femoral neck vBMD and 13 SNPs in 7 genes (*APC*, *BMPRI1*, *FOXC2*, *HOXA*, *IGFBP2*, *NFATC1*, and *SOST*) that were associated with lumbar spine vBMD in both genotyping samples ( $p < 0.05$ ). Although most associations were specific to one skeletal site, SNPs in the *APC* and *HOXA* gene regions were associated with both femoral neck and lumbar spine BMD. This analysis identifies several novel and robust genetic associations for volumetric BMD, and these findings in combination with other data suggest the presence of genetic loci for volumetric BMD that are at least to some extent skeletal-site specific.

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### INTRODUCTION

OSTEOPOROSIS, A CONDITION marked by low BMD and an increased risk of fracture, is a significant health burden in older individuals.<sup>(1)</sup> Hip and vertebral fractures are major osteoporotic fractures and occur in  $\sim 300,000$  and 750,000 individuals, respectively, each year.<sup>(2,3)</sup> Although osteoporosis is more common in women, it is also a substantial problem among older men. In contrast to our understanding of osteoporosis in women, considerably less is known about the etiology and prevention of osteoporosis in men.

Genetic factors have an established influence on BMD, with heritability estimates indicating that as much as 85% of the population variance in BMD is caused by genetic variants<sup>(4)</sup> and that some of these variants may be skeletal site and sex specific.<sup>(5)</sup> Identification of individual genetic variants by candidate gene association studies has had some success, but many studies have been limited because

they characterized a single candidate gene at a time, did not comprehensively investigate genetic variation for the candidate gene of interest, and did not validate findings in an independent sample. Further complicating the search for genetic factors contributing to BMD, many studies have had small sample sizes and/or had potential design flaws, such as failing to account for population stratification. Recently, genome-wide association studies have been completed to identify genetic variants that influence DXA measures of areal BMD.<sup>(6,7)</sup> Although these genome-wide studies have identified several genetic loci of interest, the loci identified explain very little of the variation in BMD.<sup>(8)</sup> Thus, much of the genetic variation in BMD remains to be explained. Furthermore, the majority of genetic studies have measured areal BMD by DXA, which is confounded by bone size and may be increased erroneously at the lumbar spine by spinal degenerative disease and aortic calcification.<sup>(9–13)</sup> Volumetric BMD (vBMD) measured by QCT avoids some of the limitations of DXA, but to date, little is known about the genetic determinants of vBMD.

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In this study, we assessed and subsequently validated the associations between common genetic variation in 383 biological candidate genes and vBMD of the femoral neck and lumbar spine among 2018 older white men in the Osteoporotic Fractures in Men Study (MrOS).

## MATERIALS AND METHODS

### *Study participants*

Participants for this study were selected from the MrOS. MrOS is a prospective, cohort study designed to investigate anthropometric, lifestyle, and medical factors related to bone health in older, community-dwelling men. At study entry, participants were at least 65 yr old, community dwelling, ambulatory, and had not had bilateral hip replacement.<sup>(14)</sup> In total, 5995 men were recruited from March 2000 through April 2002 primarily using population-based mailings in six geographic regions in the United States: Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR; and San Diego, CA.<sup>(15)</sup>

White participants with volumetric BMD (vBMD) were selected for genotyping in this study if they had not reported taking bone-altering medications such as androgens, anti-androgens, or oral corticosteroids and had not reported being on osteoporosis treatment. Medication use was defined as medications taken daily or almost daily for the last 30 days. Prescription medications recorded by the clinics were stored in an electronic medications inventory database, and each medication was matched to its ingredient(s) based on the Iowa Drug Information System (IDIS) Drug vocabulary.

Genotyping was completed in two phases using two independent samples from the MrOS cohort: a discovery sample and a validation sample. Specifically, the discovery sample was comprised of 862 white men with lumbar spine or femoral neck vBMD measures. These men were selected without regard to their BMD level from the Minneapolis and Pittsburgh clinic sites for genotyping. Promising SNPs identified in the discovery sample were tested for replication in 1156 additional men with vBMD measures in a validation sample that was comprised of men from the remainder of the MrOS clinic sites (Birmingham, Palo Alto, Portland, and San Diego).

### *vBMD*

vBMD was measured using QCT of the hip and lumbar spine. Because of cost restraints, only the first 65% of the MrOS cohort and all nonwhite participants were referred for QCT scans. There were few differences between the men who did and did not receive QCT scans except that those with QCT scans were slightly younger and more likely to be from a minority population.<sup>(16)</sup>

QCT measurement of the lumbar spine was obtained using an anatomical region 5 mm above the L<sub>1</sub> superior endplate to 5 mm below the L<sub>2</sub> inferior endplate and hip scans were obtained in the anatomical region defined by the femoral head to 3.5 cm below the lesser trochanter. Lumbar spine images were acquired using settings of 120

kVp, 150 mA, 1-mm slice thickness, and 512 × 512 matrices. The region of interest was the second lumbar vertebra excluding the transverse processes. Hip images were acquired at settings of 80 kVp, 280 mA, 3-mm slice thickness, and 512 × 512 matrix in spiral reconstruction mode. The femoral neck region was defined as the region from the minimum cross-sectional area to the point 25% toward the maximum cross-sectional area where cross-sectional area was measured along the neutral axis. Different scanners were used at different clinic sites. Specifically, the images were acquired on a GE Prospeed at the Birmingham clinic, a GE Hispeed Advantage at the Minneapolis clinic, a Philips MX-8000 at the Palo Alto clinic, a Siemens Somatom +4 at the Pittsburgh clinic, either a Phillips CT-Twin or Toshiba Aquilion at the Portland site, and a Picker PQ-5000 in San Diego.

QCT images were processed (by T.F.L.) at the University of California at San Francisco using a standardized protocol. Each participant scan included a calibration standard of three hydroxyapatite concentrations (150, 75, and 0 mg/cm<sup>3</sup>), and these were used to convert between Hounsfield units and vBMD. Differences between the clinic sites exist and are statistically adjusted for in all analyses.

### *Other baseline characteristics*

Participant characteristics including age, health history, and medication use were obtained by a self-administered questionnaire. Physical characteristics were obtained by clinic staff. Height was measured by Harpenden stadiometer, and weight was measured by balance beam scale except at the Portland clinic, which used a digital scale.

### *Candidate gene and single nucleotide polymorphism selection*

Physiologically defined candidate genes were identified from publicly available resources. Specifically, literature searches were conducted using PubMed, evidence of gene expression in normal human trabecular bone cells was obtained from the Skeletal Gene Database ([sgd.nia.nih.gov](http://sgd.nia.nih.gov), no longer available) and the NCBI UniGene database ([www.ncbi.nlm.nih.gov/sites/entrez?db=unigene](http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene)), genes with functions of interest (e.g., “regulation of bone mineralization” or “skeletal development”) were obtained from Entrez Gene ([www.ncbi.nlm.nih.gov/sites/entrez?db=gene](http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)) and Amigo ([amigo.geneontology.org/cgi-bin/amigo/go.cgi](http://amigo.geneontology.org/cgi-bin/amigo/go.cgi)), genes with a skeletal phenotype in mice were identified from the Jackson Laboratory Mouse Genome Informatics database (<http://www.informatics.jax.org/>), and the Online Mendelian Inheritance in Man database ([www.ncbi.nlm.nih.gov/omim/](http://www.ncbi.nlm.nih.gov/omim/)) was queried for evidence of genes implicated in skeletal conditions in humans. In total, 383 candidate genes were identified for genotyping (Table 1).

Publicly available databases were interrogated for single nucleotide polymorphism (SNP) variation in the region surrounding the candidate gene. For the first phase of genotyping (the discovery sample), two SNP selection strategies were used. In the first strategy, genetic variation

TABLE 1. Candidate Genes Screened for Association With BMD in the Discovery Sample

Chr1	<i>DVL1, TNFRSF1B, PAX7, ALPL, WNT4, ID3, CSF3R, LEPR, TGFB3, DNTTIP2, CSF1, HSD3B2, HSD3B1, NOTCH2, GNRHR2, CTSK, IL6R, ZBTB7B, BGLAP, MEF2D, NTRK1, RXRG, ADIPOR1, MYOG, HSD11B1, TGFB2, WNT9A, WNT3A</i>
Chr2	<i>ID2, NCOA1, POMC, LTBP1, CYP1B1, LHCGR, PPP3R1, IL1R2, IL1R1, IL1A, IL1B, IL1RN, EN1, GLI2, LCT, NR4A2, TANK, DLX1, DLX2, ATF2, HOXD13, HOXD12, HOXD11, HOXD10, HOXD9, HOXD8, HOXD4, HOXD3, HOXD1, FRZB, MSTN, STAT1, CASP8, FZD7, BMPR2, FZD5, IGFBP2, IGFBP5, WNT10A, IHH, PAX3, IRS1, TWIST2</i>
Chr3	<i>IRAK2, GHRL, PPARG, WNT7A, THRB, TGFB2, MYD88, ACVR2B, CTNNA1, PTHRI, WNT5A, POU1F1, GSK3B, CASR, GATA2, TRH, SHOX2, GHSR, TNFSF10, CHRDL2, AHSR, ADIPOQ, OSTN, HES1</i>
Chr4	<i>FGFR3, MSX1, NKX3-2, PPARGC1A, KDR, GNRHR, GC, BMP2K, BMP3, DMPI, IBSP, MEPE, SPPI, BMPR1B, NFKB1, DKK2, LEF1, EGF, FGF2, SMAD1, SFRP2, CASP3</i>
Chr5	<i>LIFR, PTGER4, GHR, FST, IL6ST, MAP3K1, CRHBP, MEF2C, APC, HSD17B4, CSF2, PDLIM4, TCF7, HDAC3, FGF1, NR3C1, CSF1R, SPARC, FGF18, MSX2, PROPI</i>
Chr6	<i>RIPK1, SOX4, TNF, CYP21A2, RXRB, PPARD, MAPK14, CDKN1A, VEGFA, RUNX2, OSTM1, WISP3, ENPP1, CTGF, TNFAIP3, ESRI, IGF2R</i>
Chr7	<i>TWIST1, IL6, GPNMB, HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13, CRHR2, GHRHR, SFRP4, GLI3, IGFBP1, IGFBP3, EGFR, FZD1, CDK6, CALCR, DLX6, DLX5, CYP3A4, LEP, SMO, NRF1, PTN, TRPV5, CASP2, SHH</i>
Chr8	<i>EGR3, TNFRSF10A, GNRH1, STAR, FGFRI, SFRP1, IKKKB, DKK4, CRH, NCOA2, HEY1, KLF10, FZD6, EXT1, TNFRSF11B, FBXO32, WISPI, CYP11B1</i>
Chr9	<i>CER1, IFNBI, CNTFR, OSTF1, NTRK2, ROR2, OGN, PTCHI, HSD17B3, TGFBRI, TLR4, TRAF1, WDR5, RXRA, NOTCH1, TRAF2</i>
Chr10	<i>DKK1, EGR2, BMPRIA, CHUK, CYP17A1, FGFR2</i>
Chr11	<i>IGF2, CDKN1C, DKK3, PTH, CALCA, SOX6, MYOD1, BDNF, TRAF6, EXT2, CNTF, ESRRB, LTBP3, FOSL1, TCIRG1, LRP5, CCND1, FGF3, FADD, CHRDL2, ARRBI, WNT11, FZD4</i>
Chr12	<i>WNT5B, ADIPOR2, FGF23, NTF3, TNFRSF1A, LRP6, MGP, SOX5, PTHLH, VDR, WNT10B, WNT1, IGFBP6, SP7, CYP27B1, WIF1, IRAK3, MYF6, MYF5, DCN, IGF1, TBX3, HNF1A, P2RX7</i>
Chr13	<i>FLTI, KL, POSTN, TNFSF11</i>
Chr14	<i>NFKB1A, PAX9, BMP4, ESR2, LTBP2, FOS, TGFB3, TSHR, DLK1, AKT1</i>
Chr15	<i>GREM1, CYP19A1, MAP2K1, SMAD3, CYP11A1, CYP11A2, NTRK3, IGFBP1, MEF2A</i>
Chr16	<i>AXIN1, CLCN7, IGFBP3, MAPK3, RBL2, CBFB, TRADD, HSD17B2, FOXC2</i>
Chr17	<i>ALOX15, ARRB2, DVL2, SHBG, PIK3R5, CSF3, THRA, IGFBP4, HSD17B1, SOST, MAP3K14, CRHR1, PHOSPHO1, DLX3, COL1A1, TOB1, NOG, TBX2, TBX4, GH1, SOX9</i>
Chr18	<i>MC2R, SMAD2, SMAD4, TCF4, TNFRSF11A, BCL2, NFATC1</i>
Chr19	<i>KISS1R, MAP2K2, MEF2B, CEBPA, NFKB1B, DLL3, AKT2, TGFB1, FOSB, BAX, LHB, OSCAR</i>
Chr20	<i>GNRH2, BMP2, JAG1, PAX1, ID1, E2F1, GDF5, RBL1, GHRH, SRC, WISP2, MMP9, NCOA3, CEBPB, CYP24A1, BMP7</i>
Chr21	<i>IFNAR2, IFNARI, RUNX1, ETS2</i>
Chr22	<i>COMT, KREMEN1, LIF, CSF2RB, ATF4, MCHRI, PPARA</i>
X Chr	<i>STS, RPS6KA3, GATA1, AR, BGN, IRAK1, IKKKG</i>

Gene symbols are presented by chromosome in order across the given chromosome.

in the region spanning 30 kb upstream and 10 kb downstream of each candidate gene was captured by creating a reference SNP panel of variants with a minor allele frequency (MAF) of at least 5% in phase I of the International HapMap Project ([www.hapmap.org](http://www.hapmap.org)).<sup>(17)</sup> Tag SNPs were selected using a pairwise correlation method ( $r^2 \geq 0.80$ ).<sup>(18)</sup> Candidate genes that were clustered near each other on the chromosome were tagged as a unit spanning all loci of interest. For example, IGFBP2 and IGFBP5 are located only 7.6 kb from each other on chromosome 2. Because the region of interest for these two candidates overlapped, they were tagged as a unit. In the second strategy, potentially functional SNPs that were either nonsynonymous coding variants, predicted to alter a putative transcription factor binding site in the promoter region, or a putative exon splice enhancer with MAF  $\geq 1\%$  were selected for genotyping using the PupaSNP ([pupasuite.bioinfo.cipf.es/](http://pupasuite.bioinfo.cipf.es/)) and Promolign ([polly.wustl.edu/promolign/main.html](http://polly.wustl.edu/promolign/main.html)) databases.<sup>(19,20)</sup>

Genotyping for the second phase of the project was conducted in a validation sample for promising SNP associations identified from the discovery sample. Specifically, SNPs with  $p \leq 0.015$  for either femoral neck or lumbar spine vBMD in the discovery sample were genotyped in the validation sample. Additionally, SNPs with  $p \leq 0.05$  in a gene that also had a SNP with  $p \leq 0.015$  in the discovery sample were included in the second phase of genotyping.

### Genotyping

Genomic DNA from frozen whole blood specimens was extracted using the Flexigene protocol (Qiagen, Valencia, CA, USA). Genotyping was completed using the Illumina Golden Gate custom assay. For the discovery sample, 37 participant samples were run as blind duplicates, and 4 internal controls were included per plate to ensure reproducibility. We observed 100% reproducibility among the internal controls and 99.9% reproducibility among

replicate participant samples. In the validation sample, 26 participant samples and 4 internal controls per plate were included for quality control. We observed 99.9% reproducibility among internal controls and among duplicate participant samples. To ensure maximum genotyping completeness in the validation sample, loci of interest that could not be genotyped using the Illumina Golden Gate assay were genotyped using one of two platforms: the TaqMan allelic discrimination assay system (Applied Biosystems, Foster City, CA, USA) on a 7900HT Real-time PCR instrument with probes and reagents purchased from Applied Biosystems or Sequenom MassARRAY iPLEX Gold technology (Sequenom, San Diego, CA, USA) with PCR primers purchased from Invitrogen (Carlsbad, CA, USA). A subset of participant samples were run in duplicate for these platforms, and an average reproducibility of 99.8% and 99.9% was observed for TaqMan and Sequenom, respectively.

Loci with a minor allele frequency <1% in the genotyping sample ( $N = 129$ ) that did not conform to the expectations of Hardy-Weinberg equilibrium ( $p < 0.0005$ ;  $N = 123$ ) or that had a low call rate (<85%;  $N = 248$ ) were excluded from statistical analysis. Individual samples with a low call rate (<85%;  $N = 14$ ) or that were highly correlated with another sample (indicating relatedness;  $n = 13$ ) were excluded from the analysis.

### Statistical analysis

Although only white individuals were investigated in this study, population stratification is a potential concern in large-scale genomic analyses.<sup>(21)</sup> Stratification was initially assessed using the program Structure. Structure is a model-based clustering program that parses the participants into subpopulations and assesses whether there are distinct populations or admixed individuals.<sup>(22)</sup> We found little evidence of population stratification in the discovery, validation, or pooled samples. Nevertheless, we accounted for potential fine scale population substructure by using a principal components method of analysis using uncorrelated SNPs ( $r < 0.2$ ) to calculate the principal components.<sup>(23)</sup>

Analyses of association between genotype and vBMD assumed both an additive and recessive model of inheritance. Linear regression was used to test for an additive association between the number of copies of the minor allele and vBMD. For the recessive model, regression methods were implemented to determine whether individuals having two copies of the minor allele differed from those with the other two genotypes. SNPs with 10 or less individuals having the rare genotype were not tested for the recessive model to minimize spurious findings based on small genotype specific sample sizes. All analyses adjusted for participant age and clinic site in addition to the first principal component of the population substructure analysis. SNPs with associations ( $p < 0.05$ ) in both the discovery and validation sample and that also had the same direction of association (regression coefficient + or - for both genotyping samples) were considered to be replicated. Therefore, although we did not use a strict

Bonferroni  $p$  value to adjust for multiple testing in the discovery sample, we conservatively required that the same SNP had an equivalent, significant ( $p < 0.05$ ) effect in the validation sample to be considered a replication.

Replicated SNP associations were examined further in the pooled sample of 2018 individuals from the discovery and validation samples. In addition to the analyses described above, further adjustment for height and weight was conducted in the pooled sample to determine whether body size attenuated the relationship between genotype and vBMD. Linear regression analysis was used to determine the amount of phenotypic variation explained by all of the significant (replicated) SNPs. Because SNPs in the same gene region are often correlated, the colinearity of individual SNPs in the model was assessed. One pair of SNPs in the femoral neck analysis and three in the lumbar spine analysis were highly correlated, and, in those instances, the SNP with the most missing genotypes was dropped from the regression modeling of all significant SNPs.

## RESULTS

The average age of men in the pooled analysis was 74 yr (range, 65–100 yr). The men in the discovery and validation samples were similar in age. Men in the validation sample had lower body weight, lower BMI, taller stature, and slightly lower lumbar spine and femoral neck vBMD ( $p < 0.001$  for all; Table 2).

In the discovery sample, 4108 of 4608 attempted SNPs passed quality control criteria and were analyzed for their association with lumbar spine and femoral neck vBMD. The mean SNP density for candidate genes was one SNP per 13.2 kb (range, 1 SNP/3.2 kb to 1 SNP/97 kb). Tag SNPs were selected based on phase I of the International HapMap Project. Nevertheless, the SNPs included in our analysis tagged on average 64% (range per gene, 1–100%) of the SNPs with an MAF >5% in phase II of the International HapMap project. Of the SNPs captured by our tag SNP set, the mean max  $r^2$  was 0.97.

One hundred ninety-three SNPs in 56 genes were associated with femoral neck vBMD and 173 SNPs in 59 genes were associated with lumbar spine vBMD in the discovery sample and were subsequently genotyped in the validation sample (Figs. 1A and 2A). For the femoral neck, there were several SNPs that had significant  $p$  values in both the discovery and validation samples, but the direction of association was not the same in the two samples. Specifically, this occurred for one SNP in *PAX3* (rs1367408), *IRAK2* (rs779905), *EGFR* (rs2075109), *CYP17A1* (rs12219246), *IGF1R* (rs3784606), and *BMP7* (rs6127983) and for two SNPs in *CDK6* (rs2374589 and rs3802073) (Fig. 1B). Eight SNPs in six genes (*DMPI*, *APC*, *HOXA*, *PTN*, *FGFR2*, and *FLT1*) were associated with femoral neck vBMD in both the discovery and validation sample, and the association was in the same direction (Table 3). The strongest SNP association with femoral neck vBMD in the pooled analysis was with rs4705573 in *APC* ( $p = 0.001$ ). Men who were homozygous for the minor allele (GG genotype) of

TABLE 2. Participant Characteristics (Mean and SD)

	Discovery sample (N = 862)	Validation sample (N = 1156)	Pooled sample (discovery + validation) (N = 2018)
Age (yr)	74 (5.8)	74 (6.0)	74 (5.9)
Weight (kg)	85.3 (14.1)	82.9 (12.5)*	83.9 (13.3)
Height (cm)	173.6 (6.7)	174.9 (6.7)*	174.3 (6.7)
BMI	28.3 (4.1)	27.1 (3.6)*	27.6 (3.9)
Lumbar spine vBMD (g/ml)	0.243 (0.042)	0.226 (0.038)*	0.234 (0.040)
Femoral neck vBMD (g/ml)	0.305 (0.056)	0.275 (0.052)*	0.287 (0.056)

\* Significant difference between discovery and validation sample ( $p < 0.001$ ).

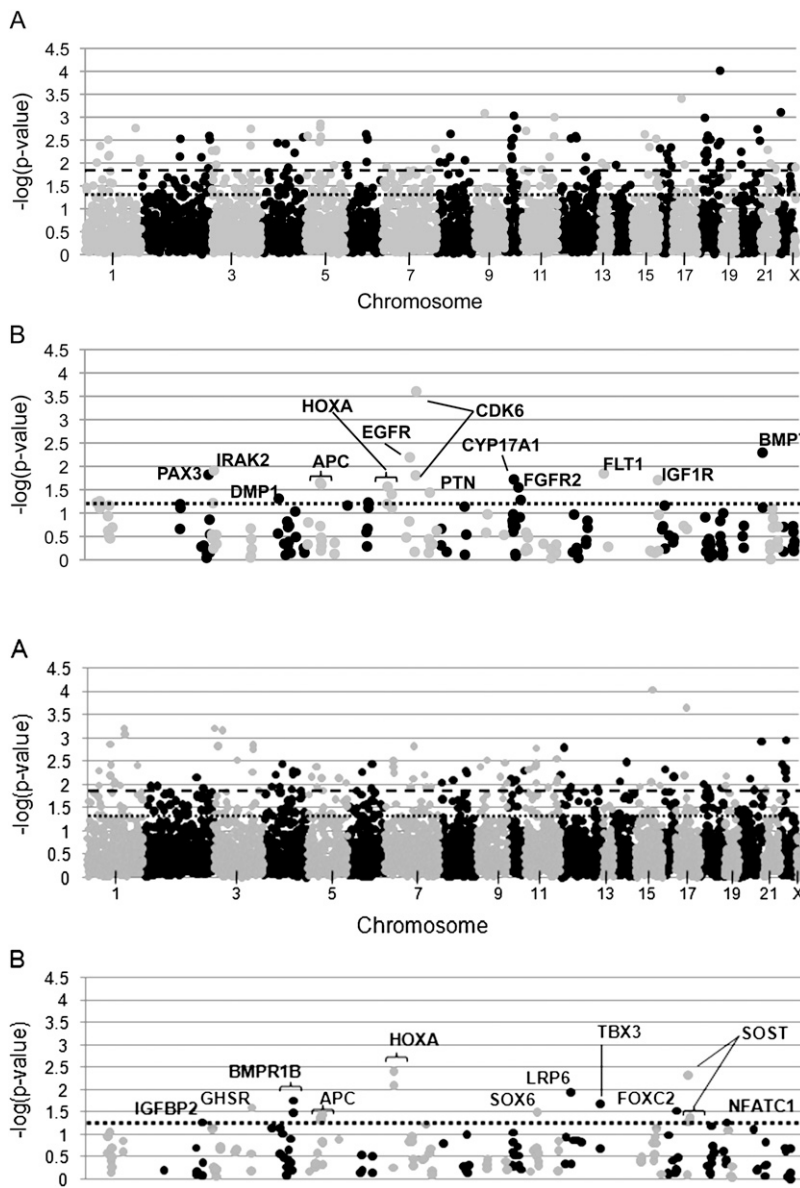


FIG. 1. SNP association results for femoral neck vBMD. Association results for the femoral neck are presented for the first phase of genotyping (discovery sample) in A and in the validation sample in B. Specifically, the  $-\log$  of the  $p$  value observed is presented on the y-axis. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. The SNPs are ordered across the x-axis by chromosome and the base pair position on the chromosome. Odd numbered chromosomes and the X chromosome are presented in light gray. Even numbered chromosomes are presented in dark gray. In A, the dark dashed line represents  $p = 0.015$  and the dotted line represents  $p = 0.05$ . The dotted line in B represents  $p = 0.05$ , and SNPs with  $p \leq 0.05$  are labeled with the gene symbol that they lie in.

FIG. 2. SNP association results for lumbar spine vBMD. Association results for the lumbar spine are presented for the discovery (A) and validation sample (B). Specifically, the  $-\log$  of the  $p$  value observed is presented on the y-axis, and SNPs are ordered across the x-axis by chromosome and base pair position. The most significant result of the two models tested (either additive or recessive) is presented for each SNP. Odd numbered chromosomes and the X chromosome are presented in light gray. Even numbered chromosomes are presented in dark gray. In A, the dark dashed line represents  $p = 0.015$  and the dotted line represents  $p = 0.05$ . The dotted line in B represents  $p = 0.05$ , and SNPs with  $p \leq 0.05$  are labeled with the gene symbol that they lie in.

this SNP had a 3.4% lower vBMD than men homozygous for the major allele (AA genotype). Although rs1381632 in DMP1 was significant in both the discovery and validation sample, different genetic models were significant, and consequently, the pooled analysis was not significant ( $p =$

0.0961). Additional adjustment for weight and height did not attenuate any of the SNP associations for femoral neck vBMD (Table 3). Each SNP only explained a small amount of the variation in femoral neck vBMD in the pooled sample (0.1–0.5%; Table 3). The two SNPs in APC

TABLE 3. Replicated Associations for Femoral Neck vBMD

Gene	SNP	Allele	Location	Frequency*	Discovery sample (adjustment 1) <sup>†</sup>			Validation sample (adjustment 1) <sup>‡</sup>			Pooled sample			r <sup>2</sup> §	
					β	p	p	β	p	p	Adjustment 1 <sup>†</sup>		Adjustment 2 <sup>‡</sup>		
											β	p	β		p
APC	rs459552	T → A	Exon (Val→Asp)	0.228	0.007	0.044	add	0.014	0.011	add	0.005	0.014	add	0.003	
APC	rs4705573	A → G	Upstream	0.480	-0.008	0.004	add	-0.008	0.001	rec	-0.009	0.001	rec	0.005	
APC	rs6594646	A → G	Intron	0.497	-0.006	0.021	add	-0.008	0.023	rec	-0.008	0.005	rec	0.004	
DMP1	rs1381632	A → T	Upstream	0.228	0.007	0.037	add	0.014	0.049	rec	0.003	0.096	add	0.001	
FLT1	rs1408245	C → G	Intron	0.166	-0.032	0.010	rec	-0.007	0.014	add	-0.021	0.003	rec	0.004	
FGFR2	rs7916940	G → A	Downstream	0.299	-0.013	0.050	rec	-0.005	0.052	add	-0.004	0.019	add	0.003	
HOXA <sup>¶</sup>	rs6951180	A → G	Upstream of HOXA13	0.133	0.010	0.018	add	0.024	0.039	rec	0.027	0.008	rec	0.003	
PTN	rs322297	A → C	Intron	0.116	0.010	0.014	add	0.007	0.037	add	0.008	0.002	add	0.004	

Additive and recessive models were tested for each SNP, and the regression parameter and p value from the most significant genetic model (additive or recessive) are shown: add, p value from the additive model; rec, p value from recessive model.

\* Minor allele frequency in the pooled sample.

<sup>†</sup> Adjustment 1: age, clinic site, population substructure.

<sup>‡</sup> Adjustment 2: age, clinic site, population substructure, height, body weight.

<sup>§</sup> Amount of variation explained after adjusting for age, site, population substructure, height, body weight.

<sup>¶</sup> HOXA genes were tagged as a cluster. They include (in order across the chromosome) HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13.

(rs6594646 and rs4705573) were in linkage disequilibrium (LD; r<sup>2</sup> = 0.979 and D' = 0.991), and rs6594646 was consequently dropped from the regression modeling. The seven replicated SNPs in APC, DMP1, FGFR2, FLT1, HOXA, and PTN included in the regression modeling explained 1.7% of the variation in femoral neck vBMD after accounting for age, clinic, population substructure, height, and weight.

Four SNPs in four genes (GHSR, rs558572; SOX6, rs1354329; LRP6, rs4477532; TBX3, rs6489968) had significant associations with lumbar spine vBMD in both the discovery and validation sample, but the direction of the association was in different directions (Fig. 2B). For the lumbar spine, there were 13 SNPs in seven genes (APC, BMPR1B, FOXC2, HOXA, IGFBP2, NFATC1, and SOST), which had a consistent direction of association in both genotyping samples (Table 4). Each individual SNP explained 0.03–0.89% of the variation in lumbar spine vBMD in the pooled sample. The SNP explaining the most variation in lumbar spine vBMD was rs1877632 in the SOST gene region, which explained 0.89% of the phenotypic variation in vBMD. Men with the less common AA genotype for rs1877632 had 6% higher lumbar spine vBMD than men with the more common GG and GA genotypes. As in the femoral neck analysis, rs6594646 and rs4705573 were in LD and rs6594646 was consequently dropped from the regression modeling of significant SNPs for lumbar spine vBMD. In addition, two SNPs in HOXA (rs6951180 and rs6964896; r<sup>2</sup> = 0.939 and D' = 1.000) and two SNPs in BMPR1B (rs3796443 and rs1434536; r<sup>2</sup> = 1.000 and D' = 0.992) were in high LD, and thus rs6964896 and rs1434536 were dropped from the regression modeling. Collectively, the 10 replicated SNP associations in APC, BMPR1B, FOXC2, HOXA, IGFBP2, NFATC1, and SOST explained 3.5% of the variation in lumbar spine vBMD after accounting for age, clinic, population substructure, height, and weight.

## DISCUSSION

Although highly heritable, little is known about the genetic variants contributing to BMD in men and vBMD in general.<sup>(24,25)</sup> This study used a two-staged genotyping strategy to investigate the association between SNPs in 383 biologically defined candidate genes and vBMD at the lumbar spine and femoral neck in a large sample of older men. We identified associations between SNPs in 11 genes and vBMD that were validated in a separate sample of older men. To our knowledge, the associations with SNPs in APC, BMPR1B, DMP1, FLT1, HOXA, IGFBP2, NFATC1, and PTN have not yet been described. We also confirmed previously identified associations in the sclerostin (SOST) and forkhead box C2 (FOXC2) genes.<sup>(26–30)</sup> Importantly, although two of the gene associations were shared between the femoral neck and lumbar spine, most were distinct for one skeletal site. These observations underscore the importance of measuring BMD at multiple skeletal sites in studies aimed at identifying osteoporosis susceptibility genes.

TABLE 4. Replicated Findings for Lumbar Spine vBMD

Gene	SNP	Allele	Location	Frequency*	Discovery sample (adjustment 1) <sup>†</sup>			Validation sample (adjustment 1) <sup>†</sup>			Pooled sample						
					β	p	p	β	p	Adjustment 1 <sup>‡</sup>			Adjustment 2 <sup>‡</sup>				
										β	p	r <sup>2§</sup>	β	p	r <sup>2§</sup>		
APC	rs4705573	A → G	Upstream	0.477	-0.005	0.007	add	-0.005	0.049	rec	-0.006	0.005	rec	-0.005	0.007	rec	0.004
APC	rs6594646	A → G	Intron	0.494	-0.004	0.035	add	-0.005	0.044	rec	-0.005	0.015	rec	-0.004	0.023	rec	0.003
BMPR1B	rs1434536	A → G	3' UTR	0.443	0.005	0.006	add	0.006	0.032	rec	0.007	0.001	rec	0.007	0.001	rec	0.005
BMPR1B	rs3796443	A → G	Intron	0.439	0.006	0.005	add	0.007	0.017	rec	0.008	3.2 × 10 <sup>-4</sup>	rec	0.008	3.0 × 10 <sup>-4</sup>	rec	0.006
FOXC2	rs3751797	T → A	Upstream	0.260	-0.016	0.007	rec	-0.004	0.029	add	-0.004	0.005	add	-0.004	0.003	add	0.004
HOXA <sup>¶</sup>	rs6951180	A → G	Upstream of HOXA13	0.134	0.009	0.003	add	0.025	0.004	rec	0.008	3.7 × 10 <sup>-5</sup>	add	0.007	2.5 × 10 <sup>-4</sup>	add	0.007
HOXA <sup>¶</sup>	rs6964896	C → A	Upstream of HOXA13	0.127	0.008	0.006	add	0.006	0.008	add	0.007	8.1 × 10 <sup>-5</sup>	add	0.006	0.001	add	0.006
IGFBP2	rs10932669	C → A	Upstream	0.134	0.007	0.026	add	0.004	0.053	add	0.005	0.002	add	0.006	0.002	add	0.005
NFATC1	rs177820	A → G	Intron	0.365	-0.005	0.027	add	-0.006	0.052	rec	-0.008	0.003	rec	-0.007	0.008	rec	0.004
SOST	rs1534401	A → G	Downstream	0.380	-0.004	0.030	add	-0.006	0.050	rec	-0.006	0.012	rec	-0.003	0.012	add	0.003
SOST	rs1877632	G → A	Downstream	0.313	0.015	0.001	rec	0.011	0.005	rec	0.013	1.5 × 10 <sup>-5</sup>	rec	0.006	1.4 × 10 <sup>-5</sup>	add	0.009
SOST	rs851054	A → G	Upstream	0.381	-0.004	0.054	add	-0.006	0.046	rec	-0.006	0.008	rec	-0.006	0.008	rec	0.004
SOST	rs851056	C → G	Upstream	0.380	-0.004	0.054	add	-0.006	0.043	rec	-0.006	0.008	rec	-0.006	0.008	rec	0.004

Additive and recessive models were tested for each SNP, and the regression parameter and *p* value from the most significant genetic model (additive or recessive) are shown; add, *p* value from the additive model; rec, *p* value from recessive model.

\* Minor allele frequency in the pooled sample.

† Adjustment 1: age, clinic site, population substructure.

‡ Adjustment 2: age, clinic site, population substructure, height, body weight.

§ Amount of variation explained after adjusting for age, site, population substructure, height, body weight.

¶ HOXA genes were tagged as a cluster. They include (in order across the chromosome) HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13.

Although most of the candidate gene associations identified were specific to either the femoral neck or lumbar spine, SNPs in the gene encoding *APC* were associated with vBMD at both sites. The minor alleles of rs4705573 in the 5' flanking region and rs6594646 in intron 1 of *APC* were associated with lower vBMD at both the femoral neck and lumbar spine. These SNPs are not known or predicted to influence *APC* function or expression and may be in LD with the causal variation. In contrast, a third SNP rs459552, which is associated only with femoral neck vBMD, is a nonsynonymous coding variant. This variant is located in the  $\beta$ -catenin downregulation domain of *APC* and may be potentially functional.<sup>(31)</sup> More commonly known for its role in cancer biology, *APC* targets  $\beta$ -catenin for degradation in the WNT signaling pathway, an important pathway in bone metabolism.<sup>(32)</sup> *APC* is expressed in osteoblasts and osteoclasts from adult human bone<sup>(33)</sup> and mice with osteoblast-specific *APC* deletions have increased bone deposition.<sup>(34)</sup>

Two SNPs upstream of the *HOXA* gene cluster in the 5' flanking region of *HOXA13* were also associated with femoral neck and lumbar spine vBMD. The *HOXA* genes are involved in the normal development of the axial skeleton and limbs.<sup>(35)</sup> The role of *HOXA* genes in determining BMD is less well characterized, but *HOXA10* contributes to osteoblastogenesis by regulating target genes for osteoblast differentiation and bone formation including RUNX2, alkaline phosphatase, bone sialoprotein, and osteocalcin.<sup>(36)</sup> Activation of *HOXA* genes has also been described during fracture repair, and a re-examination of *HOXA* gene function in adult bone and BMD regulation may be warranted.<sup>(37)</sup> The *HOXA* genes are sufficiently close to each other and oriented in such a way to enable enhancer sharing, and it is possible that SNPs in the 5' region of the gene cluster may directly or indirectly influence *HOXA* gene expression.

Four genes were uniquely associated with femoral neck vBMD. *DMP1* encodes dentin matrix protein 1, an extracellular matrix protein that regulates osteoblast gene expression and mineralization of bone matrix.<sup>(38)</sup> *DMP1*-null mice have impaired bone mineralization, mutations in *DMP1* are known to cause autosomal recessive hypophosphatemia, and there is evidence for a BMD quantitative trait locus (QTL) in mice (*Bmd2*) that encompasses the *DMP1* gene.<sup>(39–43)</sup> *FGFR2* encodes a transmembrane receptor for fibroblast growth factor that is involved in bone growth and development. Activating and dominant negative mutations in *FGFR2* are associated with altered bone mineralization and familial craniosynostosis syndromes.<sup>(44,45)</sup> *FLT1* encodes the cell surface receptor for vascular endothelial growth factor that is involved in osteoclastogenesis and osteoblast differentiation.<sup>(46–50)</sup> *FLT1*-null mice have decreased bone mineral apposition and lower trabecular bone volume.<sup>(51)</sup> Pleiotrophin (PTN) is an extracellular matrix protein released by osteoblasts that recruits, promotes adhesion of, and increases proliferation of osteoprogenitor cells.<sup>(52,53)</sup> PTN transgenic mice have greater bone calcium content compared with controls.<sup>(54)</sup>

Five gene associations were specific to vBMD at the lumbar spine. Bone morphogenetic protein receptor, type

IB (*BMPRIIB*) encodes a receptor for BMPs, which are involved in osteoblast commitment and differentiation.<sup>(55)</sup> Transgenic mice with a truncated form of *BMPRIIB* display reduced bone formation rates and BMD.<sup>(55,56)</sup> *FOXC2* encodes forkhead box C2, a member of the forkhead/winged helix transcription factor family that serves as a key regulator of embryogenesis. Mutant mice null for *FOXC2* show defects in axial skeletogenesis.<sup>(57)</sup> Association between a promoter SNP in *FOXC2* and vBMD of the radius was reported in a study of Japanese men and women.<sup>(26)</sup> *IGFBP2*, which encodes an insulin-like growth factor binding protein, is thought to target IGFs to bone and is associated with long bone growth; overexpression of this gene product results in shorter bones.<sup>(58,59)</sup> Furthermore, male *Igfbp2* knockout mice have reduced cortical and trabecular bone because of thinner trabeculae than controls.<sup>(60)</sup> Additionally, levels of circulating *IGFBP2* are negatively correlated with BMD in postmenopausal women.<sup>(61)</sup> Nuclear factor of activated T-cells 1 (*NFATC1*) is a transcription factor induced by TNF superfamily, member 11 (*RANKL*) that is involved in both osteoclast and osteoblast regulation.<sup>(62–64)</sup> Mutations in *SOST* have been associated with sclerosteosis and Van Buchem disease, and polymorphisms in *SOST* have been associated with normal variation in BMD.<sup>(27–29)</sup> Both rs851054 and rs851056 are predicted to lie in the promoter region of *SOST*, and rs851054 is predicted to abolish a sex determining region Y (SRY) binding site, whereas rs851056 is predicted to change a transcription factor binding site from the TAL1/TCF3 complex to c-MYC or *RUNX1*.<sup>(20,65)</sup>

Several significant associations were observed in both the discovery and validation samples, but the direction of the association was different. The inconsistency in direction of association may indicate spurious findings. However, others have noted a similar “flip-flop” phenomenon and have described biologically plausible scenarios that may account for this discordant pattern of association.<sup>(66,67)</sup> Some of these inconsistent associations occur in previously identified candidate genes. For example, a promoter variant (rs743572) in *CYP17A1* has been associated with BMD in some studies.<sup>(68–72)</sup> Although rs743572 was not directly genotyped in our study, it is in LD with the SNP identified in our study ( $D' = 0.915$  and  $r^2 = 0.670$ ). A nonsynonymous coding polymorphism in *LRP6* (rs2302685) has been associated with areal BMD and fracture.<sup>(73,74)</sup> This polymorphism was not associated with vBMD in our study, but an intronic variant (rs4477532) was associated with lumbar spine vBMD. Although there was inconsistent evidence for association in our study, these candidate genes may warrant further examination.

We were unable to document an association with SNPs in several widely studied candidate genes (*COL1A1*, *ESRI*, *LRP5*, and *VDR*) and vBMD. There are several reasons why we may not have been able to replicate these associations. First, our study includes only men, whereas many of the past candidate gene studies have focused on women. In addition, most of these studies investigated areal and not volumetric BMD. Finally, our study had 70% statistical power to detect a SNP association that explained 1% of the variation in vBMD in the screening stage at



$\alpha = 0.01$ . Thus, we cannot exclude the possibility of a weaker association between SNPs in the *ESRI*, *COL1A1*, *VDR*, or *LRP5* genes and vBMD at the femoral neck or lumbar spine in older men.

Three SNPs associated with vBMD in our study were also genotyped in a genome-wide association study of areal BMD but were not significantly associated with BMD in that study ( $p > 0.05$ ).<sup>(7)</sup> Although not directly genotyped in our study, other SNPs in the gene regions associated with vBMD in our study (*APC*, *BMPRI1B*, *DMPI1*, *FGFR2*, *FOXC2*, *HOXA*, *NFATC1*, and *PTN*) also showed associations ( $p < 0.05$ ) in this genome-wide association study but did not achieve genome-wide significance. Most notably, rs11984297 located just downstream of the *HOXA13* gene was associated with both hip ( $p = 0.0002$ ) and spine ( $p = 0.0014$ ) BMD, but this SNP is not in high LD with rs6951180 ( $r^2 = 0.015$ ) or rs6964896 ( $r^2 = 0.008$ ).<sup>(7)</sup>

The amount of phenotypic variation in vBMD explained by SNPs in this study was small: 1.7% for the femoral neck and 3.5% for the lumbar spine. Although small, these findings are comparable to recent genome-wide studies of BMD and other quantitative traits like height.<sup>(6,7,75)</sup> For example, in a recent genome-wide association study of areal BMD, 0.6% and 0.2% of the phenotypic variation in lumbar spine and femoral neck BMD was explained by two SNPs in two genes.<sup>(6)</sup> Given the high heritability of BMD, much of the variation in BMD explained by genetic factors remains to be identified. Future studies not only examining SNP associations, but also investigating insertion deletion mutations, copy number variants, rare variants (<5% MAF), and interactions between genetic factors and between genetic and environmental factors may explain more of the variation in BMD.

Our study has potential limitations. First, our reference SNP panel for tag SNP selection was based on phase I of the International Haplotype Map Project (HapMap), and consequently does not provide as comprehensive a set of tag SNPs as current projects based on phase II. However, the SNPs included in this analysis tagged an average of 64% of the SNPs with >5% MAF in phase II of HapMap with a mean maximum  $r^2$  of 0.97. To put this in perspective, our tag SNP set captured 82% and 95% of the SNPs with MAF >5% in the *HOXA* and *APC* gene regions, whereas a recent genome wide study of BMD examining >300,000 SNPs captured 68% and 74% for these gene regions.<sup>(7)</sup> Our analysis also focused on SNPs with a MAF of  $\geq 5\%$  (or  $\geq 1\%$  for potentially functional SNPs) and thus cannot exclude the possible contributions of less common variants. Our study is also limited to white men  $\geq 65$  yr of age, and our results may not be generalizable to other ethnic groups or to women. Our analysis of vBMD was cross-sectional, and future studies of bone loss may give further insight into the genetics of osteoporosis in men. Furthermore, a genome-wide association study, which has the advantage of being hypothesis free, may yield additional insight on the genetics of volumetric BMD. Additional genotyping in the gene regions of interest will be needed in this and in other ethnically diverse populations to refine the association signals and to inform future in vitro functional studies.

Although limited to studying only older white men, this study provided the first large-scale assessment of the genetic contributions to a unique skeletal trait (QCT volumetric BMD), assessed and adjusted for potential population stratification, identified a number of novel and robust genetic associations by including a two-stage internal replication design and collectively, and explained more of the phenotypic variance in BMD than other genetic association studies to date. We identified several novel genetic associations with vBMD, and our findings suggest that distinct genetic factors may contribute to lumbar spine and femoral neck vBMD in older men. Additional studies will be needed to confirm and extend these findings.

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