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Robust and Comprehensive Analysis of 20 Osteoporosis Candidate Genes by Very High-Density Single-Nucleotide Polymorphism Screen Among 405 White Nuclear Families Identified Significant Association and Gene–Gene Interaction

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Abstract

Many “novel” osteoporosis candidate genes have been proposed in recent years. To advance our knowledge of their roles in osteoporosis, we screened 20 such genes using a set of high-density SNPs in a large family-based study. Our efforts led to the prioritization of those osteoporosis genes and the detection of gene–gene interactions.

Introduction—We performed large-scale family-based association analyses of 20 novel osteoporosis candidate genes using 277 single nucleotide polymorphisms (SNPs) for the quantitative trait BMD variation and the qualitative trait osteoporosis (OP) at three clinically important skeletal sites: spine, hip, and ultradistal radius (UD).

Materials and Methods—One thousand eight hundred seventy-three subjects from 405 white nuclear families were genotyped and analyzed with an average density of one SNP per 4 kb across the 20 genes. We conducted association analyses by SNP- and haplotype-based family-based association test (FBAT) and performed gene–gene interaction analyses using multianalytic approaches such as multifactor-dimensionality reduction (MDR) and conditional logistic regression.

Results and Conclusions—We detected four genes (*DBP*, *LRP5*, *CYP17*, and *RANK*) that showed highly suggestive associations (10,000-permutation derived empirical global $p \leq 0.01$) with spine BMD/OP; four genes (*CYP19*, *RANK*, *RANKL*, and *CYP17*) highly suggestive for hip BMD/OP; and four genes (*CYP19*, *BMP2*, *RANK*, and *TNFR2*) highly suggestive for UD BMD/OP. The associations between *BMP2* with UD BMD and those between *RANK* with OP at the spine, hip, and UD also met the experiment-wide stringent criterion (empirical global $p \leq 0.0007$). Sex-stratified analyses further showed that some of the significant associations in the total sample were driven by either male or female subjects. In addition, we identified and validated a two-locus gene–gene

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interaction model involving *GCR* and *ESR2*, for which prior biological evidence exists. Our results suggested the prioritization of osteoporosis candidate genes from among the many proposed in recent years and revealed the significant gene–gene interaction effects influencing osteoporosis risk.

Keywords

osteoporosis; BMD; single nucleotide polymorphism; haplotype; association; gene–gene interaction

INTRODUCTION

Osteoporosis is characterized by low bone mass and deteriorated bone microarchitecture leading to the increase in bone brittleness and susceptibility to fractures. Its etiology is complex and involves a wide spectrum of endogenous (genetic and hormonal) and environmental factors. In osteoporosis genetics, BMD is the predominant study phenotype because it has a high heritability of ~70%,⁽¹⁾ and low BMD can serve as a good surrogate for osteoporosis and low/no-trauma fractures.⁽²⁾ Linkage evidence for BMD loci has been detected across every human chromosome. However, no consensus stable BMD-linkage regions have been established. In addition, >50 candidate genes have been associated with various bone phenotypes—mainly BMD. However, similar to linkage studies, no conclusive consensus associations have been found until now, nor have any causal variants been identified definitely.

Factors that may significantly contribute to the inconsistencies in osteoporosis genetic studies include inadequate statistical power, population stratification, genetic heterogeneity, experimental errors, and limited coverage of candidate genes or genomic regions. Ignoring gene–gene interaction (i.e., epistasis) also can contribute to the failure of replicating single-locus findings. To minimize the above problems, we performed a large-scale family-based association study, in which 1873 whites from 405 nuclear families (Table 1) were genotyped and analyzed with a high-density set of single nucleotide polymorphisms (SNPs) across 20 osteoporosis (OP) candidate genes. We studied human OP at three clinically important skeletal sites, the spine, hip, and ultradistal radius (UD), either as a quantitative trait, BMD variation, or as a qualitative trait, OP. We exploited the advanced and more-affordable multiplex SNP genotyping technology (Illumina BeadArray system) and the greatly enriched public SNP information to achieve a more complete coverage of the genetic variation of the selected candidate genes to increase the relative power to detect associations. Gene–gene interactions were studied as well using multianalytic approaches for model selection and validation, with the aim of distinguishing true epistatic interactions from joint effects or chance findings. Our strategy yielded a number of interesting findings in the osteoporosis genetics field.

MATERIALS AND METHODS

Subjects

The study was approved by the Creighton University Institutional Review Board. Signed informed-consent documents were obtained from all study participants before they entered the study. People with chronic diseases and conditions that might potentially affect bone mass, structure, or metabolism were excluded as detailed before.⁽³⁾ All of the 1873 participants from 405 nuclear families were U.S. whites of European origin and recruited for BMD study by advertising. The general related characteristics of them are listed in Table 1. The sample composition was the same as our previous study,⁽³⁾ yielding 1512 sib pairs and 2266 parent-offspring pairs in total.

BMD values at spine, hip, and UD were measured using a Hologic QDR 2000 + or 4500 DXA (Hologic, Bedford, MA, USA). Both machines were calibrated daily. The CV values of the DXA measurements for BMD were 1.87% on Hologic 2000 + and 1.98% on Hologic 4500,

respectively. At each skeletal site (spine, hip, or UD), the qualitative osteoporosis phenotype (denoted as “OP”) was defined by low BMD,⁽⁴⁾ namely BMD Z scores (adjusted by significant covariates including age, sex, height, and weight) within the lowest 10% of the population distribution. We also defined OP at each site following the WHO criterion of BMD T score less than -2.5 to cross-validate the association results with those obtained from Z scores.

Gene and SNP selection

We determined our list of osteoporosis candidate genes for study on the basis of the following criteria: (1) evidence of significant linkage and/or association from recent studies of osteoporosis, (2) biological significance, namely the chosen genes are involved in osteoblast or osteoclast proliferation, differentiation, and activation in bone-related hormone (e.g., calcitonin, estrogen, steroid, vitamin D) metabolism or calcium homeostasis in vivo or in bone matrix formation or degradation in vivo, and (3) evidence of importance to bone suggested by the functional genomics or molecular genetic studies. The full names of the studied genes are tabulated in Table 2. They were located across 17 chromosomes in humans (Table 3).

We selected SNPs mainly according to the public information available in dbSNP (genome build 34; <http://www.ncbi.nlm.nih.gov/Omim/>). An initial list of 384 SNPs in and around our candidate genes was determined on the basis of the following criteria: (1) validation status, especially in whites, (2) an average density of one SNP per 3 kb, (3) degree of heterozygosity (i.e., minor allele frequencies [MAF] > 0.05), (4) functional relevance and importance, and (5) reported to dbSNP by various sources of confirmation. Furthermore, Illumina (San Diego, CA, USA) helped us revise the list by screening all of the SNPs through a proprietary algorithm that predicts performance on the Illumina platform. Our final pre-experiment set of 384 SNPs contained 15 coding SNPs: 10 synonymous and 5 nonsynonymous changes.

Genotyping

Genomic DNA was extracted from whole blood using a commercial isolation kit (Gentra Systems, Minneapolis, MN, USA). DNA concentration was assessed by a DU530 UV/VIS Spectrophotometer (Beckman Coulter, Fullerton, CA, USA). We contracted the genotyping work to Illumina, which conducted high-throughput genotyping using their Integrated BeadArray System. We organized all of the 1932 DNA samples (including duplicates; each delivered for 4 μg with final concentration of 100 $\text{ng}/\mu\text{l}$) into 21 96-well barcoded DNA microtiter plates. Finally, we received 620,308 genotypes from Illumina, each with a quality metric “GC_Score” indicating reliability of genotypes called. The allele frequencies for each SNP were estimated in all the subjects of the nuclear families through a maximum likelihood method implemented in the program SOLAR (<http://www.sfbr.org/sfbr/public/software/solar>). Hardy-Weinberg equilibrium was tested using the PEDSTATS procedure embedded in Merlin (<http://www.sph.umich.edu/csg/abecasis/Merlin/>).

According to Illumina, the overall sample success rate, locus success rate, and genotype call rate were 98.24% (1898/1932), 85.16% (327/384), and 99.95% (620,308/620,646), respectively. The reproducibility rate as revealed through blind duplicating was 100%. Of the 327 successfully genotyped SNPs, 50 were discarded (12 were monomorphic, 10 significantly departed from Hardy-Weinberg equilibrium [HWE] at the $p < 0.01$ level among parents and 28 had MAF $< 5\%$). The GC_Scores across all SNP genotypes ranged from 0.53 to 0.97, with a mean \pm SD of 0.86 ± 0.08 . Thus, we incorporated a total of 277 (84.7%) of the original 327 Illumina-produced SNPs into the final analyses. The detailed information of all the 277 SNPs is shown in Table A1 (available online at <http://orlinux.creighton.edu/donghai.htm>).

PedCheck⁽⁵⁾ was used to check Mendelian consistency of SNP genotype data, and any inconsistent genotypes were removed. The error checking option embedded in Merlin⁽⁶⁾ was run to identify and disregard the genotypes flanking excessive recombinants, further reducing genotyping errors. Less than 0.02% of the overall 525,483 genotypes analyzed (<105) were omitted because of the violation of any of the above two rules.

Statistical analysis

Population haplotypes and their frequencies were inferred for each of the 20 genes using PHASE v2.1.1 (<http://www.stat.washington.edu/stephens/software.html>) software among the group of 703 unrelated parents. Based on the inferred haplotype data, we used HaploBlockFinder (<http://cgi.uc.edu/cgi-bin/kzhang/haploBlockFinder.cgi>) to identify block structures and select haplotype-tagging SNPs (htSNPs) of each candidate gene.⁽⁷⁾ To generate graphical representation of linkage disequilibrium (LD) structure as measured by $|D'|$, we adopted the program Haploview⁽⁸⁾ (<http://www.broad.mit.edu/mpg/haploview/index.php>), which yielded similar haplotype block structures compared with HaploBlockFinder.

We conducted family-based association test (FBAT)^(9,10) (<http://biosun1.harvard.edu/~fbat/fbat.htm>) for (1) the quantitative trait BMD residuals adjusted by significant covariates including age, sex, height, and weight and (2) the qualitative OP phenotypes defined by the criteria of Z or T scores. The reason of studying OP is that genes controlling low BMD in families that are significant enough to cause osteoporotic fracture may have no effect on normal BMD variation⁽¹¹⁾ and therefore will be missed if only BMD is studied. Simultaneously testing associations with both BMD and OP will thus deepen our understanding of the bone effects of the studied genes. The haplotype version of FBAT (HBAT)⁽¹²⁾ was performed to obtain empirical global p values for single SNP and haplotype markers (with adjustment for all possible alleles or haplotypes) by means of the Monte-Carlo permutation procedures implemented in HBAT (10,000 permutations were conducted). Haplotype analyses were conducted in two ways: (1) using sliding windows defined by four SNPs consecutively across each gene and (2) using haplotypes constructed by htSNPs that are selected by HaploBlockFinder. Adopting both approaches may better exploit the information contained in haplotypes. In addition, we conducted the global association test between marker genotypes at each SNP locus with the studied phenotypes using the $-g$ flag in FBAT. All of the above statistical procedures were also done in the male and female subsamples separately to detect the potential sex linked bone effects of the studied genes.

It is difficult to correct the complex multiple testing issues in this study because of the biological interdependence of the selected candidate genes and the various correlations (LD) over all the SNPs. Therefore, we chose to rank the results for each phenotype separately by empirical global p values following a recent study of the same nature.⁽¹³⁾ The genes with any marker (SNP or haplotype) having empirical global p values in the range of (0.01, 0.05) or ≤ 0.01 were defined as “suggestive” or “highly suggestive,” respectively, for the corresponding phenotypes.

We used the multifactor dimensionality reduction (MDR; <http://www.epistasis.org>) method to test for potential high-order gene–gene interactions⁽¹⁴⁾ influencing OP risk and thereby to provide specific interaction models for further confirmation. Briefly, MDR first pooled multilocus genotypes with high dimensions into only one dimension; it combined “cross-validation” and “permutation-testing” procedures to minimize false-positive results and evaluate the model fitness by multiple examination of the data.⁽¹⁴⁾ Cross-validation consistency and prediction error were calculated for each combination of a pool of genetic polymorphisms. The final best model was selected as the one having the maximal cross-validation consistency and minimal prediction error. If the two criteria could not be met simultaneously, the model meeting any one of the criteria and involving the fewest loci/factors was chosen as the best. The statistical significance was assessed by comparing the average prediction error from the

observed data with the distribution of average prediction errors under the null hypothesis of no associations derived empirically from 1000 permutations. The null hypothesis was rejected when the permutation-derived Monte Carlo $p \leq 0.05$.

In this study, to generate a balanced case-control sample used in MDR, we selected 143 cases and 143 controls from the 405 families. All of the 286 subjects were independent in terms of family relatedness. Cases were the subjects having OP at any of the three studied skeletal sites (spine, hip, and UD). Here, cases were defined based on all the skeletal sites instead of separately at each site as in FBAT. Such an approach had the advantage of increasing the number of cases to improve the power of MDR while also being feasible in osteoporosis research. Controls were the subjects having no OP at any of the skeletal sites examined.

We applied 137 htSNPs representing the 20 genes (see *Descriptive analyses*) into the MDR analyses. Because true effects can be overwhelmed when many markers are analyzed, we adopted the staged approach to study the extremely large amount of combinations among the 137 loci. First we tested interactions using MDR within each of the five gene groups (Table 2) separately; the markers of the best model from each group were selected to build the final cross-group model. The much reduced number of markers for the final MDR analysis guarantees reasonable power to detect potential gene–gene interaction effects. To exclude the possibility that the significant final best models for each group identified by MDR represent only the gene–gene joint effects driven by the main effects from the component loci instead of the epistatic interactions, conditional logistic regression was performed using SPSS (version 13.0 for windows). If an interaction term is not significant ($p > 0.05$) by logistic regression, the corresponding significant MDR interaction model could be simply caused by the additive main effects from the component loci.

RESULTS

Descriptive analyses

Table 3 summarizes the characteristics of the 20 candidate genes. These genes range in size from 3 to 254 kb, and the range of the number of SNPs per gene was 4–29, with an average of 14 SNPs per gene and an average density of one SNP per 4.0 kb. We summarized the information for all of the 277 analyzed SNPs such as their names, cytogenetic position, locations on dbSNP build 34, and MAF into Table A1. The average heterozygosity across all the 277 SNPs was 0.37 and their MAFs were approximately uniformly distributed (see Figure A1, available online at <http://orlinux.creighton.edu/donghai.htm>), showing that our selected SNPs were informative.

We identified 137 htSNPs representing 73 blocks across the 20 genes in total. SNPs that could not be classified into any LD block containing more than one SNP were considered as a separate block of size 1 (there were 20 such “orphan” SNPs in our data set). The average length of the 53 blocks composed of more than one SNP was 18.1 kb, ranging from 2.5 to 106.4 kb. The average number of blocks per gene was 3.7, and the range was one to seven blocks per gene. We plotted graphs of pairwise LD values and haplotype block delineations for each gene in Figure A2 (available online at <http://orlinux.creighton.edu/donghai.htm>).

Association analyses in the total sample

Figures 1–3 show the empirical global p values for single SNP and haplotype sliding window (defined by four SNPs consecutively across each gene) analyses for all of the 277 SNPs for BMD variation at the three studied skeletal sites, respectively. For spine BMD, the highly suggestive genes were *DBP* and *LRP5*, whereas the suggestive genes were *TNFR2*, *BMP2*, *CYP19*, and *RANKL* (Table 3; Fig. 1). For hip BMD, *RANKL* was highly suggestive, whereas

BMP2, *LRP5*, *CTR*, and *DBP* were suggestive (Table 3; Fig. 2). For UD BMD, the highly suggestive genes were *BMP2* and *RANK*, whereas the suggestive genes were *DBP*, *CYP19*, *HDC*, *ALOX12*, *CYP17*, and *CTR* (Table 3; Fig. 3). Detailed illustrations of BMD associations with each of the above genes are presented in Fig. 4. Among the significant genes for BMD variations, association results of haplotype blocks defined by the LD structures were similar to those yielded by analyses of single SNPs and haplotype sliding windows (Fig. 4). Within each highly suggestive BMD genes, multiple markers showed empirical global $p \leq 0.05$, with the most significant markers giving $p \leq 0.01$. Specifically, in the *DBP* gene, the most significant $p = 0.003$ was for spine BMD; in *LRP5*, the most significant $p = 0.005$ was for spine BMD. In *RANKL*, the most significant $p = 0.001$ was for hip BMD. In *BMP2*, the most significant $p = 0.0005$ was for UD BMD; in *RANK*, the most significant $p = 0.009$ was for UD BMD (Fig. 4).

Family-based association tests for the qualitative osteoporosis phenotype (OP) detected 8 significant genes (Table 4), which were among the list of 11 significant genes for BMD phenotypes. The association results for OP defined by either Z score or T score were the same. Briefly, for spine OP, *LRP5* and *RANK* were highly suggestive, whereas *CTR*, *CYP19*, *HDC*, and *TNFR2* were suggestive; for hip OP, *CYP19* and *RANK* were highly suggestive, whereas *BMP2*, *HDC*, *LRP5*, and *TNFR2* were suggestive; for UD OP, *BMP2*, *CYP19*, *RANK*, and *TNFR2* were highly suggestive, whereas *CYP17*, *HDC*, and *LRP5* were suggestive.

Overlapping genes significant for both BMD and OP were also found at each skeletal site. In summary, at the spine, *LRP5* was highly suggestive, whereas *CYP19* and *TNFR2* were suggestive for BMD and OP; at the hip, *BMP2* and *LRP5* met the suggestive criterion for BMD and OP; at the UD, *BMP2* and *RANK* were highly suggestive, whereas *CYP17*, *CYP19*, and *HDC* were suggestive for both traits. Within the same genes, the SNPs that were associated with BMD were usually different from those associated with OP, with the exception of *LRP5* (SNP1 and 2 were suggestive for both BMD and OP at hip) and *HDC* (SNP10 and 11 were suggestive for both BMD and OP at UD). However, when considering three kinds of markers together (SNPs, haplotype windows, and blocks), at each skeletal site, the BMD-associated and OP-associated regions overlap for most of the BMD- and OP-double significant genes (Table 4; Fig. 4).

For the three studied BMD phenotypes, the global genotypic association results at each SNP locus (data not shown) matched very well with nearly all the results of the global allelic association tests, with the exception of *CYP17*. In *CYP17*, its SNP1 to SNP5 were all significant for BMD variation at the spine and hip, with global genotypic p values ranging from 0.002 to 0.032 for spine and from 0.004 to 0.027 for hip. However, the corresponding global allelic p values were >0.05 for those marker-phenotype combinations (Fig. 4). For the OP phenotypes studied at the three skeletal sites, all of the global genotypic association results were consistent with the global allelic association results.

In total, we found four, four, and four genes that were highly suggestive for BMD and/or OP (BMD/OP) at the spine, hip, and UD, respectively. At the suggestive significance level, there were an additional six, seven, and six significant genes for BMD/OP at the spine, hip, and UD, respectively (Table 5). All of the 11 significant genes could play a role in BMD regulation and/or OP risk at multiple skeletal sites (Table 5). We also compared our candidate gene findings with previous reports in Table 5.

Sex-stratified association analyses

The significant association results of the analyses in the male and female subsamples for BMD and OP were tabulated in Tables A2 and A3 separately (available online at <http://orclinux.creighton.edu/donghai.htm>) and summarized in Table 6. At the spine, the significant associations of *CTR* and *RANKL* with either BMD variation or OP phenotype

observed in the total sample were mainly driven by the male subjects; the associations for *ALOX12*, *BMP2*, *DBP*, *HDC*, and *TNFR2* were mainly driven by the female subjects; and the associations for *CYP19*, *LRP5*, and *RANK* were present in both male and female subsamples. At the hip, the associations of *ALOX12*, *BMP2*, *CYP19*, *LRP5*, and *TNFR2* with BMD or OP phenotypes observed in the total sample were mainly driven by the female subjects, and the associations of *CTR*, *HDC*, *RANK*, and *RANKL* with hip phenotypes were present in both male and female subjects. At the UD, the associations of *ALOX12*, *CTR*, and *RANKL* with BMD or OP were mainly driven by the male subjects; the associations for *CYP17*, *DBP*, *LRP5*, and *TNFR2* were mainly driven by the female subjects; and the associations for *BMP2*, *CYP19*, *HDC*, and *RANK* were observed in both males and females. In addition, *IGF1* polymorphisms were significantly associated with BMD variations at the spine and hip only in men, whereas *CCR3* polymorphisms were significantly associated with BMD variations at the spine, hip, and UD only in females. The associations of either *IGF1* or *CCR3* with bone phenotypes cannot be found in the total sample.

Gene by gene interaction analyses

MDR was run to detect gene–gene effects influencing OP risk for both within- and cross-group models. All of the best models identified were significant ($p \leq 0.05$ at least). The best within-group models were *RANK4*, *TNFR2_10*, and *TNFR2_12*; *CYP17_1*, *ESR2_4*, and *ESR2_10*; *BMP2_7* and *DBP3*; *GCR9*, *GCR29*, and *HDC10*; and *ALOX12_2* and *SOST1* from groups 1–5, respectively. The final cross-group best model was *GCR9* and *ESR2_4* (Table 7). To evaluate whether the above gene–gene effects are caused by epistatic interactions, conditional logistic regression analyses were conducted. The results supported the significant gene–gene interactions for the best models in group 1 (i.e., *RANK4*, *TNFR2_10*, and *TNFR2_12*), group 4 (i.e., *GCR9*, *GCR29*, and *HDC10*), and the final group (i.e., *GCR9* and *ESR2_4*); the p values for the interaction terms of the group 1, group 4, and the final group were 0.012, 0.022, and 0.047, respectively; the ORs (high risk versus low risk) for the corresponding models were 1.2, 1.1, and 1.3, respectively. The potential gene–gene interactions for the best models of groups 2, 3, and 5 were not supported by the conditional logistic regression analysis. Interestingly, the HBAT analysis among all of the 405 nuclear families for the interacting loci identified by MDR found the significant global association with OP risk for haplotypes reconstructed on the basis of the best cross-group model (*GCR9* and *ESR2_4*; namely rs6188–rs1256061 combination, $p = 0.021$). Two individual haplotypes defined by these two loci were also associated with OP risk ($p = 0.0026$ and 0.050, respectively, for haplotype AA and CA of *GCR9* and *ESR2_4*). Therefore, the gene–gene interaction of the best cross-group model suggested by MDR was supported by both conditional logistic regression and HBAT.

DISCUSSION

To our best knowledge, this study is the first comprehensive study of the allelic, haplotypic, genotypic association, and gene–gene interaction of a large group of osteoporosis candidate genes. This study also had a number of strengths with regard to minimizing the limitations often afflicting association studies in osteoporosis. First, given the sample size adopted, this study has ~90% power to detect association assuming a marker is in strong LD ($r^2 > 0.8$) with a functional mutation locus that accounts for $\geq 2\%$ of phenotypic variation.⁽³⁾ Second, we performed the family-based association study for both quantitative BMD measurements and dichotomous OP phenotypes at various skeletal sites. In the genetic research of either osteoporosis⁽⁴⁾ or other complex diseases such as obesity and hypertension,⁽¹⁵⁾ studying both the major predicative quantitative trait and its related dichotomous qualitative phenotype is gradually becoming common. Third, the experimental errors had been controlled to a very low level according to the Illumina report. Fourth, multianalytic approaches were adopted to facilitate interpreting our findings and comparing with previous work. Fifth, we achieved much

greater coverage than previously reported for the majority of the studied candidate genes. Across the 20 genes, an average of ~14 SNPs were analyzed per gene; the average density of one SNP for 4 kb was achieved; almost every gene and its proximal regulatory regions (5'-promoter, 3'-untranslated region [3'-UTR]) were fully covered by LD blocks detectable with the studied SNPs. In addition, our LD findings provided valuable information about the LD structures across the 20 studied osteoporosis candidate genes for the white population of European origin. We found the mean block size to be 18.1 kb in our population with the working SNP density of 4 kb, which is smaller than the previous reported size of 22 kb in the European population.⁽¹⁶⁾ This may be because of the more sparse SNP density of 7.8 kb genotyped in their study.⁽¹⁶⁾ Finally, we studied the potential complex gene–gene interactions influencing OP using a large number of tagging SNPs. Additional significant insights were gained in such interaction analyses as shown.

Prioritization

There is currently no appropriate and established method to correct for the highly correlated tests in our study. One suggestion is to adjust for multiple testing by the number of LD blocks,⁽¹⁷⁾ and this approach has been used in practice.⁽¹³⁾ Given the assumption that 73 blocks identified for the 20 genes represent independent information, the corrected single-test threshold may be $\alpha = 0.05/73 = 0.0007$ for experiment-wide significance at the $\alpha = 0.05$ level. SNP2 in *BMP2* (rs1980499) met this stringent criterion for UD BMD (Fig. 4); SNP7 in *RANK* (rs11664594) and its associated haplotype windows and block 3 reached this level for OP at the spine, hip, and UD simultaneously (Table 4). In addition, haplotype window 1 of *RANK* covering SNP1 to SNP4 reached the strict threshold for hip OP (RANK_SW1; Table 4). However, the above adjustment could be too strict to allow for the identification of significant association signals. To best present and explain our results, we ranked our findings according to the empirical global *p* values and focused on highly suggestive genes. Whereas there may still be false-positive results, our approaches provide a relatively comprehensive and reliable list of novel osteoporosis candidate genes prioritized by their significance levels for further replication and functionality studies.

Genes experiment-wide significant for BMD/OP phenotypes

We found that *BMP2* block 1 was highly suggestive for UD BMD; particularly, the SNP2 of *BMP2* reached the experiment-wide significance level (Fig. 4). *BMP2* is biologically important to bone because of its crucial role in bone formation and osteoblast differentiation. It was reported as a susceptibility gene for osteoporotic fractures and low BMD in Icelandic and Danish populations.⁽¹¹⁾ Because SNP2 lies in the proximal promoter region of *BMP2*, its variants per se or highly correlated (i.e., in strong LD) functional variants could influence the mRNA transcription rate of *BMP2*, which may finally affect UD BMD variation in whites. In addition, we detected the highly suggestive association between SNP7 of *BMP2* with UD OP (Table 4). Residing within 3'-UTR, SNP7 variants may directly or indirectly influence the mRNA stability of *BMP2*, similar to the role of 3'-UTR functional polymorphisms within the *VDR* gene.⁽¹⁸⁾

Another experiment-wide significant gene is *RANK*, which is the central regulator of osteoclast development and function, bone resorption, and calcium homeostasis. The unanimous experiment-wide significant associations of SNP7 and its related haplotypes with OP risk at the spine, hip, and UD strongly support the existence of nearby causal functional loci that may lie within the exon 3/intron 3 boundary or exon 3, whose causal alleles could result in abnormal *RANK* mRNA splicing or protein structure and ultimately lead to increased OP risk independent of skeletal sites. Taken together, it is clear that the surrounding areas of SNP7 should be thoroughly studied to dissect out the associated causal variants of *RANK*.

Genes highly suggestive for BMD/OP

Two genes important to estrogen biosynthesis, *CYP17* and *CYP19*, were highly suggestive for BMD and OP at various skeletal sites. The *CYP17* gene encodes cytochrome P450c17 α —the enzyme with both 17 α -hydroxylase and 17,20-lyase activities crucial for the biosynthesis of gonadal hormones like estrogens and androgens, which have beneficial effects on bone remodeling. Deleterious mutations in *CYP17* can cause retarded skeletal growth and diffuse osteoporosis.⁽¹⁹⁾ The genotypes of T²⁷-C (also denoted as -34TC) in the 5'-UTR of *CYP17* had been associated with BMD.^(20,21) Although that SNP was not directly studied here, the highly suggestive associations between the genotypes of *CYP17* SNPs 1–4 with spine and hip BMD (empirical global genotypic *p* values ranged from 0.002 to 0.004) still lent some support to the previous studies, because the entire *CYP17* gene is actually in one block (Fig. 4). The data from ours and others all suggested that the genetic effects of *CYP17* on BMD variations at spine and hip may be dominant or recessive instead of additive.

The *CYP19* gene encodes the aromatase enzyme that converts androgens into estrogens and is crucial for normal bone development. Two commonly studied *CYP19* polymorphisms are (1) the TTTA repeat polymorphism in intron 4 and (2) the silent polymorphism (GA at Val⁸⁰) in exon 3. These two polymorphisms were in nearly complete LD ($|D'| = 0.98$)⁽²²⁾ and previously associated with osteoporosis risk⁽²³⁾ and BMD at the total hip, hip component sites, or radius.^(22,24) The Val⁸⁰ polymorphism was also analyzed in this study, which is SNP7 (rs700518) and suggestively significant for hip OP (CYP19_SW7; Table 4). The highly suggestive polymorphisms for OP at hip and UD in our study were SNP11 (rs17523527) and its associated haplotypes within the 5'-UTR. (The translation of *CYP19* starts from exon 2. The *CYP19* 5'-UTR contains at least nine variants of exon 1 and spans the region from SNP9 to SNP28 in this study. For simplicity, we only showed the most distant exon 1 that is ~90 kb upstream from exon 2 in Fig. 4.) The association evidence of *CYP19* 5'-UTR polymorphisms with osteoporosis phenotypes has emerged recently.^(22,25,26) The underlying mechanism could be related to the differences in *CYP19* transcription rate caused by the hidden functional variants.

DBP and *LRP5* met the highly suggestive criterion for spine BMD/OP in this study. *DBP* is a key factor for regulating calcium homeostasis. Interestingly, the highly suggestive *DBP* haplotype window 3 (DBP_SW3) in block 1 contains the (TAAA)(n)-Alu polymorphism that was previously associated with the same trait, spine BMD, in the same ethnic group, whites.^(27,28) In addition, we detected a novel highly suggestive variant, SNP2 (rs17467825; Fig. 4), within the 3'-UTR of the *DBP* gene. This SNP could be in strong LD with causal functional variants (or be such variant per se) affecting the mRNA stability of *DBP*, similar to the recently identified role of *VDR* 3'-UTR polymorphisms in influencing both *VDR* mRNA stability and osteoporotic fracture risk.⁽¹⁸⁾

LRP5 encodes a transmembrane protein mediating WNT- β -catenin signaling pathway important in the regulation of bone metabolism. Its block 5 polymorphisms were highly suggestive for spine BMD variation (Fig. 4). The block 5 region ranged from intron 7 to intron 19 and contained a number of spine BMD-associated polymorphisms reported before.^(29–33) We also identified a new 3'-UTR polymorphism (SNP26; rs7105218) of *LRP5* that nearly reached the highly suggestive threshold for spine BMD (Fig. 4), the functionality of which may also resemble the *VDR* 3'-UTR polymorphisms.⁽¹⁸⁾ In addition, two polymorphisms in intron 1, SNP6 (rs634008) and SNP7 (rs312023), showed highly suggestive association with spine OP (Table 4), which was partially supported by a previously study.⁽³¹⁾

RANKL is essential for osteoclastogenesis by binding and activating RANK. Highly suggestive associations with hip BMD were detected for two *RANKL* SNPs (SNP3 and 8, namely rs9525641 and rs3742257) and all of the *RANKL* haplotypes (Fig. 4). The recently reported

SNP rs9594782 that was highly significant for extremely low hip BMD⁽⁴⁾ was localized to the block 1 of *RANKL* and near SNP3—the most and highly significant SNP for hip BMD in this study. No prior reports studied *RANKL* block 2 region, whose polymorphisms were also highly suggestive for hip BMD as shown by us (Fig. 4). The ubiquitous highly suggestive associations of *RANKL* with hip BMD indicate that the gene-based replication and functional analysis is needed to unravel the causal variants within *RANKL*.

TNFR2 is critical for mediating the effect of TNF- α on osteoclastogenesis. In *TNFR2*, we detected a haplotype window, TNFR2_SW5, that was highly suggestive for UD OP (Table 4). This window included two BMD-associated variants reported by others: exon 6 T676G and intron 4 CA repeat polymorphisms.^(34,35) It was also suggested that the haplotype containing the specific alleles of those two loci may define low BMD phenotype.⁽³⁵⁾ All of those lent support to our finding in *TNFR2*.

Other findings in relation to previous studies

In addition to the highly suggestive results, the suggestive findings, either replicating previous ones or being reported for the first time, were also tabulated in Table 5. For example, the prior associations of the *BMP2* and *CTR* genes with BMD/OP phenotypes at spine and hip^(11,36,37) were mainly supported by the *BMP2* SNP1 (rs235710) within the 5'-promoter region and the *CTR* SNP1 (rs2374634) within the 3'-UTR (Table 4; Fig. 4); the significance of the *LRP5* gene to BMD/OP phenotypes at hip and UD^(32,33,38,39) was mainly supported by SNP6 (rs634008) and SNP7 (rs312023) of *LRP5* (Table 4). Among the novel suggestive findings, the most notable ones were the associations of the *HDC* and *ALOX12* genes with BMD/OP at multiple skeletal sites (Table 5). *HDC* is the only enzyme for the synthesis of human histamine,⁽⁴⁰⁾ which can stimulate osteoclastogenesis and osteoclast activity. A high level of circulating histamine was associated with osteoporosis and excessive bone resorption.⁽⁴¹⁾ Our recent functional genomics study also showed that the expression of the *HDC* gene was significantly upregulated in subjects with low BMD.⁽⁴²⁾ Here we supported those findings by showing the polymorphisms of *HDC*, especially SNP10 (rs12901373) and SNP11 (rs2114447) in the promoter region that may influence *HDC* gene expression, were associated with OP risk at all of the three skeletal sites and BMD variation at UD. Another gene, *ALOX12*, in humans is functionally similar to the mouse *ALOX15* gene because both encode the enzyme of 12/15-lipoxygenase (12/15-LO). *ALOX15* has been established as an important regulator of peak bone mass in mice, suggesting the same bone effect of *ALOX12* in human.⁽⁴³⁾ This study first reported the associations of *ALOX12* markers with human BMD variations at hip and UD. Specifically, the haplotype block covering the entire *ALOX12* gene was associated with hip BMD, whereas SNP2 (rs2073438) in the intron1/exon2 boundary of *ALOX12* was associated with UD BMD. These results partially supported the speculation that *ALOX12* may be a human BMD regulatory gene.^(1,43)

Because associations with p values in the 0.05–0.10 range may reflect lack of power rather than lack of replication, no evidence of association was defined according to the criterion of $p > 0.10$ for all of the tests of a specific gene–phenotype combination. We found no support for two previous associations based on our data (Table 5). First, the significant associations between *TNF* polymorphisms with spine and hip BMD variations observed previously^(44,45) were not supported. However, those studies had very small sample sizes and did not control for population stratification. In addition, their subjects were white women at specific physiological stages such as postmenopause and puberty. Therefore, the lack of replication may reflect that the prior associations were false positive or population specific. Second, the association between *MTHFR* with hip BMD in whites⁽⁴⁶⁾ was not observed. However, this discrepancy was easy to explain because the bone effects of *MTHFR* depend on folate

status,⁽⁴⁶⁾ for which we did not measure. Ignoring such gene–environment interaction could cause lack of power to detect the effect of *MTHFR* on hip BMD variation.

Sex-specific associations

The best examples of sex-specific associations were the associations of *IGF1* and *CCR3* with BMD variations only in men and women, respectively. Interestingly, we previously showed that the gene expression of *CCR3* was significantly upregulated in women with low BMD.⁽⁴²⁾ However, the male-specific association pattern of *IGF1* with BMD is not supported by the previous literature because the *IGF1* gene promoter CA repeat polymorphism has been associated with BMD in both men and women.⁽¹⁾ This may be because of the lack of strong correlation between our testing markers within *IGF1* and the promoter CA repeat polymorphism. Because we only genotyped one *IGF1* SNP in its ~10-kb promoter region, it is very likely that the CA repeat polymorphism was not captured in our marker set. This suggested that our sex-specific results have limitations and should be taken with caution.

Gene–gene interactions for OP

The most promising gene–gene interaction detected was that between *GCR* and *ESR2*. It was identified from the cross-group models and further confirmed by conditional logistic regression and HBAAT. The accumulation of findings across all analyses indicated the existence of epistatic interaction between *GCR* and *ESR2* influencing OP risk. Biological studies also shed some light on explaining this interaction model. In bone, glucocorticoids induce bone resorption, whereas estrogens inhibit it. Therefore, *GCR* and *ESR2* play counteracting yet critical roles in bone metabolism. More interestingly, it has been shown that the molecular cross-talk between the *GCR* and *ESR* signaling pathways exists because the in vivo levels of *GCR* are downregulated by *ESR*-dependent proteasomal degradation.⁽⁴⁷⁾ Thus, the different genotype combinations from these two genes, if representing part of the *GCR–ESR* cross-talk, could result in different *GCR* regulation of downstream gene transcriptions associated with different bone effects. Taken together, the statistical and biological evidence all supported the existence of *GCR–ESR2* epistatic interaction on OP risk.

The other two interactions, one between *RANK* and *TNFR2* and the other between *GCR* and *HDC*, were the significant best models within their respective groups. The biological nature of these two gene–gene interactions that may influence OP risk is not clear and remains an interpretive challenge. Nevertheless, our data provided some clues for future studies.

Other pertinent issues

Several issues and limitations of this study should be considered. First, although the SNP density and block coverage are already very high (Table 3), we concede that not all of the genetic variants of the 20 selected genes were studied. An exhaustive representation of all the possible common variants within those genes will be feasible in the near future by the use of HapMap (<http://www.hapmap.org>) tag-SNPs.⁽⁴⁸⁾ Examining the HapMap tagSNPs that are not in strong LD with our studied SNPs and haplotypes ($r^2 < 0.8$) can close the coverage gaps when conducting further studies on those genes among whites.

Second, similar to any other candidate gene studies on complex diseases/traits, this work only reveals part of the whole genetic architecture underlying the etiology of osteoporosis. With the nearly completion of the International HapMap Project and the rapid improvements in high-throughput SNP genotyping technology, the ultimate solution to completely understanding the genetic basis of osteoporosis may come from the genome-wide association approach in which a dense set of tagSNPs across the whole human genome is genotyped to survey the common genetic variation for a role in osteoporosis or the related heritable quantitative traits such as

BMD. This study, however, represents a precursor to the genome-wide association studies of osteoporosis.

Third, despite the overlapping significant genes for both BMD and OP at each studied skeletal site, the association outcomes for BMD and OP did not completely match. For example, *RANK* was experiment-wide significant for OP at spine and hip but not significant for BMD at those two skeletal sites (Table 4, Fig. 4). On the other hand, *RANKL* was highly suggestive significant for hip BMD but not significant for hip OP (Table 4, Fig. 4). These results supported the recent notion that genes controlling low BMD in families that are significant enough to cause osteoporotic fracture (OF) are not necessarily the same as those that control the normal BMD variation in families.⁽¹¹⁾ Another prior notion—that there are common and specific genetic factors underlying determination of BMD or OP in various skeletal sites⁽⁴⁹⁾—was also supported (Fig. 4). Both of these notions were also supported by the sex-stratified analyses (Tables A2 and A3), implying that (1) different association pattern of a given gene with BMD and OP may not be a function of sex and (2) genetic variants influencing BMD or OP at various distinct skeletal sites exist in both men and women.

Fourth, the best epistatic effect detected for OP is that between *GCR* and *ESR2*—two genes in different yet related signaling pathways and having no main effects individually as shown by FBAT and HBAT (p values for all the markers of *GCR* and *ESR2* were > 0.05). This is not surprising because both theoretical and empirical studies showed the existence of epistasis without any independent main effects.⁽¹⁵⁾ In the research of human diseases such as breast cancer, atrial fibrillation, type 2 diabetes, and hypertension, the statistically significant evidence for epistasis in the absence of detectable independent main effects of any one polymorphism was found.⁽¹⁵⁾ Therefore, such a phenomenon does not seem to be exception in the genetic etiology of human complex diseases.⁽¹⁵⁾ Our examples suggested that some candidate genes may exert their bone effects only through interactions with other genes in either the same or related molecular pathways.

The prioritized 11 genes, plus the gene–gene interactions detected in this study, deserve further study to confirm their importance to OP. In addition, the general importance of these genes to BMD variation and/or OP risk may need to be evaluated in other nonwhite populations. Other important topics such as gene–environment interactions and parent-of-origin effects regarding these genes are also worth pursuing. Eventually, functional genomics and molecular genetics approaches are needed to improve the understanding of the underlying mechanisms driving the statistically significant evidence from the epidemiological studies.

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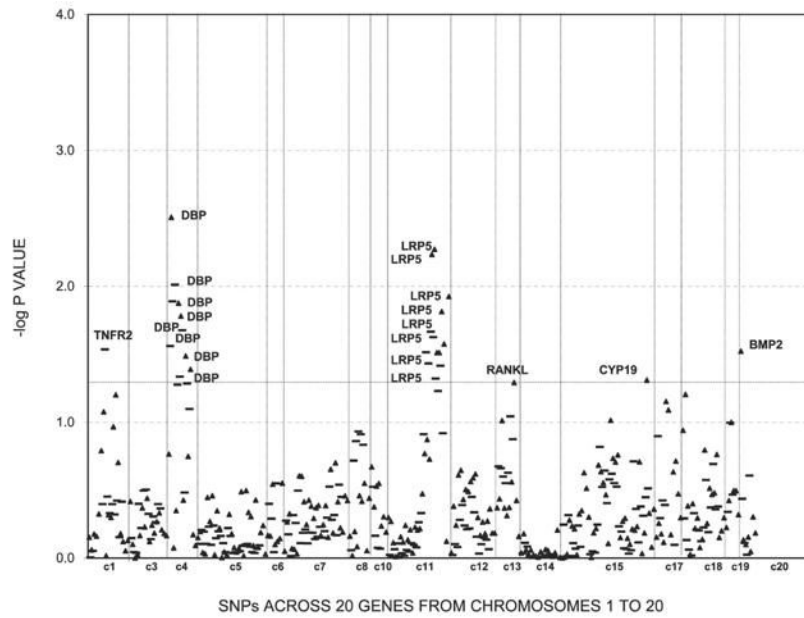


FIG. 1. Association results for spine BMD using single SNP markers and haplotype windows. Empirical global p values from HBAT analyses are calculated after 10,000 permutations. Triangles, SNP results; bold lines, 4-SNP haplotype sliding window results. Any suggestive significant results ($p \leq 0.05$) were marked with associated genes.

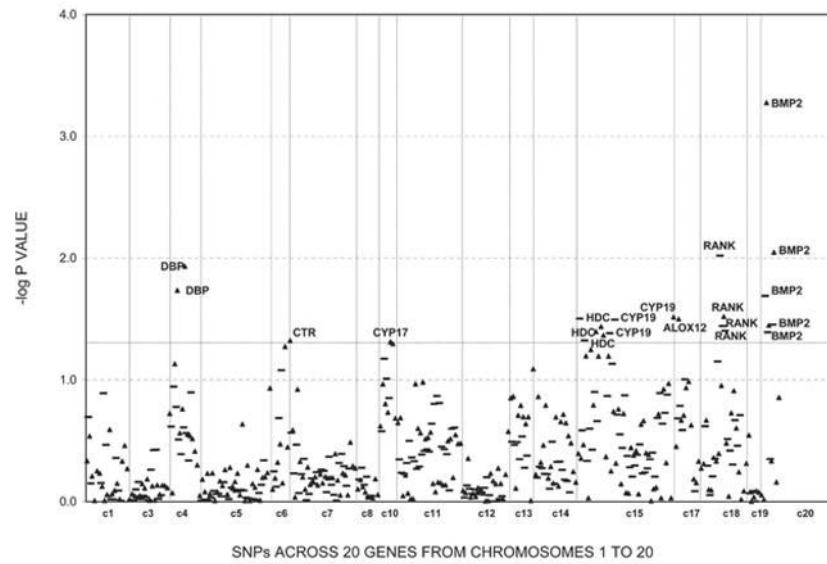
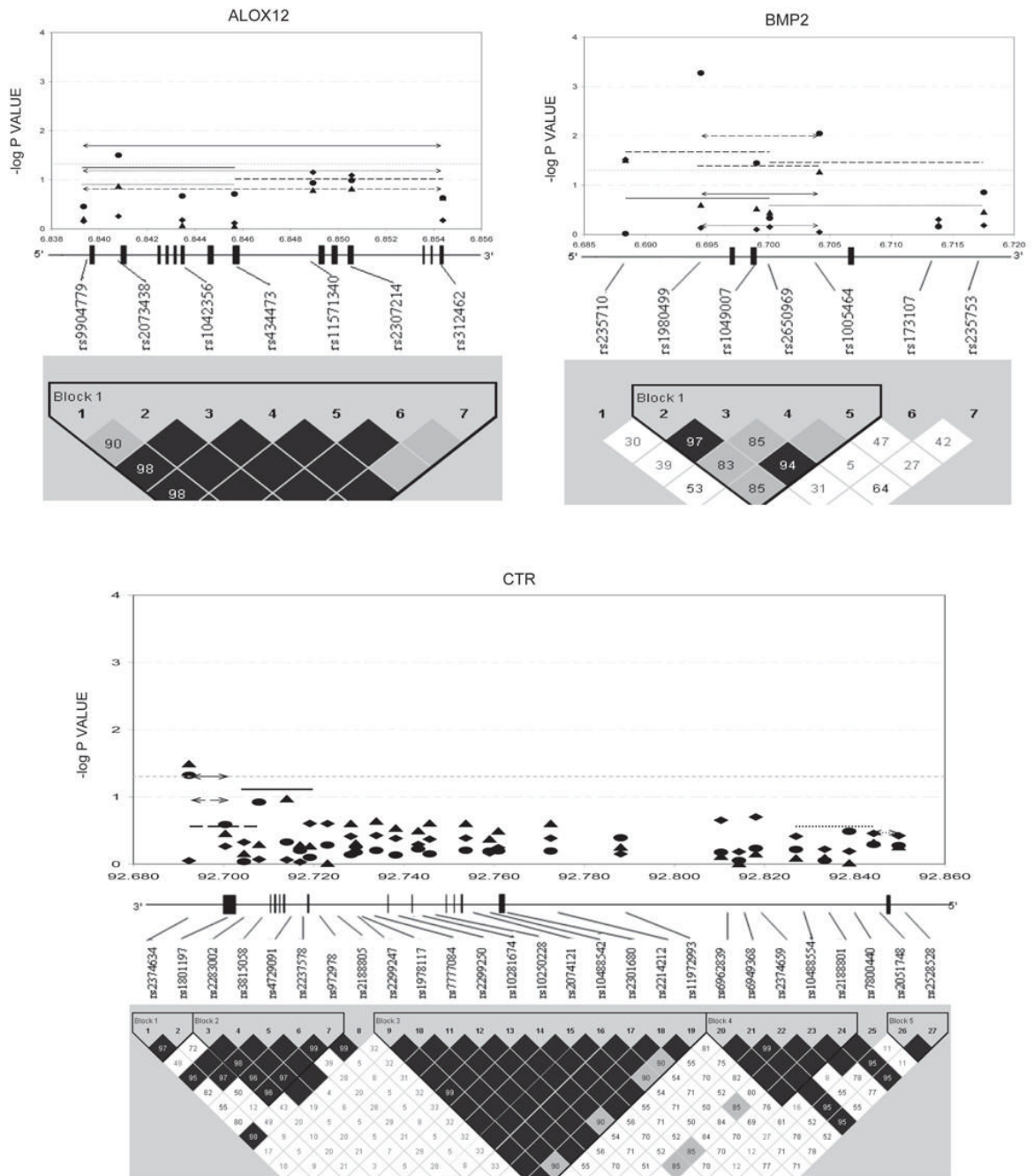
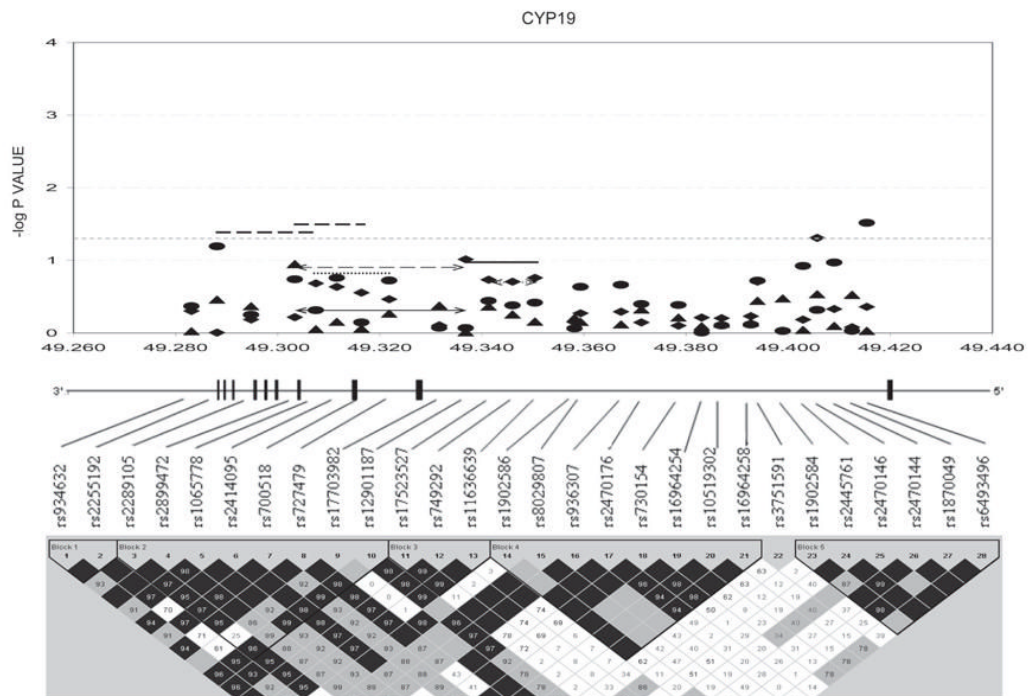
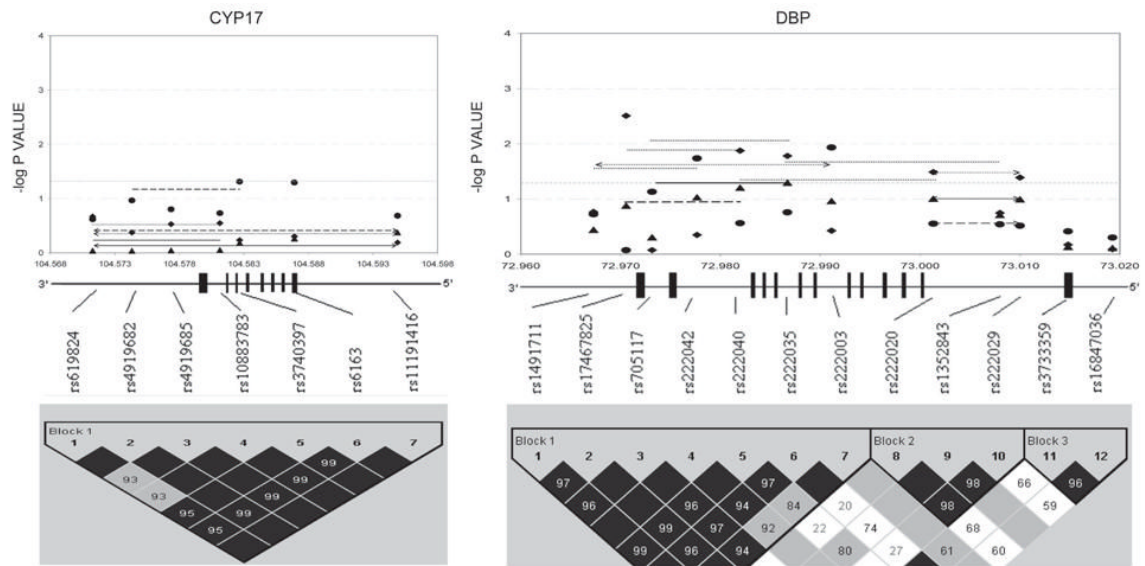


FIG. 3. Association results for UD BMD using single SNP markers and haplotype windows. Empirical global p values from HBAT analyses are calculated after 10,000 permutations. Triangles, SNP results; bold lines, 4-SNP haplotype sliding window results. Any suggestive significant results ($p \leq 0.05$) were marked with associated genes.





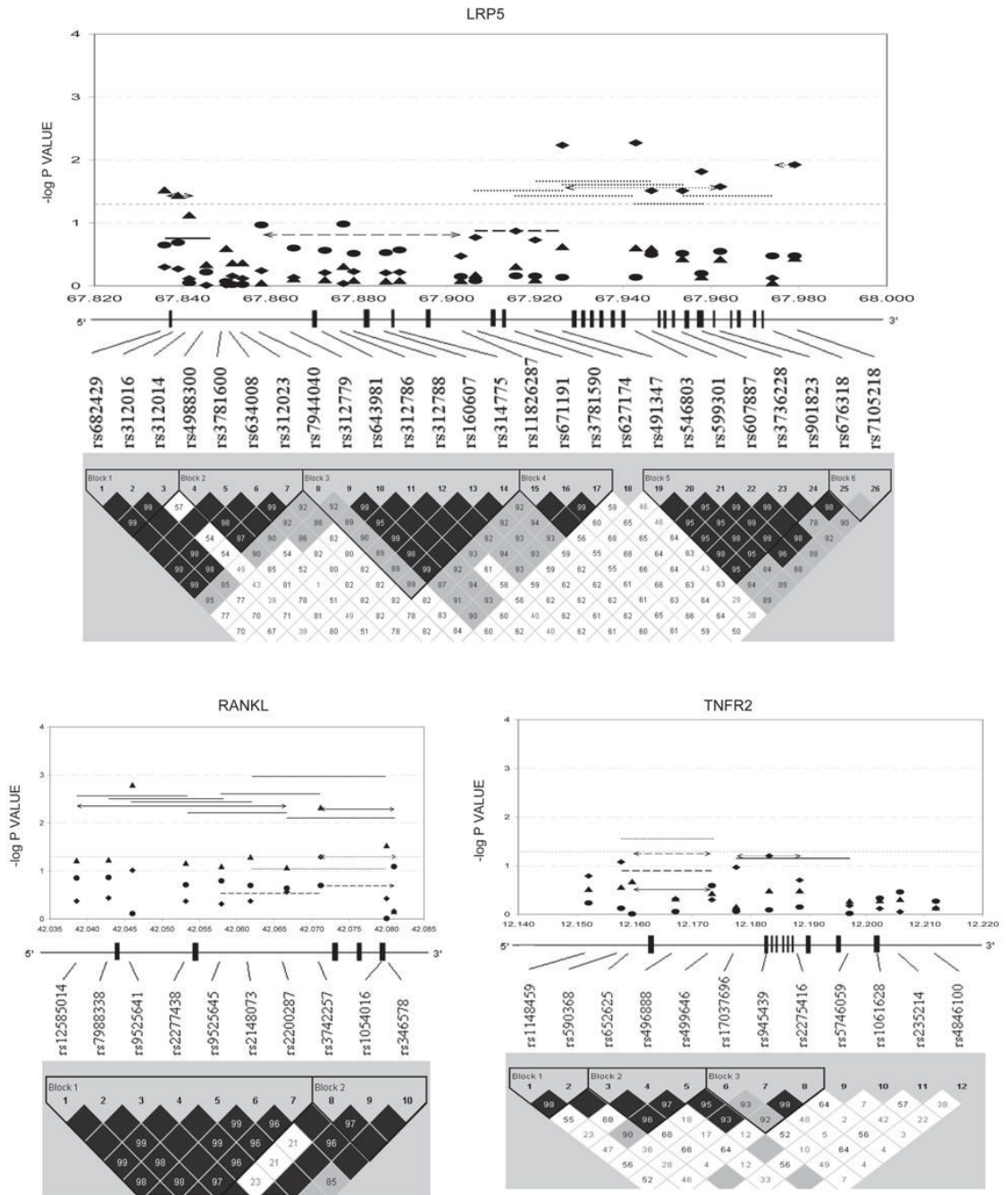


FIG. 4. Gene-specific results for BMD variation. Empirical global p values are plotted. For spine BMD: SNP, diamond; haplotype window, dotted line; haplotype, dotted line with arrow. For hip BMD: SNP, triangle; haplotype window, solid line; haplotype, solid line with arrow. For UD BMD: SNP, circle; haplotype window, long-dashed line; haplotype, long-dashed line with arrow. Only haplotypes with suggestive evidence ($-\log p \geq 1.3$, i.e., $p \leq 0.05$) or haplotypes showing best signals (if no significant haplotypes exist) were plotted. x -axes were in the unit of megabases; vertical bars represent exons.

Table 1
Basic Characteristics of the Study Subjects From the 405 Nuclear Families

Trait	Sons (n = 359)	Daughters (n = 697)	Fathers (n = 308)	Mothers (n = 324)
Age (yr) [*]	35.7 (10.7)	37.6 (10.3)	63.0 (10.7)	62.5 (10.5)
Height (cm) [*]	179.7 (6.9)	165.3 (6.1)	176.5 (6.7)	161.9 (6.3)
Weight (kg) [*]	86.8 (15.0)	70.1 (6.0)	90.2 (15.2)	73.7 (15.4)
Spine BMD (g/cm ²) [*]	1.07 (0.13)	1.05 (0.13)	1.07 (0.18)	0.99 (0.18)
Hip BMD (g/cm ²) [*]	1.08 (0.14)	0.96 (0.13)	1.02 (0.15)	0.89 (0.16)
UD BMD (g/cm ²) [*]	0.53 (0.07)	0.44 (0.06)	0.50 (0.08)	0.41 (0.08)
Menopause [†]	—	5.9 (41)	—	41.4 (133)

Phenotypes are missing for 30 sons, 47 daughters, 52 fathers, and 56 mothers.

^{*}Data are unadjusted means (SD).

[†]Data are the percentages of postmenopausal women (number of such women).

Table 2
Full Names of the 20 Studied Candidate Genes

Gene symbol	Gene full name	Group*
<i>ALOX12</i>	<i>Arachidonate 12-Oxidoreductase</i>	5
<i>BMP2</i>	<i>Bone morphogenetic protein 2</i>	3
<i>CCR3</i>	<i>Chemokine receptor 3</i>	4
<i>CTR</i>	<i>Calcitonin receptor</i>	3
<i>CYP17</i>	<i>17-α-hydroxylase</i>	2
<i>CYP19</i>	<i>Aromatase</i>	2
<i>DBP</i>	<i>Vitamin D-binding protein</i>	3
<i>ESR2</i>	<i>Estrogen receptor-β</i>	2
<i>GCR</i>	<i>Glucocorticoid receptor</i>	4
<i>HDC</i>	<i>Histidine decarboxylase</i>	4
<i>IGF1</i>	<i>Insulin-like growth factor I</i>	3
<i>LRP5</i>	<i>Low-density lipoprotein receptor-related protein 5</i>	3
<i>MTHFR</i>	<i>Methylene tetrahydrofolate reductase</i>	5
<i>OPG</i>	<i>Osteoprotegerin</i>	1
<i>RANK</i>	<i>Receptor activator of NF-κB</i>	1
<i>RANKL</i>	<i>Receptor activator of NF-κB ligand</i>	1
<i>SOST</i>	<i>Sclerostin</i>	5
<i>TGFB1</i>	<i>Transforming growth factor β1</i>	3
<i>TNF</i>	<i>Tumor necrosis factor α</i>	1
<i>TNFR2</i>	<i>Tumor necrosis factor receptor 2</i>	1

* The candidate genes studied could be roughly grouped into five groups according to their roles in bone biology: (1) constituting or regulating RANKL/RANK/OPG signaling pathway, including *OPG*, *RANK*, *RANKL*, *TNF*, and *TNFR2*; (2) involved in estrogen metabolism and function, including *CYP17*, *CYP19*, and *ESR2*; (3) regulating calcium homeostasis, namely *DBP*; mediating the inhibitive effect of calcitonin on bone resorption, namely *CTR*; or promoting bone formation, including *BMP2*, *IGF1*, *LRP5*, and *TGFB1*; (4) implicated in a novel pathophysiological mechanism for osteoporosis by our functional genomics research,⁽⁴²⁾ including *CCR3*, *GCR*, and *HDC*; and (5) decreasing the structural integrity of bone, *MTHFR*, or negatively regulating bone mass, including *ALOX12* and *SOST*.

Table 3

Characteristics of 20 Candidate Genes and SNP and Haplotype Results for Spine, Hip, and UD BMD

Gene symbol	Region	Length (kb)	Exons	SNPs intended	SNPs used	SNFs/ kb	Median D'	Median r^2	LD blocks	Block coverage*	Highly suggestive†	Suggestive‡
<i>MTHFR</i>	1p36.3	21	12	11	5	4.7	0.99	0.05	2	1.33	—	—
<i>TNFR2</i>	1p36.3- p36.2	42	10	16	12	4.6	0.48	0.02	7	1.42	UD OP	Spine BMD, OP at spine and hip
<i>CCR3</i>	3p21.3	144	4	18	16	2.6	1.00	0.10	2	0.31	—	—
<i>DBP</i>	4q12- q13	64	13	15	12	4.0	0.95	0.06	3	0.81	Spine BMD	BMD at hip and UD
<i>GCR</i>	5q31.3	158	10	49	29	4.7	1.00	0.16	5	0.89	—	—
<i>TNF</i>	6p21.3	3	4	10	7	2.4	1.00	0.07	3	6.30	—	—
<i>CTR</i>	7q21.3	150	13	36	27	5.6	0.53	0.09	7	1.05	—	BMD at hip and UD, spine OP
<i>OPG</i>	8q24	29	5	11	9	3.9	0.94	0.20	2	1.35	—	—
<i>CYP17</i>	10q24.3	7	8	7	7	3.0	1.00	0.64	1	3.38	BMD at spine and hip	UD BMD, UD OP
<i>LRP5</i>	11q13.4	137	23	41	26	5.3	0.64	0.18	7	1.04	Spine BMD, spine OP	Hip BMD, OP at hip and UD
<i>IGF1</i>	12q22- q23	85	4	27	19	5.1	0.98	0.16	4	1.20	—	—
<i>RANKL</i>	13q14	45	5	13	10	3.9	0.98	0.17	2	0.94	Hip BMD	Spine BMD
<i>ESR2</i>	14q23.2	254	9	22	17	4.3	0.98	0.47	2	0.30	—	—
<i>CYP19</i>	15q21.1	130	10	33	28	4.6	0.83	0.03	6	1.02	OP at hip and UD	BMD at spine and UD, spine OP
<i>HDC</i>	15q21- q22	24	12	23	11	3.6	0.68	0.12	5	1.79	—	UD BMD, OP at spine, hip, and UD
<i>ALOX12</i>	17p13.1	15	14	9	7	1.9	1.00	0.06	1	1.00	—	BMD at hip and UD
<i>SOST</i>	17q12- q21	5	2	7	4	4.1	0.94	0.48	1	4.13	—	—
<i>RANK</i>	18q22.1	66	10	18	18	3.5	0.48	0.06	7	0.99	UD BMD, OP at spine, hip, and UD	—
<i>TGFB1</i>	19q13.2	52	7	9	6	5.0	1.00	0.03	2	0.67	—	—
<i>BMP2</i>	20p12.3	13	3	9	7	3.6	0.53	0.07	4	2.24	UD BMD, UD OP	BMD at spine and hip, hip OP
Total or average	—	1,444	8.95	384	277	4.0	0.85	0.16	3.65	1.61	—	—

* Fraction of gene length covered by block defined by genotyped SNPs. Genes entirely within a larger block have block coverage > 1.

† Genes were defined as highly suggestive or suggestive significant if any SNP or haplotype showed $p \leq 0.01$ or $0.01 < p \leq 0.05$, respectively.

Table 4
Association to OP as a Qualitative Trait at Spine, Hip, and UD in HBBAT Analyses

Marker ID	Component SNP(s) [*]	Empirical global p value [†]			Marker ID	Component SNP(s)	Empirical global p value		
		Spine OP	Hip OP	UD OP			Spine OP	Hip OP	UD OP
BMP2_SNP7	rs235753	0.130	0.050	0.005	LRP5_SW4	LRP5_4-7	0.018	0.092	0.096
CTR_SNP1	rs2374634	0.050	0.183	0.086	LRP5_SW5	LRP5_5-8	0.015	0.056	0.050
CTR_SNP2	rs1801197	0.049	0.150	0.104	LRP5_SW6	LRP5_6-9	0.010	0.032	0.053
CTR_SNP3	rs2283002	0.034	0.118	0.096	LRP5_SW7	LRP5_7-10	0.023	0.090	0.096
CYP17_SW1	CYP17_1-4	0.068	0.061	0.032	LRP5_Block2	LRP5_4-7	0.050	0.163	0.216
CYP19_SNP2	rs2255192	0.028	0.014	0.074	RANK_SNP1	rs4941125	0.024	0.009	0.004
CYP19_SNP3	rs2289105	0.044	0.044	0.040	RANK_SNP2	rs7235803	0.131	0.049	0.025
CYP19_SNP10	rs12901187	0.102	0.037	0.060	RANK_SNP4	rs8086340	0.001	0.00097	0.002
CYP19_SNP11	rs17523527	0.033	0.007	0.010	RANK_SNP7	rs11664594	0.00017	0.00014	0.00026
CYP19_SNP12	rs749292	0.047	0.015	0.020	RANK_SNP10	rs4303637	0.086	0.106	0.044
CYP19_SNP13	rs11636639	0.045	0.018	0.019	RANK_SNP15	rs9646629	0.174	0.192	0.016
CYP19_SNP24	rs2445761	0.068	0.050	0.153	RANK_SNP16	rs884205	0.092	0.094	0.037
CYP19_SNP26	rs2470144	0.092	0.030	0.098	RANK_SNP17	rs2957127	0.148	0.158	0.00087
CYP19_SW7	CYP19_7-10	0.090	0.030	0.206	RANK_SW2	RANK_1-4	0.001	0.00033	0.021
CYP19_SW8	CYP19_8-11	0.050	0.017	0.014	RANK_SW3	RANK_2-5	0.015	0.014	0.006
CYP19_SW9	CYP19_9-12	0.011	0.003	0.006	RANK_SW4	RANK_3-6	0.009	0.009	0.00035
CYP19_SW10	CYP19_10-13	0.022	0.007	0.006	RANK_SW5	RANK_4-7	0.002	0.002	0.00032
CYP19_SW11	CYP19_11-14	0.077	0.023	0.029	RANK_SW6	RANK_5-8	0.001	0.00057	0.00092
CYP19_SW13	CYP19_13-16	0.084	0.035	0.054	RANK_SW7	RANK_6-9	0.00042	0.00031	0.00045
HDC_SNP1	rs10519263	0.064	0.030	0.044	RANK_SW9	RANK_7-10	0.001	0.00030	0.007
HDC_SNP2	rs1802536	0.055	0.024	0.031	RANK_SW10	RANK_9-12	0.019	0.028	0.00093
HDC_SNP10	rs12901373	0.024	0.015	0.016	RANK_SW12	RANK_10-13	0.004	0.005	0.012
HDC_SNP11	rs2114447	0.023	0.014	0.017	RANK_SW13	RANK_12-15	0.010	0.019	0.028
LRP5_SNP1	rs682429	0.017	0.050	0.082	RANK_SW14	RANK_13-16	0.023	0.031	0.016
LRP5_SNP2	rs312016	0.019	0.050	0.079	RANK_SW1	RANK_1-3	0.091	0.043	0.029
LRP5_SNP4	rs4988300	0.023	0.055	0.096	RANK_Block1	RANK_4-5	0.004	0.004	0.006
LRP5_SNP6	rs634008	0.008	0.035	0.040	RANK_Block2	RANK_6-7	0.00009	0.00009	0.00004
LRP5_SNP7	rs312023	0.008	0.027	0.034	RANK_Block3	RANK_10-14	0.004	0.005	0.001
					RANK_Block5				
					TNFR2_SW5	TNFR2_5-8	0.033	0.017	0.003
					TNFR2_SW6	TNFR2_6-9	0.085	0.046	0.053

Only those genes having markers suggestively significant for OP (10,000 permutation-derived empirical global $p \leq 0.05$) in at least one skeletal site are listed.

* For single SNPs, the corresponding cell in this column shows its dbSNP rs number; for haplotype sliding window (SW), the cell shows the component SNPs of the window (e.g., CYP17_1-4 means the window consists of SNP1,2,3,4 of CYP17); for haplotype block, the cell shows all the SNPs within the block.

[†] p values are obtained by 10,000-time permutations. $p \leq 0.05$ are in boldface italic.

[‡] p values significant after Bonferroni correction by the number of independent LD blocks (single-test threshold $\alpha = 0.0007$).

Table 6
Candidate Gene Associations for BMD/OP at Spine, Hip, and UD in the Male and Female Subsamples Separately

Category	Spine	Hip	UD
Male-specific	<i>CTR</i> [*]	<i>IGF1</i> ^{†‡}	<i>ALOX12</i> [†]
	<i>IGF1</i> ^{†‡}		<i>CTR</i> [*]
Female-specific	<i>RANKL</i> ^{*†}		<i>RANKL</i> [*]
	<i>ALOX12</i> [†]	<i>ALOX12</i> [†]	<i>CCR3</i> ^{†‡}
	<i>BMP2</i> [†]	<i>BMP2</i> [†]	<i>CYP17</i> [†]
	<i>CCR3</i> ^{†‡}	<i>CCR3</i> ^{†‡}	<i>DBP</i> [†]
	<i>DBP</i> [†]	<i>CYP19</i> [*]	<i>LRP5</i> ^{*†}
	<i>HDC</i> [*]	<i>LRP5</i> ^{*†}	<i>TNFR2</i> [*]
	<i>TNFR2</i> ^{*†}	<i>TNFR2</i> ^{*†}	
Significant in both male and female subsamples	<i>CYP19</i>	<i>CTR</i>	<i>BMP2</i>
	<i>LRP5</i>	<i>HDC</i>	<i>CYP19</i>
	<i>RANK</i>	<i>RANK</i>	<i>HDC</i>
		<i>RANKL</i>	<i>RANK</i>

* Significant for the OP phenotype at the corresponding skeletal site.

† Significant for the BMD variation at the corresponding skeletal site.

‡ Significant only in the sex-specific subsamples, not in the total sample.

Table 7
Within-Group and Cross-Group Best Gene–Gene Effect Models for OP Identified by MDR Method

Loci number	Group	1	2	3	4	5	Final
2-locus	Loci combination	<i>TNF7,</i> <i>TNFR2_1</i>	<i>CYP19_4,</i> <i>ESR2_6</i>	<i>[BMP2_7,</i> <i>DBP3]</i>	<i>HDC7,</i> <i>HDC10</i>	<i>[ALOX12_2,</i> <i>SOST1]</i>	<i>[GCR9,</i> <i>ESR2_4]</i> *
	CV consistency	10	10	10	10	10	10
	Prediction error (%)	38.27 [†]	36.97 [‡]	35.32 [‡]	36.82 [‡]	36.82 [‡]	34.55 [‡]
3-locus	Loci combination	<i>[RANK4,</i> <i>TNFR2_10</i> <i>TNFR2_12]</i>	<i>[CYP17_1,</i> <i>ESR2_4,</i> <i>ESR2_10]</i>	<i>CALCR1,</i> <i>CALCR24,</i> <i>LRP5_4</i>	<i>[GCR9,</i> <i>GCR29,</i> <i>HDC10]</i> *	<i>MTHFR3,</i> <i>MTHFR4,</i> <i>SOST1</i>	<i>ALOX12_2,</i> <i>GCR29,</i> <i>HDC10</i>
	CV consistency	10	10	9	10	8	10
	Prediction error (%)	37.86 [‡]	35.59 [§]	35.17 [‡]	33.68 [§]	39.46 [‡]	35.02 [§]
4-locus	Loci combination	<i>RANK10,</i> <i>RANKL8,</i> <i>TNFR2_4,</i> <i>TNFR2_12</i>	<i>CYP17_4,</i> <i>CYP19_10,</i> <i>CYP19_24,</i> <i>ESR2_6</i>	<i>CALCR1,</i> <i>BMP2_1,</i> <i>BMP2_2,</i> <i>LRP5_4</i>	<i>CCR3_2,</i> <i>CCR3_7,</i> <i>GCR1,</i> <i>GCR29</i>	<i>ALOX12_1,</i> <i>ALOX12_2,</i> <i>MTHFR1,</i> <i>SOST1</i>	<i>BMP2_7,</i> <i>CYP17_1,</i> <i>RANK4,</i> <i>TNFR2_12</i>
	CV consistency	9	9	9	9	9	10
	Prediction error (%)	36.1 [‡]	36.76 [‡]	34.13 [§]	38.81 [‡]	35.33 [§]	35.48 [‡]

In each group, the best gene–gene effect models regardless of the number of interacting loci are shown in brackets.

* The best models that were validated for interaction effects by conditional logistic regression implemented in SPSS.

[†] Empirical $p > 0.05$ on the basis of 1000 permutations.

[‡] Empirical p value in the range of (0.01, 0.05) on the basis of 1000 permutations.

[§] Empirical $p \leq 0.01$ on the basis of 1000 permutations.