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Gene-gene interaction between *RBMS3* and *ZNF516* influences bone mineral density

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Abstract

Osteoporosis is characterized by low bone mineral density (BMD), a highly heritable trait that is determined, in part, by the actions and interactions of multiple genes. While an increasing number of genes have been identified to have independent effects on BMD, few studies have been performed to identify genes that interact with one another to affect BMD. In this study, we performed gene-gene interaction analyses in selected candidate genes in individuals with extremely high vs. low hip BMD (20% tails of the distributions), in two independent US Caucasian samples. The first sample contained 916 unrelated subjects with extreme hip BMD Z-scores selected from a population composed of 2,286 subjects. The second sample consisted of 400 unrelated subjects with extreme hip BMD Z-scores selected from a population composed of 1,000 subjects. Combining results from these two samples, we found one interacting gene pair (*RBMS3* vs. *ZNF516*) which, even after Bonferroni correction for multiple testing, showed consistently significant effects on hip BMD. *RMBS3* harbored two SNPs, rs6549904 and rs7640046, both of which had significant interactions with a SNP, rs4891159, located on *ZNF516* (*P* values: 7.04×10^{-11} and 1.03×10^{-10}). We further validated these results in two additional samples of Caucasian and African descent. The gene pair, *RBMS3* vs. *ZNF516*, was successfully replicated in the Caucasian sample (*P* values: 8.07×10^{-3} and 2.91×10^{-3}). For the African sample, a significant interaction was also detected (*P* values: 0.031 and 0.043), but the direction of the effect was opposite to that observed in the three Caucasian samples. By providing evidence for genetic interactions underlying BMD, this study further delineated the genetic architecture of osteoporosis.

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All authors state that they have no conflicts of interest.

Keywords

interaction; association; BMD; osteoporosis

Introduction

Osteoporosis is associated with an increased risk of low-trauma osteoporotic fractures, and is recognized as a major public health problem (1). Low bone mineral density (BMD) serves as a diagnostic parameter in the assessment of osteoporosis and fracture risk, and is the single best predictor of osteoporotic fracture (2). Hip fracture is the most common and severe form of osteoporotic fracture. It has a high associated morbidity and mortality, and contributes substantially to health care expenditures within the U.S., and elsewhere (3). Consequently, studies evaluating risk of osteoporotic fracture often assess BMD at the hip, as this is often considered to be the most important risk phenotype for osteoporosis.

BMD is a highly heritable quantitative trait for which approximately 50% to 85% of BMD variability is genetically determined (4,5). In recent years, genome-wide association studies (GWASs) have evolved into powerful tools for dissecting the genetic basis for osteoporosis. GWASs have successfully identified a number of genetic loci which, individually, have modest effects on BMD, and collectively account for only approximately 5% of the overall heritability of BMD (6–13). One significant limitation of utilizing GWASs to identify genetic loci associated with BMD, osteoporosis, or other complex human diseases is that GWASs examine the effects of each individual single nucleotide polymorphism (SNP) independently. Complex diseases and phenotypes, however, often arise from the joint effects or interactions of multiple genes (14). Consequently, GWASs designed to identify only those individual SNPs that have a statistically significant impact on a specific phenotypic trait are unlikely to identify genetic variants that are dependent upon interactions with one another to impact that trait (15). In order to elucidate the joint effects or interactions of multiple genes on phenotypic traits, it has become important, and necessary, to model gene-gene interactions, particularly within the context of analyzing data generated from GWASs. Incorporating analyses of gene-gene interactions into GWASs has proven to increase statistical power, thereby contributing to the discovery of missing variants for complex diseases (16,17).

In this study, we performed gene-gene interaction analyses in selected candidate genes to identify genetic variants impacting hip BMD variation. By considering statistically interacting SNPs, our results have provided new insights that enhance our understanding of the genetic architecture of osteoporosis.

Materials and Methods

Ethics Statement

Each study was approved by the required Institutional Review Board or Research Administration of the institutions involved. Signed informed-consent documents were obtained from all study participants before entering the study.

Subjects

The study was initially performed with a discovery stage for detection of pairwise SNP interactions in two GWAS samples (Kansas City and Omaha samples). Significant SNP pairs derived from both GWAS samples in the discovery stage, were further confirmed through a replication stage in two additional independent samples (Framingham Heart Study (FHS) sample and Women's Health Initiative (WHI) sample). The basic characteristics of all study samples are summarized in Table 1, with additional descriptive detail below.

Kansas City *sample*—The Kansas City sample contained 2,286 unrelated US Caucasians of Northern European origin living in Kansas City and its surrounding areas. Subjects with chronic diseases and conditions that might potentially affect bone mass, structure, or metabolism were excluded from the study to minimize the influence of known environmental and therapeutic factors on bone variation. Exclusion criteria have been detailed in our earlier publication (18).

BMD (g/cm^2) at the total hip for each subject was measured with dual energy x-ray absorptiometry (DXA) using Hologic 4500W machines (Hologic Inc., Bedford, MA, USA) that were calibrated daily. The coefficient of variation (CV) value of the DXA measurements for hip BMD was approximately 1.87%. A Z-score was calculated by comparing the measured BMD to the mean BMD values obtained in a population of the same age and gender (19). Based on the distribution of the hip BMD Z-scores, we selected 914 subjects with extreme phenotypes (those who fell within the highest and lowest 20% of the population distribution in this sample) for subsequent statistical analyses.

Omaha *sample*—The Omaha sample included 1,000 US Caucasians living in Omaha, Nebraska and its surrounding areas. Exclusion criteria were the same as those adopted in the above Kansas City sample. BMD at the hip was again measured using Hologic 4500W machines (Hologic Inc., Bedford, MA, USA). Similarly, we selected 400 subjects with extreme hip BMD Z-score (those who fell within the highest and lowest 20% of the population distribution in this sample) for subsequent statistical analyses.

FHS sample—The FHS sample was derived from the Framingham Heart Study (FHS) SNP Health Association Resource (SHARe) project, for which genotyping was conducted in over 9,300 phenotyped subjects from three generations (including over 900 families). Details and descriptions about the FHS have been reported previously (20,21). From the FHS sample, we had data from 3,240 phenotyped Caucasian subjects from 904 families. BMD at the hip was measured using DXA machine (Lunar DPX-L). Since information on Z-scores was not available to us for this sample, we selected extreme phenotypes based on hip BMD values after adjustment by age and sex. Therefore, 1,296 subjects with extreme phenotypes (those falling within the highest and lowest 20% of the population distribution in this sample) were selected. Since the subsequent interaction analyses could not consider familial relationships, we further extracted unrelated subjects (parental generation or only one child from each family) from these 1,296 subjects. Finally, 697 subjects (335 subjects with high BMD and 362 subjects with low BMD) were included for subsequent statistical analyses.

WHI sample—The WHI sample came from the Women's Health Initiative (WHI), which is a long-term national health study for preventing heart disease, cancer, and osteoporotic fractures. All women enrolled in the WHI were between 50 and 79 years old and were postmenopausal. Details regarding the WHI study have been reported elsewhere (22,23). From the WHI sample, we had data from 710 phenotyped subjects, whose self-reported ethnicity was African American. BMD at the hip was measured using DXA (DXA QDR; Hologic Inc., Waltham, Mass) using a standard protocol. The criteria for selecting subjects with extreme phenotypes were the same as those adopted in the above FHS sample. 284 subjects with extreme phenotypes were included for subsequent statistical analyses.

Genotyping and Quality Control

For the discovery stage, genomic DNA was extracted from peripheral blood leukocytes using standard protocols. The Kansas City sample was genotyped using Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA), according to the Affymetrix protocol. Briefly, approximately 250 ng of genomic DNA was digested with restriction enzyme NspI or StyI. Digested DNA was adaptor-ligated and PCR-amplified for each sample. Fragment PCR products were then labeled with biotin, denatured, and hybridized to the arrays. Arrays were then washed and stained using Phycoerythrin on a Affymetrix Fluidics Station, and scanned using the GeneChip Scanner 3000 7G to quantitate fluorescence intensities. Data management and analyses were conducted using the Genotyping Command Console. For the Omaha sample, SNP genotyping was performed using the Affymetrix Human Mapping 500K array set, which had been completed for our previous experiments (24).

Quality control procedures were as follows. First, only samples with a minimum call rate of 95% were included. Due to efforts of repeat experiments, all samples (Kansas City sample: n = 2,286; Omaha sample: n = 1,000) met this criteria and the final mean call rate reached a high level of 98.93% for the Kansas City sample and 99.14% for the Omaha sample, respectively. Second, prior to association analyses, we filtered SNPs based on genotyping call rate < 95%, Hardy-Weinberg equilibrium (HWE) (P < 0.001) and minor allele frequencies (MAF) < 0.1. Therefore, a total of 562,024 SNPs in the Kansas City sample and 292,859 SNPs in the Omaha sample passed these filters and were used in subsequent analyses.

For the replication stage, the FHS sample was genotyped using approximately 550,000 SNPs (Affymetrix 500K mapping array plus Affymetrix 50K supplemental array). For details of the genotyping method, please refer to FHS SHARe at NCBI dbGaP website (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v3.p2). The WHI sample was genotyped using Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA). The details of the genotyping method can be found at NCBI dbGaP website (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gap).

Since the Affymetrix 500K array (used for the Omaha sample) has less SNP coverage than the Affymetrix Array 6.0 (used for the Kansas City sample), we performed SNP imputation in the Omaha sample. Based on HapMap data (release 22), the IMPUTE program (25) was utilized to impute genotypes of SNPs detected with the 6.0 array, that were not detected with

the 500K array . To ensure the reliability of imputation, all imputed SNPs reached a calling threshold of 0.90, i.e., there was a 90% probability that an imputed genotype is true.

Population Stratification

To correct for potential stratification that may lead to spurious association results, principal component analysis (PCA) implemented in EIGENSTRAT (26) was used to estimate population substructure. We applied PCA to all available genotypic data for the Kansas City and Omaha samples separately, retaining the top ten principal components (PCs). These ten PCs, along with height and weight, were included as covariates to adjust for hip BMD Z-scores before performing single SNP and pairwise interaction analyses. For the replication analyses, since the FHS sample is family-based, the top 10 PCs were first built using a subset of 200 biologically unrelated subjects and projected to all study samples (27). The top ten PCs, along with age, sex, height, and weight, were included in the regression model to adjust for hip BMD for both the FHS and WHI samples.

Statistical analyses

For pairwise SNP interaction analyses at the discovery stage, we followed a two-stage strategy which had been previously established (16,28). We first conducted single-SNP genome-wide association analysis in the Kansas City sample using the logistic regression model in PLINK software (29). SNPs showing highly or marginally significant effects ($P < 0.05$, $n = 27{,}890$) were selected for subsequent pairwise interaction analysis. Moreover, in order to decrease the burden of multiple testing, one of two SNPs in completely linkage disequilibrium (LD, $r^2 = 1$) with each other was pruned out randomly by PLINK ($n = 102$). Therefore, 27,788 SNPs were included (about 1/20 of the SNPs in the genome-wide scan). We limited the analyses to these SNPs because these SNPs have already been implicated in osteoporosis, and because analyzing all combinations of pairwise interactions of genome-wide SNP data would be computationally exhaustive. This strategy was envisioned to effectively lessen the computational load, while producing a high probability of generating significant results. Pairwise SNP interaction analyses were conducted using the logistic regression model implemented in PLINK. Briefly, PLINK considers allelic by allelic epistasis, which fits a logistic regression model in the following equation:

$$\mathrm{Y} \sim \beta + \beta 1 * \mathrm{SNP1} + \beta 2 * \mathrm{SNP2} + \beta 3 * \mathrm{SNP1} \times \mathrm{SNP2} + e$$

For “two copies” of A allele (minor allele) of SNP2 (SNP2=2), the equation is:

$$\mathrm{Y} (\beta + 2\beta 2) + (\beta 1 + 2\beta 3) * \mathrm{SNP1} + e$$

For “one copy” of A allele of SNP2 (SNP2=1), the equation is:

$$\mathrm{Y} \sim (\beta + \beta 2) + (\beta 1 + \beta 3) * \mathrm{SNP1} + e$$

For "zero copy" of A allele of SNP2 (SNP2=0), the equation is:

$$Y \sim \beta + \beta 1 * SNP1 + e$$

For the odds ratios (OR), the term of exp(β1 + 2β3) is the OR for the effect of SNP1 when subjects carry two copies of A allele (AA) of SNP2. The term of exp(β1 + β3) is the OR for the effect of SNP1 when subjects carry one copy of A allele (AB) of SNP2. Exp(β1) is the OR for the effect of SNP1 when subjects carry BB of SNP2. Therefore, the OR for the interaction can be represented by the term of exp(β3), which means the fold changes for the effect of SNP1 along with increasing per one copy of A allele of SNP2. As Plink does not give the 95% confidence interval (CI) of OR values, we used MINITAB to calculate 95% CI. The Pairwise SNP interaction results with *P* value less than 10^{-4} in the Kansas City sample were validated in the Omaha sample. Combining the results from these two samples by meta-analysis, we further replicated the most promising results in the FHS and WHI samples.

Meta-analysis calculations were done using the METAL software package (http://genome.sph.umich.edu/wiki/METAL_Documentation) using inverse-variance weighted fixed-effect model. Combining results from the samples at discovery stage by meta-analysis, we set the significance threshold at $P < 1.30 \times 10^{-10}$ after adjustment for multiple testing by Bonferroni correction ($0.05/C^2_{27788} \approx 1.30 \times 10^{-10}$).

Results

The study design included a discovery stage in two sample sets from GWAS, denoted as the Kansas City and Omaha samples. We initially screened a large quantity of pairwise SNP-SNP interactions in the Kansas City sample. For the most significant pairwise interactions (*P* values < 10^{-4}), we conducted follow-up validation analyses in the Omaha sample. Combining results from these two sample sets at the discovery stage, we listed the most significant pairwise interaction results with meta-analysis *P* values < 10^{-6} in Supplementary Table S1. The most significant interaction between a pair of genes involved *RBMS3* and *ZNF516*. We subsequently confirmed this interaction through a replication stage in two additional independent samples, denoted as the FHS and WHI samples. We focused on subjects with extremely low BMD, aiming to identify genes involved in osteoporosis producing the highest risk for osteoporotic fractures. The major pairwise interaction results are summarized in Table 2a. In Table 2b, the number of individuals with extremely low vs. high BMD for the Kansas City and Omaha samples are presented by genotype for each of the SNP pairs presented in Table 2a.

For the pair of interacting genes with the highest significance, *RBMS3* vs. *ZNF516*, statistical significance was achieved at the discovery stage after applying the Bonferroni correction for multiple testing (combined $P < 1.30 \times 10^{-10}$) (Table 2a). *RBMS3* harbored two SNPs, rs6549904 and rs7640046, both of which had significant interactions with a single SNP, rs4891159 located in *ZNF516* (combined $P = 7.04 \times 10^{-11}$ and $P = 1.03 \times 10^{-10}$, respectively). SNPs rs6549904 and rs7640046 in *RBMS3* were in high LD with an r^2 of

0.94. The directions of the effect for these two pairs of interactions were shown to be congruent between the Kansas City and Omaha samples in METAL software. Taking rs6549904 vs. rs4891159 as an example, the interaction OR was estimated to be 3.19 (95% CI: 2.01–5.04) and 4.82 (95% CI: 2.39–9.72) in the Kansas City and Omaha samples, respectively. This means that the effect of the minor allele in SNP rs4891159 (A-allele, MAF = 0.413) increased 3.19-fold (interaction OR value) and 4.82-fold in the Kansas City and Omaha samples, respectively, for each copy of the minor allele in rs6549904 (C-allele, MAF = 0.139).

At the replication stage, these two pairs of interacting SNPs were successfully replicated in the FHS sample, with *P* values of 8.07×10^{-3} for rs6549904 vs. rs4891159, and 2.91×10^{-3} for rs7640046 vs. rs4891159 (Table 2a). The interaction OR for rs6549904 vs. rs4891159 was estimated to be 1.83 (95% CI: 1.17–2.88) and the direction of this effect was the same as it was for the Kansas City and Omaha samples. Namely, the effect of the A-allele in SNP rs4891159 increased 1.83-fold for each copy of the C-allele in rs6549904. In the WHI sample, the *P* values for the two pairs of interactions were significant ($P = 0.031$ and 0.043), however, the direction of this effect was opposite to that observed in the above three samples. For rs6549904 vs. rs4891159, the effect of the A-allele in SNP rs4891159 showed fold-decrease for each copy of the C-allele in rs6549904 (Interaction OR: 0.06, 95% CI: 0.01–0.79). This difference in directional effect could be due to fact that the MAF for these three SNPs were markedly different in blacks in the WHI sample vs. whites in the other three samples ($P < 0.001$). Detailed information for these three SNPs is presented in Table 3.

In order to compare our results with previous studies, we briefly reviewed the published gene-gene interaction studies on osteoporosis. Then, using the available genotypes in our two GWAS samples, we performed candidate gene-gene interaction analyses for the important genes identified in those studies, including *ESR1*, *ESR2*, *VDR*, *COL1A1*, *RANK*, *RANKL*, *OPG* etc.(30–41). The major results are summarized in Table 4. Since the analysis was driven by the hypothesis, SNP pairs with $P < 0.05$ for both the Kansas City and Omaha samples were considered significant. We validated six pairs of genes with interaction effects, including *ESR1* vs. *VDR*, *ESR1* vs. *COL1A1*, *ESR1* vs. *ESR2*, *ESR1* vs. *IL6*, *OPG* vs. *RANKL*, and *RANK* vs. *RANKL* (Table 4).

Discussion

The major contribution of the research reported here was our successful identification of one pairwise interaction, *RBMS3* vs. *ZNF516* that contributes to variations in BMD in humans. This interaction achieved statistically significant levels even after applying the highly conservative Bonferroni correction, and statistically significant signals were obtained with all four sample populations in this study. Interestingly, an ethnic difference in the directional effect for this pairwise interaction was revealed between whites and blacks. One potential explanation for this ethnic difference could be that the MAF's for the SNPs identified in these interacting genes are quite different between whites vs. blacks ($P < 0.001$). Alternatively, the relatively small sample size of blacks may have impacted results. Consequently, further studies with a larger sample size are needed to validate the ethnic difference detected in the current study.

The *RBMS3* gene is located on chromosome 3p24. The protein encoded by this gene has the capacity to bind DNA/RNA. RBMS3 was first identified as a DNA-binding protein that bound the promoter sequence of the collagen α2(I) gene *in vitro* (42). Recently, RBMS3 has also been found to bind Prx1 mRNA and increase expression of Prx1 protein, which could stimulate transcription of the collagen α1(I) gene (43). Collagen type α1 is the most abundant component of bone tissue. Importantly, *RBMS3* has been identified as a potential candidate gene for osteoporosis by a previous GWAS using Affymetrix 100K SNP GeneChip (44). Specifically, *RBMS3* was identified to have suggestive association with trochanter BMD in 1,141 subjects selected from the same FHS sample (44). This collective evidence suggests that RBMS3 might be a potentially key factor contributing to the pathogenesis of osteoporosis.

The *ZNF516* gene, which is located on chromosome 19q23, encodes a zinc finger protein. This gene, of unknown function, is expressed in bone, indicating a potentially unidentified role in the biologic characteristics of bone. Although the biological nature of the *RBMS3* vs. *ZNF516* interaction is not clear, our statistical analyses provide evidence to support the hypothesis that one mechanism by which *RBMS3* influences osteoporosis risk is through its interaction with *ZNF516*. Consequently, future efforts will be focused on determining the mechanism by which these interactions influence osteoporosis risk.

A recent study by Zuk et al. (45) indicated that a substantial proportion of the missing heritability for complex diseases/traits could be due to genetic interactions that have escaped current methods of analysis. Consequently, it is important to develop and apply tools that can decipher interconnected networks of genes and their relationships with variations in phenotypic traits or disease susceptibility. Such tools represent a potentially valuable approach for discovering the genetic basis for the missing heritability associated with these traits/diseases that has eluded identification using traditional genetic association studies. Although recent GWASs have contributed greatly to the identification of individual SNP underlying osteoporosis (7–9,12,13), studies utilizing pairwise gene interaction analyses for complex diseases/traits, particularly on a genome-wide scale, have been relatively rare. One potential reason for the relative rarity of this approach might be the low statistical power of these methods for detecting significant interactions at the genome-wide level. Zuk et al. (45) showed that a sample size of ∼500,000 was needed to detect genome-wide genetic interactions, and the likelihood of accumulating a sample of this magnitude is extremely low. In order to compensate for this relative deficiency in statistical power, we considered that it would be efficient to limit analysis of potential interactions to a subset of specific SNPs. Specifically, in order to increase statistical power and avoid extremely intensive computations demanded by genome-wide interaction analysis, we only screened SNPs shown to have independent effects on BMD ($P < 0.05$) for potentially significant interactions with other genes across the genome. Through this approach, we successfully identified an interaction between *RBMS3* and *ZNF516* that impacted variations in BMD. In the current study, no individual SNPs from these two genes achieved statistical significance at the genome-wide level in single SNP analysis. Consequently, our successful identification of interactions between *RBMS3* and *ZNF516* that impacted variations in BMD suggests that gene-gene interaction analysis might be a complementary approach to traditional GWAS for

detecting new genes associated with complex human diseases and traits. It is important to recognize, however, that for SNPs without epistasis which show strong associations in single SNP analysis, signals might disappear in gene-gene interaction analysis.

Previous candidate gene association studies have identified several gene-gene interactions influencing osteoporosis, such as *RANK*/*RANKL*/*OPG* (40,41), *ESR1*/*ESR2* (38), and *ESR1*/*VDR* (30). In the present study, we confirmed several pairwise interactions identified by previous candidate gene-gene interaction studies at the replication level, including *RANK*/*RANKL*, *OPG*/*RANKL*, *ESR1*/*ESR2* and *ESR1*/*VDR*, et al. (Table 4). We also examined pairwise interactions between genes identified by previous GWAS and other genes in our discovery sample. Suggestive interaction results ($P < 10^{-4}$) are summarized in Supplementary Table S2, which may serve as a reference for future investigators.

Our study was designed differently from most traditional GWAS of BMD, in that we used an extreme-truncated scheme to select subjects with extremely high or low BMD to increase the computing efficiency for interaction analyses. Selection of study subjects in this manner has proven to be an efficient and powerful approach for the study of quantitative traits, as demonstrated by two recent GWAS on BMD (46,47). In this study, based on power scenarios at different cutoffs for truncation, assuming a marker-disease-associated allele LD of r^2=0.9, alpha=0.0001 and variants contributing 1.5% of the additive genetic variance of BMD, a 20% cutoff generated the highest statistical power compared to other cutoffs (cutoff: power; 10%: 0.54; 15%: 0.70; 20%: 0.79; 25%: 0.75; and 30%: 0.75), and produced virtually no loss in power compared to the whole distribution (power: 0.81). Moreover, we intentionally focused on BMD at a single skeletal site, the hip. BMDs measured at different skeletal sites are highly correlated with one another, and the genes associated with variations in BMD at different sites overlap to a large extent, but are not identical. Our study was designed to reduce heterogeneity due to skeletal site specific effects. Further justification for choosing only “hip BMD” as the studied phenotype is that hip BMD is directly relevant to risk of hip fracture, the most severe and fatal consequence of osteoporosis. Consequently, findings based on hip BMD might be more clinically relevant than other osteoporosis phenotypes.

Although we are convinced that the approach that we have used to study gene-gene interactions has significant potential to further delineate the genetic basis for complex human diseases, our study has significant limitations. First, the study design might miss some potential significant interactions for SNPs without major independent effects, since they might have significant effects when they interacted with each other. Second, we only considered two-locus interactions and many genes and/or their products often work together in groups of three or more; these more complex interactions would have evaded detection by the current approach. Pathway-based or gene sets analyses are optimally effective for identifying pathophysiologically significant pathways underlying complex traits. However, pathway-based or gene sets analyses need prior knowledge to define which genes are involved in a pathway or gene set. Since our knowledge of all gene networks and pathways is not even close to being comprehensive, gene-gene interaction analyses, as performed in the current study, may find novel epistasis effects between genes in unidentified pathways. Third, the 95% CIs of OR for the significant results were relatively wide, indicating that the

sample size of our study was not large enough to obtain an accurate estimate for the interaction term. Consequently, further study with a larger sample size is needed to validate our results.

In conclusion, we identified a promising pairwise genetic interaction, *RBMS3* vs. *ZNF516*, which may influence susceptibility to osteoporosis. Our findings demonstrated that association analyses that take gene-gene interactions into account may enhance detection of genetic variants that can be missed by routine (single SNP) association analyses. Thus, interaction analysis provides an additional tool to help understand the genetic basis of osteoporosis, and other complex diseases/traits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Author's roles: Study design: TLY and HWD. Study conduct: TLY. Data collection: HS, SML, SKL, QT, and YJL. Data analysis: TLY, YG, JL, and LZ. Drafting manuscript: TLY and YG. Revising manuscript content: CJP. Approving final version of manuscript: TLY, YG, JL, LZ, HS, SML, SKL, QT, YJL, CJP and HWD. HWD takes responsibility for the integrity of the data analysis.

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Table 1

Basic characteristics of the study subjects

Trait	Kansas City sample		Omaha sample		Framingham sample		WHI sample	
	Low BMD	**High BMD**	**Low BMD**	**High BMD**	**Low BMD**	**High BMD**	**Low BMD**	**High BMD**
Number	458	458	200	200	362	335	142	142
Age (years)	51.19 (12.86)	51.24 (14.19)	49.34 (18.49)	50.22 (18.81)	61.86 (10.82)	62.04 (10.68)	60.40 (6.74)	60.76 (7.05)
Weight (kg)	75.78 (18.99)	75.95 (16.63)	81.44 (19.5)	80.38 (16.50)	78.94 (18.67)	77.54 (15.30)	81.61 (19.60)	81.89 (15.31)
Height (cm)	166.92 (8.42)	166.51 (8.16)	171.74 (10.56)	171.10 (9.38)	168.13 (10.64)	166.80 (9.26)	162.47 (6.02)	162.52 (5.62)
Female/Male	348/110	351/107	80/120	81/119	171/191	186/149	142/0	142/0
Z-score	−1.09 (0.56)	1.25 (0.62)	−1.08 (0.62)	1.21 (0.73)	-	-	-	-
Hip BMD (g/cm^2)	0.77 (0.07)	1.17 (0.08)	0.82 (0.11)	1.15 (0.12)	0.81 (0.13)	1.15 (0.12)	0.78 (0.10)	1.12 (0.10)

Note: Data are shown as mean (standard deviation, SD).

Table 2

a. The interaction SNP pairs identified by gene-gene interaction analyses for hip BMD

SNP 1	Gene 1	SNP 2	Gene 2	Kansas City sample			Omaha sample			Combined *P* value[a]	Framingham sample		WHI sample	
			SNP1 *P*	SNP2 *P*	Interaction *P*	OR (95% CI)	Interaction *P*	OR (95% CI)	Interaction *P*		OR (95% CI)	Interact -ion *P*	OR (95% CI)	
rs6549904	RBMS3	rs4891159	ZNF516	0.015	0.025	3.46×10^{-6}	3.19 (2.01-5.04)	7.21×10^{-7}	4.82 (2.39-9.72)	3.64×10^{-11}	8.07×10^{-3}	1.83 (1.17-2.88)	0.031	0.06 (0.01-0.79)
rs7640046	RBMS3	rs4891159	ZNF516	0.020	0.025	5.31×10^{-6}	3.07 (1.96-4.82)	4.40×10^{-7}	4.90 (2.46-9.73)	5.06×10^{-11}	2.91×10^{-3}	1.94 (1.25-3.01)	0.043	0.17 (0.03-0.95)

b. The number of individuals with extremely low vs. high BMD for the Kansas City and Omaha samples presented by genotype, for each of the SNP pairs identified in Table 2a.

Kansas City sample								Omaha sample							
rs6549904 (missing 1*)	rs4891159 (missing 7)	Low BMD	High BMD	rs7640046 (missing 3)	rs4891159 (missing 7)	Low BMD	High BMD	rs6549904 (missing 2)	rs4891159 (missing 2)	Low BMD	High BMD	rs7640046	rs4891159 (missing 2)	Low BMD	High BMD
TT	GG	123	107	CC	GG	122	107	TT	GG	54	30	CC	GG	55	28
	AG	167	150		AG	163	148		AG	63	72		AG	62	72
	AA	53	65		AA	53	65		AA	17	36		AA	17	35
CT	GG	20	58	TC	GG	21	57	CT	GG	7	20	TC	GG	7	22
	AG	60	55		AG	62	57		AG	24	11		AG	26	11
	AA	28	12		AA	26	12		AA	10	5		AA	10	6
CC	GG	0	4	TT	GG	0	5	CC	GG	1	3	TT	GG	1	2
	AG	2	4		AG	3	4		AG	1	2		AG	1	2
	AA	0	0		AA	1	0		AA	2	0		AA	2	0

Abbreviations: OR, odds ratio; CI, confidence interval

[a] The *P* value was combined by including the Kansas City sample and Omaha sample at the discovery stage.

Note: Minor alleles were underlined.

* The means the number of subjects with missing genotypes.

Table 3

The information of identified significant SNPs, rs4891159, rs6549904 and rs7640046.

SNP	Allele[a]	Kansas City sample			Omaha sample			Framingham sample			WHI sample		
		HWE	MAF	Call rate	HWE	MAF	Call rate	HWE	MAF	Call rate	HWE	MAF	Call rate
rs4891159	A/G	0.681	0.413	0.992	0.748	0.436	0.995	0.639	0.392	1	0.873	0.245	1
rs6549904	C/T	0.037	0.139	0.999	0.245	0.132	0.995	1	0.14	1	1	0.032	1
rs7640046	T/C	0.138	0.144	0.992	0.375	0.139	0.997	1	0.147	1	1	0.046	1

Abbreviations: HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency.

[a]The former allele represents the minor allele.

Table 4

Validation for the previously reported gene-gene interactions in our two GWAS samples

SNP 1	Gene 1	SNP 2	Gene 2	Kansas City sample	Omaha sample	Reference
				Interaction *P*	Interaction *P*	
ESR1 vs. *VDR*						(30,31)
rs1856057	ESR1	rs2239180	VDR	0.048	8.26×10^{-3}	
rs7740686	ESR1	rs2239180	VDR	0.035	0.011	
rs7763637	ESR1	rs2239180	VDR	0.037	0.011	
rs2046210	ESR1	rs2239180	VDR	0.043	0.030	
rs13207030	ESR1	rs1544410	VDR	0.045	0.032	
rs851998	ESR1	rs2525046	VDR	0.026	0.039	
ESR1 vs. *COL1A1*						(32)
rs2982561	ESR1	rs2075555	COL1A1	0.014	0.037	
rs2207396	ESR1	rs17639446	COL1A1	0.047	0.050	
ESR1 vs. *ESR2*						(38)
rs3003917	ESR1	rs8017441	ESR2	0.044	9.73×10^{-3}	
rs3003917	ESR1	rs2987983	ESR2	0.044	0.026	
rs3003917	ESR1	rs3020444	ESR2	0.035	0.027	
rs2248586	ESR1	rs1256056	ESR2	0.049	0.021	
rs3020393	ESR1	rs8017441	ESR2	0.033	0.024	
ESR1 vs. *IL6*						(37)
rs851998	ESR1	rs2069837	IL6	7.27×10^{-3}	0.042	
rs980280	ESR1	rs2069837	IL6	0.017	0.047	
rs3798577	ESR1	rs2066992	IL6	0.021	0.026	
rs11155819	ESR1	rs2069835	IL6	0.037	0.039	
OPG vs. *RANKL*						(35,41)
rs11775992	OPG	rs9533128	RANKL	2.46×10^{-3}	0.050	
rs4355804	OPG	rs9533128	RANKL	2.98×10^{-3}	0.050	

SNP 1	Gene 1	SNP 2	Gene 2	Kansas City sample	Omaha sample	Reference
				Interaction *P*	Interaction *P*	
rs3134079	OPG	rs1886214	RANKL	4.74×10^{-3}	0.012	
rs11775992	OPG	rs9533103	RANKL	5.37×10^{-3}	0.035	
rs4355804	OPG	rs9533103	RANKL	6.48×10^{-3}	0.028	
rs7823265	OPG	rs7491228	RANKL	7.74×10^{-3}	0.012	
rs7823265	OPG	rs9594780	RANKL	8.04×10^{-3}	0.017	
rs3890832	OPG	rs9533103	RANKL	0.011	0.036	
rs1485289	OPG	rs912100	RANKL	0.014	0.044	
rs1485289	OPG	rs430586	RANKL	0.021	0.048	
rs1493942	OPG	rs7491228	RANKL	0.023	8.16×10^{-3}	
rs1493942	OPG	rs9594780	RANKL	0.024	0.011	
rs12545780	OPG	rs7491228	RANKL	0.024	4.93×10^{-3}	
rs12541149	OPG	rs7491228	RANKL	0.024	5.12×10^{-3}	
rs12545780	OPG	rs9594780	RANKL	0.025	7.62×10^{-3}	
rs12541149	OPG	rs9594780	RANKL	0.025	7.89×10^{-3}	
rs3134078	OPG	rs1886214	RANKL	0.029	0.012	
rs11573897	OPG	rs9533103	RANKL	0.032	0.048	
rs11573870	OPG	rs9533103	RANKL	0.033	0.047	
RANK vs. *RANKL*						(40,41)
rs17069845	RANK	rs17536328	RANKL	0.028	0.047	
rs17069845	RANK	rs9525641	RANKL	0.047	0.037	

Note: SNP pairs with interaction $P < 0.05$ both in the Kansas City and Omaha samples were included.