

CLINICAL STUDY

Single nucleotide polymorphisms in new candidate genes are associated with bone mineral density and fracture risk

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Abstract

Objective: Osteoporosis (OP) is a multifactorial disease with high heritability but its exact genetic background is still poorly understood. We examined the effect of 24 single nucleotide polymorphisms (SNPs) located in five genes – alkaline phosphatase, matrix metalloproteinase-2, tissue inhibitor of metalloproteinases-2 (*TIMP2*), fibroblast growth factor receptor-1 (*FGFR1*), and fatty acid-binding protein-3 (*FABP3*) – previously not associated with OP.

Design: We performed a genotype–phenotype association study at a university hospital.

Methods: A total of 360 Hungarian postmenopausal women were involved in the study. Bone mineral density (BMD) was determined at spine, hip, and distal radius. Genomic DNA was extracted from venous blood samples and a high-throughput genotyping method based on single-based primer extension was applied for allelic discrimination. Robust statistical tools were utilized for multiplex data analysis.

Results: SNP *rs6996321* in *FGFR1* was significantly related to spine BMD ($P=0.002$) and *rs10914367* in *FABP3* was associated with hip BMD ($P=0.028$). Non-vertebral fracture risk was significantly increased in carriers of 'A' allele of *rs9900972* in *TIMP2* (odds ratio=2.06, $P=0.0187$). We could also identify validated gene–gene interactions significantly affecting BMD and fracture risk.

Conclusions: We identified two previously unreported SNPs in *FGFR1* and *FABP3* associated with BMD and a third SNP in *TIMP2* related to risk for non-vertebral osteoporotic fractures. This is the first report about the association between these allelic variants and the phenotypes of postmenopausal OP. Further studies need to clarify the role of these genes and their polymorphisms in the process of bone loss.

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Introduction

Osteoporosis (OP) is a common, multifactorial disease with high heritability (1). Linkage studies have identified several loci associated with OP across every human chromosome. More than 200 candidate genes influencing the disease have already been reported (2, 3), but the exact genetic background and the interactions among suspected genes as well as genes and environmental or lifestyle factors (4) are still poorly understood (5). Previous studies have also shown that genetic influences can differ among distinct populations and between genders (6–8). Bone mineral density (BMD) and risk of osteoporotic fracture, predominant study phenotypes in genetic studies, are affected by several genes and their polymorphisms. Previously reported single nucleotide polymorphisms (SNPs) have small individual effects and the genetic correlation with the phenotypes is low (9). Therefore, more haplotype and

gene–gene interaction cohort studies should be performed for the investigation of the genetic background of OP (5, 10, 11).

Previously, less investigated genes came to our attention in our previous studies regarding the relationship between deer antler development and human OP (12). Based on literature data and on our pilot studies, we selected five genes with potential roles in human OP: alkaline phosphatase (*ALPL*), matrix metalloproteinase-2 (*MMP2*), tissue inhibitor of metalloproteinases-2 (*TIMP2*), fibroblast growth factor receptor-1 (*FGFR1*), and fatty acid-binding protein-3 (*FABP3*). In a previous human gene expression study (13), significant differences in gene expression of *ALPL* and *MMP2* were determined between the healthy and the porotic human bone tissue. The change was in trend in the case of *FGFR1* gene in this study. We supposed that different gene expression patterns determined in healthy and porotic bone tissue could be related to the

allelic variants of these genes. In this work, we aimed to investigate the effect of multiple SNPs in *ALPL*, *FABP3*, *FGFR1*, *MMP2*, and *TIMP2* on BMD and osteoporotic fracture risk in Hungarian postmenopausal women using a high-throughput genotyping method.

Materials and methods

Study population

Three hundred and sixty Hungarian unrelated postmenopausal women were recruited to the study through our clinic. Subjects visited the clinic for a standard dual energy X-ray absorptiometry (DXA) screening. All the subjects underwent physical examination and completed a detailed questionnaire on family and medical histories and lifestyle habits. Exclusion criteria were history of bone, metabolic, or endocrine disease; any chronic illness; hormone replacement, steroid therapy, or any medication known to influence bone metabolism; premature menopause (before 40 years of age); and alcohol consumption greater than two units per day. Subjects with biochemical abnormalities such as increased levels of serum *ALPL*, thyrotrophin, parathyroid hormone, or reduced level of 25-OH vitamin D (< 30 ng/ml) were not included in the study. BMD values at the lumbar spine (L2–L4) and total hip were measured using a Lunar Prodigy DXA (GE Medical Systems, Diegem, Belgium). BMD at the distal radius was determined by a Norland pDEXA (Cooper-Surgical Inc., Trumbull, CT, USA) densitometer. Coefficient of variation was below 1% at both sites. OP was defined according to the WHO criteria, i.e., *T*-score less than -2.5 s.d. at any measured site. Detailed fracture history was obtained from each subject. Non-vertebral osteoporotic fracture was defined as low-trauma fractures after the age of 45 years excluding the fractures of the face, skull, fingers, toes, and spine. Vertebral compression fractures were not investigated in this study. The study was approved by the local ethical committee, and written informed consent was obtained from all participants. Characteristics of the study population are shown in Table 1.

Gene and SNP selection

Based on our previous study (13) and on online data mining (<http://www.ncbi.nlm.nih.gov/OMIM/>, <http://www.hapmap.org>), we chose five genes expressed in human bones that are likely to play an essential role in bone metabolism; *ALPL*, *MMP2*, *TIMP2*, *FGFR1*, and *FABP3*. We used the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) and data from HapMap project (<http://www.hapmap.org>) to extract the available information on the SNPs of these genes. We chose 24 SNPs from the sites of the genes with optimal (40–65%) GC content using the following criteria: a)

Table 1 Characteristics of study population ($n=353^a$).

| Variable | Mean \pm sd (range) |
|--|-----------------------|
| Age (years) | 61.6 \pm 7.9* |
| Menopausal age (years) | 13.5 (1–42) |
| Height (cm) | 158.1 (139–176) |
| Weight (kg) | 68.9 \pm 12.1* |
| Body mass index (kg/m ²) | 27.6 \pm 4.5* |
| Smokers (%) | 45 (12.5%) |
| Alcohol consumption (%) ^b | 16 (4.44%) |
| Calcium intake (mg/day) | 642 (350–2000) |
| Lumbar spine BMD (g/cm ²) | 0.887 \pm 0.176* |
| Total hip BMD (g/cm ²) | 0.785 \pm 0.168* |
| Distal radius BMD (g/cm ²) | 0.689 \pm 0.161* |
| Subjects with osteoporosis at either site (%) | 198 (55%) |
| Subjects with non-vertebral osteoporotic fractures (%) | 76 (21.5%) |

*Distribution of the variable is not different from normal distribution ($P > 0.05$ in Kolmogorov–Smirnov test).

^aSeven subjects from 360 were excluded due to their genotyping failure. This table shows the parameters of the final study cohort.

^bCurrent alcohol consumption is more than one drink per week but less than one drink per day.

polymorph alleles are C/T or A/G (these two types of SNPs can be genotyped on the same plate at the same time by the high-throughput method used in our study); b) validation status in Caucasians is available at public databases; c) reported minimal allele frequency (MAF) is more than 5%; and d) SNP site (promoter, intron, exon, and 3'-UTR) and function (non-synonymous coding, synonymous coding, and splice site) according to general rules of SNP selection for association studies (14). The most of the genotyped SNPs are either haplotype-tagging SNPs identified by HapMap or strong linkage disequilibrium (LD) with tag SNPs. The full coverage could be realizable with genotyping at least 30, 5, 11, 9, and 20 tag SNPs across the *ALPL*, *FABP3*, *FGFR1*, *MMP2*, and *TIMP2*. The 24 genotyped SNPs in this study tagged with an $r^2 > 0.8$ the 29, 80, 64, 41, and 55% of the all allelic variants located in the intragenic as well as upstream and downstream regions of *ALPL*, *FABP3*, *FGFR1*, *MMP2*, and *TIMP2* respectively. Selected SNPs in candidate genes are shown in Fig. 1 and Table 2.

Genotyping

Blood samples were collected from each subjects and genomic DNA was extracted using High Pure PCR Template Purification kit (Roche Diagnostics, GmbH, Mannheim, Germany). DNA quality and quantity was determined with NanoDrop B-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and all the samples were diluted to a DNA concentration of 10 ng/ μ l. Large-scale genotyping process was performed at the SNP Core Facility of Semmelweis University using a GenomeLab SNPstream Genotyping System (Beckman Coulter, Fullerton, CA, USA). This automated ultra-high-throughput genotyping system utilizes multiplexed

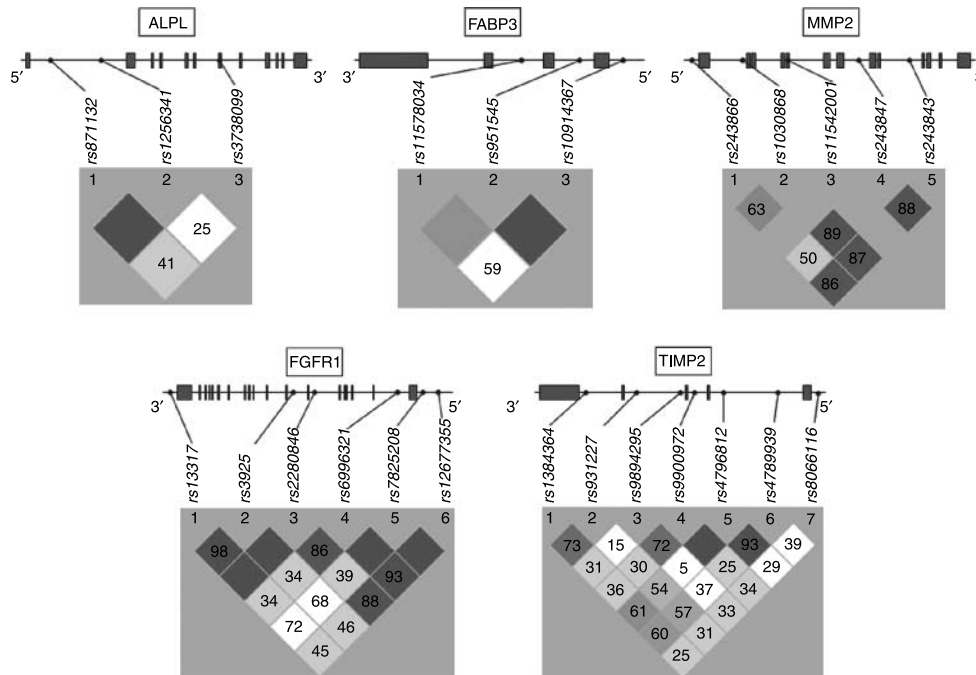


Figure 1 Position of genotyped SNPs and the pattern of linkage disequilibrium in selected genes. Pairwise linkage disequilibrium (LD) statistics in examined genes were calculated with Haploview. Squares are colored darker if the D' value is high (i.e., LD is strong), lighter shades indicate less LD. Empty dark squares mean $D' = 1$ (i.e., complete LD between two SNPs).

Table 2 Descriptive statistics of genotyped single nucleotide polymorphisms (SNPs).

| Genes | SNP number | Alleles | SNP position | SNP site | Success rate (%) | HWP | MAF |
|--|-------------------|---------|----------------|---------------|------------------|--------------|--------------|
| ALPL alkaline phosphatase | <i>rs871132</i> | G/A | chr1:21607931 | Intron | 97.4 | 0.509 | 0.116 |
| | <i>rs1256341</i> | G/A | chr1:21612135 | Intron | 100 | 0.822 | 0.305 |
| | <i>rs3738099</i> | G/A | chr1:21640041 | Exon (263Y/H) | 100 | 0.086 | 0.113 |
| FABP3 fatty acid-binding protein-3 | <i>rs11578034</i> | C/T | chr1:31511246 | Intron | 99.7 | <i>0.003</i> | 0.055 |
| | <i>rs951545</i> | C/T | chr1:31513758 | Intron | 99.1 | 1.0 | 0.119 |
| | <i>rs10914367</i> | G/A | chr1:31515299 | Promoter | 100 | 0.716 | 0.166 |
| FGFR1 fibroblast growth factor receptor-1 | <i>rs13317</i> | C/T | chr8:38388671 | 3'-UTR | 99.1 | 0.646 | 0.212 |
| | <i>rs3925</i> | G/A | chr8:38400815 | Intron | 97.4 | 0.942 | 0.204 |
| | <i>rs2280846</i> | G/A | chr8:38401451 | Intron | 100 | 1.0 | 0.082 |
| | <i>rs6996321</i> | G/A | chr8:38441503 | Intron | 98 | 0.506 | 0.322 |
| | <i>rs7825208</i> | G/A | chr8:38446444 | Promoter | 99.1 | 0.362 | 0.137 |
| | <i>rs12677355</i> | C/T | chr8:38449100 | Promoter | 100 | 0.921 | 0.350 |
| MMP2 matrix metalloproteinase-2 | <i>rs243866</i> | G/A | chr16:54069038 | Promoter | 100 | 0.701 | 0.262 |
| | <i>rs1030868</i> | C/T | chr16:54074268 | Intron | 97.7 | 0.747 | 0.390 |
| | <i>rs11542001</i> | C/T | chr16:54077073 | Exon (239F/L) | 99.4 | 1.0 | <i>0.000</i> |
| | <i>rs243847</i> | C/T | chr16:54081499 | Intron | 99.7 | 0.919 | 0.317 |
| | <i>rs243843</i> | G/A | chr16:54084799 | Intron | 100 | <i>0.004</i> | 0.156 |
| TIMP2 tissue inhibitor of metalloproteinases 2 | <i>rs1384364</i> | C/T | chr17:74364423 | Intron | 99.7 | 1.0 | 0.195 |
| | <i>rs931227</i> | G/A | chr17:74370134 | Intron | 98.8 | 0.697 | 0.173 |
| | <i>rs9894295</i> | G/A | chr17:74373700 | Intron | 100 | 0.063 | 0.122 |
| | <i>rs9900972</i> | G/A | chr17:74380209 | Intron | 99.7 | 0.720 | 0.184 |
| | <i>rs4796812</i> | C/T | chr17:74386781 | Intron | 99.7 | 0.656 | 0.058 |
| | <i>rs4789939</i> | G/A | chr17:74393298 | Intron | 100 | 1.0 | 0.133 |
| | <i>rs8066116</i> | C/T | chr17:74441474 | Promoter | 98.8 | 0.064 | 0.119 |

Success rate means the call percentage of genotypes, HWP, P value of the χ^2 test for Hardy–Weinberg equilibrium (numbers in italics indicate SNPs in Hardy–Weinberg disequilibrium), MAF, minimal allele frequency.

PCR in conjunction with tagged-array, multiplexed, single-base primer extension technology (15). Briefly, the workflow steps were as follows: 1) A multiplex PCR was applied to amplify the selected genes. 2) After a clean-up step (enzymatic degradation of unconsumed primers and dNTPs present in PCR products with a specialized formulation of two hydrolytic enzymes, exonuclease I and shrimp ALPL), a single-base primer extension was performed. 3) Due to the SNP primers with unique 5'-tagged sequence, extended products were hybridized to the glass-bottom SNPware Tag Array Plate. 4) Genotypes were detected with direct allele-specific, two-color fluorescent detection method using the SNPstream Imager. 5) Data analysis and genotype determination were performed with SNPstream software. All PCR primers and tagged SNP primers for primer extension were designed by the web-based primer design tool of Beckman Coulter (Autoprimer.com, <http://www.autoprimer.com>). Each step of genotyping was performed on 384-well plates and the pipetting procedures were automated using Biomek FX Dual Arm system (Beckman Coulter). Ten random samples were genotyped in three parallel runs for testing technical errors.

Data pre-processing and statistical analysis

Samples with more than 15% missing genotypes (less than 20 successfully genotyped polymorphic sites) were removed from the database to minimize the false results due to genotyping error. A 10-nearest neighbor method was used to assign the missing genotypes in the final database (16). We used Haploview 3.0 software (Broad Institute of MIT and Harvard, Cambridge, MA, USA) (17) for descriptive analyses (Table 2) and its 'Tagger' option for tagging process. SNPs with MAFs less than 5% and SNPs in Hardy-Weinberg disequilibrium (P value of χ^2 test <0.05) were excluded from further analyses. Pairwise LD between genotyped SNPs was determined with Haploview.

Kolmogorov-Smirnov test was applied to analyze the distribution of the study phenotypes. Stepwise regression analyses were applied to spine, total hip, radius BMD (linear regression), and non-vertebral osteoporotic fracture (logistic regression), as phenotypes; and age, menopausal age, body mass index (BMI), smoking status, and alcohol consumption, as covariates that were used to identify significant covariates with phenotypes. Spine, total hip, and radius BMD values were also entered into logistic regression model to determine their influence on fracture risk. Computed regression residuals, representing covariate-adjusted study phenotypes, were used in subsequent analyses (8). Association analyses were performed using the program PedGenie (18), statistical software developed for analyses of pedigrees and independent individuals, computing a statistic equivalent to ANOVA under three genetic models (additive, dominant, and recessive). We

performed the 'quantitative' and the 'odds ratio (OR)' statistics built into the software in this study. To assess the reliability of the results, permutation procedures were performed to generate empirical P values. P values reported in individual SNP analyses are empirical P values after 10 000 Monte Carlo permutations. Permuted global $P < 0.05$ was considered significant. The statistical power of the study was calculated with the Quanto 1.1 software (University of Southern California, Los Angeles, CA, USA) (19).

We conducted haplotype analyses using the 'haplo.stats' software in the statistical environment 'R' (<http://cran.r-project.org/>). Sliding windows of 2, 3, and 4 loci were used to construct haplotypes in each gene and score statistics were applied to test the association between haplotypes and study phenotypes (20). This method computes permuted P values of the associations. Effect of haplotypes on covariate-adjusted study phenotypes was calculated in a regression model using the 'haplo.glm' function built in the software.

Gene-gene interactions (epistasis) were tested using 'SNPassoc' software published by Gonzalez *et al.* (21). This 'R' package, developed for genetic studies, determines epistasis effects performing log-likelihood ratio tests (LRTs). The graphical output of this command visualizes the P values of the interactions between each pair of SNPs and the examined trait. To avoid false-positive results, only highly suggestive interactions characterized by a P value less than 0.001 were selected and gene-gene interactions were validated under conditional regression models. Interactions with a $P < 0.01$ in the regression model were considered significant. The regression analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Sequence analysis

Genomic sequences of the regions next to the SNPs were obtained from the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). Two sequences containing the two allelic variants of each SNP were used to search for putative transcription factor binding sites in the TRANSFAC database, using the PROMO web-based software (<http://alggen.lsi.upc.es/>) (22).

Results

Descriptive analyses

The reproducibility of the high-throughput genotyping method called SNPstream was 100% in our study (genotypes were the same in each parallel run). We excluded seven subjects from the statistical analyses, because their genotyping success rate was lower than 85%. MAF was lower than 5% in the case of *rs11542001* in *MMP2* and two SNPs were not in Hardy-Weinberg equilibrium (*rs11578034* in *FABP3*

and *rs243843* in *MMP2*; Table 2). These markers were excluded from further evaluations. The final statistical study cohort consisted of 21 SNPs and 353 subjects. In this dataset, 54 genotypes were imputed. Age, menopausal age, and BMI proved to be significant covariates in the regression model fitting of BMDs. Fracture risk was significantly influenced by age, BMI, and BMDs. Regression residuals representing covariate-adjusted study phenotypes were used in all genetic analyses.

Association analyses

The power of each significant association was also computed and only statistically significant associations with a power of study higher than 80% (0.80) are discussed in this paper. For spine BMD (mean ± s.d. = 0.887 ± 0.176) at a significance level of 0.05, MAF of 0.322, and 353 individuals, there would be a power of 83% to detect differences in BMD of 0.055 g/cm² under a dominant model. For hip BMD (mean ± s.d. = 0.785 ± 0.168), there would be a power of 82% to detect difference of 0.157 g/cm² under the recessive model (MAF=0.166). For the fracture risk, the sample size of this study (76 cases and 277 controls) with a MAF of 0.166 and an OR of 2.06 would give a power of 86%.

The *rs6996321* in *FGFR1* was associated with BMD at lumbar spine (unadjusted mean BMD ± s.e.m. was 0.858 ± 0.013, 0.912 ± 0.015, and 0.916 ± 0.029 in subjects with G/G, A/G, and A/A genotypes respectively). Adjusted BMD significantly differed between genotypes in the dominant genetic model (mean of adjusted spine BMD ± s.e.m. was -0.031 ± 0.011 and 0.031 ± 0.011 in groups of G/G and A/G+A/A respectively, *P*=0.002; Fig. 2A). The homozygous recessive genotype of *rs10914367* in *FABP3* was related to higher BMD at the total hip (mean BMD ± s.e.m. was 0.783 ± 0.009, 0.776 ± 0.028, and 0.945 ± 0.029 in subjects with G/G, A/G, and A/A genotypes respectively; mean of covariates adjusted BMD ± s.e.m. in the recessive model was -0.004 ± 0.008 and 0.143 ± 0.037 in G/G+A/G and A/A groups respectively, *P*=0.028; Fig. 2B). Table 3 shows the OR of the non-vertebral fracture risk in relation to *rs9900912* (*TIMP2*) genotypes. The risk for fractures was twofold higher in carriers of the recessive 'A' allele of this SNP (*P*=0.018). None of the SNPs significant for OP phenotypes were associated with body weight or BMI.

Thirty four haplotypes across the genes were constructed and tested in 'haplo.stats'. We identified a four loci haplotype in *FGFR1* constructed by *rs13317*, *rs3925*, *rs2280846*, and *rs6996321* that was significantly related to spine BMD in score tests (permuted *P*=0.007 of global association). The common haplotype (TCGG) was associated with significantly decreased spine BMD while a less common haplotype (CTGA) was related to higher spine BMD (Table 4). *rs6996321* was also significantly associated with spine BMD in individual analyses. Therefore, we also performed LRTs

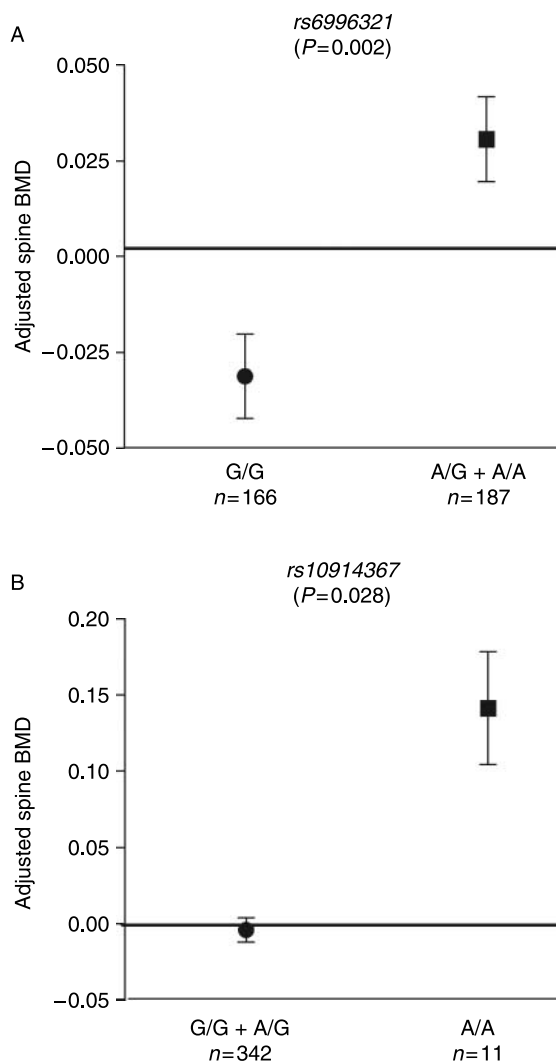


Figure 2 (A) Association of *rs6996321* in *FGFR1* with spine BMD, (B) Association of *rs10914367* in *FABP3* with hip BMD. Genotype-specific BMD means with standard errors of means (mean ± s.e.m.) are represented. The data were adjusted for significant covariates.

to analyze whether the *rs6996321* is associated with the spine BMD in the different haplotype structures. The effect of this SNP proved to be constant across the different haplotypic backgrounds in which it is carried. The effect of *rs6996321* on the TCG background (*Ht1-Ht2*) did not significantly differ from its effect on the CTG

Table 3 The association of tissue inhibitor of metalloproteases-2 (*TIMP2*) *rs9900972* with risk for non-vertebral osteoporotic fractures in the recessive model.

| Genotype | Controls | Cases | OR (95% CI) | P value |
|-----------|----------|-------|------------------|---------|
| G/G | 196 | 41 | 1.00 | 0.018 |
| A/G + A/A | 81 | 35 | 2.06 (1.12–3.58) | |

Controls and cases mean the number of subjects without and with fragility fracture. Odds ratio (OR), 95% confidence intervals and permuted *P* value are represented.

Table 4 Haplotypes built by *rs13317*, *rs3925*, *rs2280846*, and *rs6996321* in fibroblast growth factor receptor-1 (*FGFR1*) and their association to spine bone mineral density (BMD).

| Haplotype | Sequence | Frequency | Score test | | GLM model | |
|-----------|----------|-----------|------------|--------------|----------------------|--------------|
| | | | Hap-score | P value* | Coefficient ± s.e.m. | P value |
| Ht1 | TCGG | 0.506 | -2.934 | 0.003 | -0.032 ± 0.016 | 0.042 |
| Ht2 | TCGA | 0.206 | 1.062 | 0.309 | 0.023 ± 0.017 | 0.167 |
| Ht3 | CTGA | 0.107 | 2.595 | 0.012 | 0.069 ± 0.022 | 0.002 |
| Ht4 | CTGG | 0.083 | -1.121 | 0.269 | -0.016 ± 0.028 | 0.568 |
| Ht5 | TCAG | 0.078 | 0.964 | 0.346 | 0.034 ± 0.026 | 0.189 |

Haplotypes with a frequency >5% and their effect on covariate adjusted spine BMD are represented. *P values of score tests are simulated P value resulted by 10 000 permutations. #P values of GLM model sign the significance of t-statistic of regression variables characterized by the estimated regression coefficients and their standard errors in the generalized linear model, where the most frequent haplotype is the intercept. Bold numbers indicate significant individual haplotype effect (P<0.05).

background (*Ht4-Ht3*) ($\chi^2 = 2.38$, $df = 2$, $P > 0.25$). We also compared the effects of the two similar haplotypes with the two different *rs6996321* alleles (underlined). The effect of *Ht1* (TCGG) was not different from the effect of *Ht2* (TCGA) ($\chi^2 = 1.48$, $df = 2$, $P > 0.25$). Similar results were observed comparing the effect of *Ht4* (CTGG) with the effect of *Ht3* (CTGA) ($\chi^2 = 5.6$, $df = 2$, $P > 0.05$). These tests showed that the significant association between the spine BMD and the *FGFR1* haplotypes was not driven by the effect of the *rs6996321*.

Analyses of gene-gene interactions

We found that some gene-gene interactions associated with BMD or fracture risk where the P value of LRT

analyses was lower than 0.001 (P_1). These interactions were indicated with black symbols on the graphical output of SNPAssoc (Fig. 3). We validated all of these strongly significant gene-gene interactions using conditional regression analyses, and we confirmed interactions only in cases where regression analysis was also strongly significant ($P_2 < 0.01$). Table 5 shows highly suggestive gene-gene interactions and the mean BMDs and OR for non-vertebral fracture risk of each genotype combinations. We identified a gene-gene interaction between *FABP3* and *MMP2* (*rs10914367* and *rs1030868*) highly suggestive for spine BMD, and an interaction between *ALPL* and *TIMP2* (*rs3738099* and *rs9894295*) significantly associated with hip BMD. Interaction of *ALPL* and *TIMP2* (*rs871132* and *rs9894295*) was also related to radius BMD. Interaction

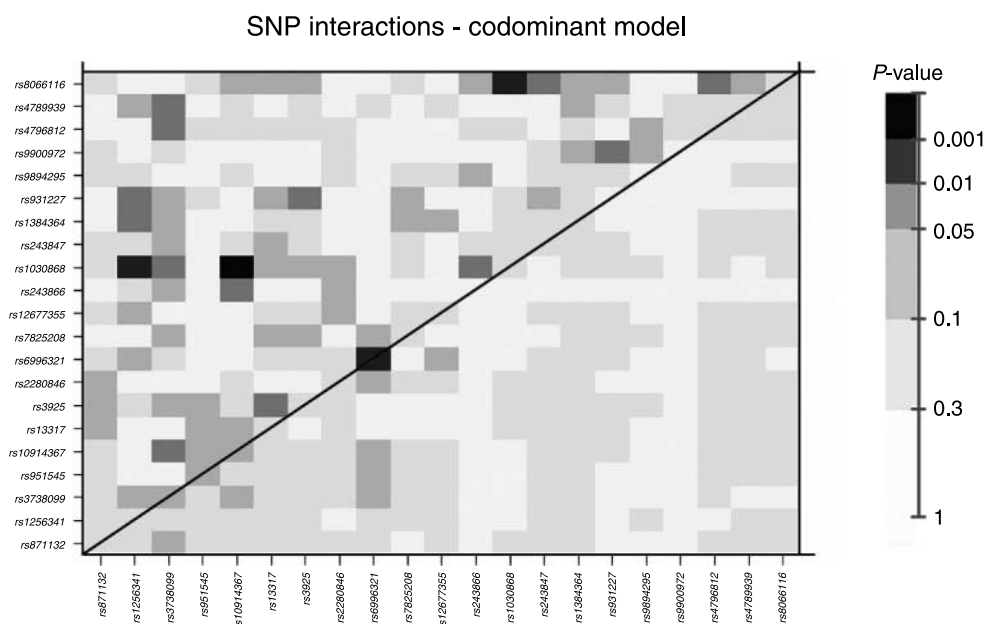


Figure 3 Gene-gene interaction plot generated by SNPAssoc. This representative plot shows the significance levels of gene-gene interactions and spine BMD in the codominant model. Each plot contains the P values obtained from different likelihood ratio tests. Different colors indicate different statistical significance levels. The diagonal line contains the P values from likelihood ratio test for the crude effect of each SNP. The upper triangle in the matrix contains the P values for the interaction (epistasis) log-likelihood ratio test. Finally, the lower triangle contains the P values from likelihood ratio test comparing the two-SNP additive likelihood to the best of the single-SNP models.

Table 5 Validated gene–gene interactions associated with (A) bone mineral density, (B) non-vertebral fracture risk.

| (A) Validated gene–gene interactions associated with bone mineral density | | | | |
|---|-----|-------------------------|------------------------|-------------------------------------|
| Spine BMD Mean \pm s.e.m. (n) | | FABP3 rs10914367 | | $P_1 = 0.0006$ $P_2 = 0.003$ A/A |
| | | G/G | A/G | |
| MMP2 rs1030868 | C/C | 0.922 \pm 0.020 (86) | 0.871 \pm 0.026 (41) | 0.812 \pm 0.104 (3) |
| | C/T | 0.869 \pm 0.014 (120) | 0.902 \pm 0.028 (44) | 1.021 \pm 0.089 (8) |
| | T/T | 0.831 \pm 0.028 (41) | 1.031 \pm 0.087 (10) | (0) |
| Hip BMD Mean \pm s.e.m. (n) | | ALPL rs3738099 | | $P_1 = 0.0008$ $P_2 = 0.003$ |
| | | A/A-G/G | A/G | |
| TIMP2 rs4796812 | C/C | 0.801 \pm 0.011 (242) | 0.746 \pm 0.022 (70) | |
| | C/T | 0.727 \pm 0.019 (30) | 0.829 \pm 0.052 (11) | |
| Radius BMD Mean \pm s.e.m. (n) | | ALPL rs871132 | | $P_1 = 0.0008$ $P_2 = 0.008$ A/A |
| | | G/G | A/G | |
| TIMP2 rs9894295 | A/A | 0.695 \pm 0.012 (203) | 0.671 \pm 0.023 (63) | 0.483 \pm 0.000 (1) |
| | A/G | 0.668 \pm 0.022 (70) | 0.768 \pm 0.057 (15) | 1.210 \pm 0.000 (1) |
| (B) Validated gene–gene interactions associated with non-vertebral fracture risk | | | | |
| Fracture risk OR (95% CI) | | MMP2 rs243847 | | $P_1 = 0.0006$ $P_2 = 0.004$ C/C |
| | | T/T | C/T | |
| TIMP2 rs931227 | G/G | 1.00 | 0.79 (0.39–1.60) | 0.43 (0.13–1.38) |
| | A/G | 0.06 (0.01–0.47) | 1.91 (0.79–4.61) | 0.91 (0.27–3.10) |
| | A/A | 0.00 (0.00) | 0.66 (0.04–11.7) | 0.47 (0.04–5.65) |

Gene–gene interactions are represented where P value of interaction in LRT analysis (P_1) was lower than 0.001 and interaction showed strong significance level in validation procedure ($P < 0.01$).

between *MMP2* and *TIMP2* (*rs243847* and *rs931227*) was highly suggestive for the risk of non-vertebral osteoporotic fracture.

Discussion

In this report, we investigated the association of postmenopausal OP and multiple allelic variants of five potential candidate genes. Polymorphisms of four of the selected genes were not previously reported in this field. Three individual SNPs associated with osteoporotic phenotypes have been identified in this study. *FGFR1* (member of receptor tyrosine kinases) is expressed on both osteoclasts and osteoblasts, and influences osteogenic differentiation of stem cells (23–25). Its mutations and haplotypes are associated with skeletal deformities seen in Pfeiffer and Kallmann syndromes, human tooth agenesis (26), and normal variations in craniofacial shape (27). In a recent study, decreased expression of *FGFR1* in osteoporotic bone tissue was demonstrated (13). *rs6996321* is a tag SNP located in the first intron of *FGFR1* tagging the whole promoter region, the first exon and the first intron with an $r^2 > 0.8$, suggesting that this polymorphism may be an important marker rather than a direct contributor to the genetic functions. It is also in

strong LD with the *rs2568231*, which is located in a splice site of the gene, 7 bp from the first exon. In our study, this marker was associated with lumbar BMD at a high level of significance. Haplotype of *FGFR1* constructed by four loci across the gene was also related to the variance in BMD at the lumbar spine suggesting a major role for this gene in bone metabolism.

Homozygous recessive genotype of *rs10914367* in the promoter region of *FABP3* was significantly correlated with increased hip BMD. This polymorphism tags the promoter region, the first exon and the first intron of the gene. In sequence analyses, we could identify putative transcription binding sites containing this locus. In the case of the 'A' allele, four transcription factors can bind to this sequence with a similarity of more than 90%. These transcription factors with their binding sites (*rs10914367* is underlined) are as follows: forkhead box protein P3, (GCCAAC); C/EBP- β , CCAAT-enhancer-binding protein- β , (CCAA); and PR-A and PR-B, progesterone receptor isoforms A and B, (AACACCA). Interestingly, these physical transcription factor binding sites seem to disappear in the case of the 'G' allele, suggesting a transcription-regulating role of this polymorphism. *FABP3* participates in fatty acid metabolism, particularly in the regulation of fatty acid uptake and intracellular transport (28) and it is also

expressed by bone-derived mesenchymal stem cells (29). *FABP3* has been previously reported to have an impact on the development of diabetes mellitus and obesity (28, 30). Expression of this gene is also modulated by the peroxisome proliferator-activated receptor (PPAR)- γ 2 transcription factor that has been shown to interfere with age-related bone loss via the activation of adipogenic and suppression of osteogenic programs of mesenchymal stem cells (31). Our results strengthen the putative connection between bone and lipid metabolism (32–34); however, further studies are needed to clarify the role of *FABP3* and its genetic variations in the pathomechanism of bone loss.

We have identified a SNP in the *TIMP2* gene (rs9900972) that has shown a significant correlation with non-vertebral osteoporotic fracture risk. Subjects carrying the 'A' allele of this polymorphism have twofold risk for fragility fracture compared with non-carriers. This intronic polymorphism is in high LD with the middle region of the gene, suggesting a marker role for fracture risk. *TIMP2* is a specific inhibitor of MMPs, matrix degradation enzymes liable for normal (35) and pathological metabolism (36, 37) of the organic compounds of bone extracellular matrix (ECM). ECM produced by osteoblasts has a crucial role in the determination of quality and fracture susceptibility of the bone tissue. Allelic variants in members of the MMP-cascade system were previously related to bone metabolism. SNPs in *MMP1* (38) and *MMP9* (39) have been suggested to be associated with BMD. Polymorphisms in *MMP2* have been described as predictors for multicentric osteolysis and arthritis syndrome (36). This enzyme may have an essential function in malignant diseases (40) as well as an impact on bone fracture healing (41). In our study, *MMP2* alone was not linked with BMD or fracture risk in individual SNP analyses; however, it appeared to contribute to gene–gene interactions significantly (two highly suggestive interactions out of four). A highly suggestive interaction between *MMP2* and its inhibitor, *TIMP2*, was found to influence fracture risk in our study population. The allelic variants of *TIMP2* might alter the inhibition process of MMPs. The decrease in the inhibition of MMPs could result in accelerated degradation of the ECM and consequently, increased fragility of the bone.

TIMP2 also appeared to have a gene–gene interaction with *ALPL* affecting BMD at the hip and radius. *ALPL* is essential for the mineralization of the osteoid matrix, and its serum level is related to the speed of bone turnover. *ALPL* expression is decreased in the subjects characterized by low bone mass in mice and human studies (42, 13). Allelic variants of this gene have been associated with various forms of hypophosphatasia (43) and a previous study reported two SNPs of these genes related to BMD in the Japanese population (44). Our results may suggest a putative role of *ALPL* allelic variants in the pathogenesis of postmenopausal OP, but further studies need to clarify it.

Sample size might be a limitation in genetic studies. Our work is a medium size multiple association study. Considering the false-positive results due to sample size and multiple tests, we conducted robust statistical analyses with rigorous criteria. In the case of individual SNP analyses, where 21 markers were analyzed with four phenotypes, a permutation procedure was performed to calculate empirical *P* values. Significant results were discussed only if the power of study was more than 80%. In haplotype analyses, empirical *P* value was also obtained in score tests and a validation procedure was performed using generalized linear method. Gene–gene interactions were accepted as 'highly suggestive' if log-likelihood analyses showed high level of significance and the *P* value of the validation procedure using regression models was also less than 0.01. We were able to collect a vast array of clinical data on our subjects, thus exclusions and statistical adjustments were considerably effective. Another limitation of our study is that we could not provide full coverage of the selected genes with these 24 genotyped SNPs. Based on the results of this first report, further studies are required to clarify the relationship between these genes and the pathological processes leading to osteoporosis.

Two phenotypes of OP (low BMD and non-vertebral fractures) have been investigated in our study. Low BMD is an important risk factor for fragility fracture; however, the recent studies suggest that genetic background of the two phenotypes can be diverse (9). We did not find any individual SNP or gene–gene interaction affecting both BMD and fracture risk. Nevertheless, some of the investigated genes (*TIMP2* and *MMP2*) participated in highly significant gene–gene interactions influencing BMD and fracture risk, as well. Our results suggest that phenotypes of OP can be affected by a number of different genomic patterns. These patterns (gene–gene interactions, SNP combinations) may result in similar phenotypes. Genetic background might be different in distinct populations and similar among people of the same sex and geographical region. From this point of view, age-related OP – despite its similar appearance – might be a genetically diverse disease. Large-scale studies with multiplex genomic analyses should be performed to draw the interaction map of the different genomic patterns related to bone loss. Furthermore, the role of protective SNPs or gene–gene interactions should be emphasized. The balance or imbalance between protecting and harmful genomic effects could be essential in the development of postmenopausal OP, and the knowledge of these mechanisms might be useful in the diagnosis and treatment of this disease.

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