Genome-wide copy number variation association study suggested VPS13B gene for osteoporosis in Caucasians

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Abstract

Summary Osteoporotic fracture (OF) is a serious outcome of osteoporosis. Important risk factors for OF include reduced bone mineral density and unstable bone structure. This genome-wide copy number variation association study suggested VPS13B gene for osteoporosis in Caucasians. Introduction Bone mineral density (BMD) and femoral neck cross-sectional geometric parameters (FNCSGPs) are under strong genetic control. DNA copy number variation (CNV) is an important source of genetic diversity for human diseases. This study aims to identify CNVs associated with BMD and FNCSGPs.

Methods Genome-wide CNV association analyses were conducted in 1,000 unrelated Caucasian subjects for BMD at the spine, hip, femoral neck, and for three FNCSGPs—cortical thickness (CT), cross-section area (CSA), and buckling ratio (BR). BMD was measured by dual energy X-ray absorptiometry (DEXA). CT, CSA, and BR were estimated using DEXA measurements. Affymetrix 500K arrays and copy number analysis tool was used to identify CNVs.

Results A CNV in VPS13B gene was significantly associated with spine, hip and FN BMDs, and CT, CSA, and BR (p<0.05). Compared to subjects with two copies of the CNV, carriers of one copy had an average of 14.6%, 12.4%, and 13.6% higher spine, hip, and FN BMD, 20.0% thicker CT, 10.6% larger CSA, and 12.4% lower BR. Thus, a decrease of the CNV consistently produced stronger bone, thereby reducing osteoporotic fracture risk.

Conclusions VPS13B gene, via affecting BMD and FNCSGPs, is a novel osteoporosis risk gene.

Keywords Bone geometry · Bone mineral density · Copy number variation · Osteoporosis

Introduction

Osteoporosis is a common skeletal disease in the elderly. The most serious outcome of osteoporosis is osteoporotic fracture (OF) [1]. Bone mineral density (BMD) is a major determinant of both osteoporosis and OF [2] and is commonly used by geneticists to search for genes underlying osteoporosis due to its high heritability (up to 70%). To date, no more than 10% of the total genetic variance in BMD has been explained by identified candidate genes. Consequently, the major genetic factors contributing to BMD variation remain to be identified [3].
Hip fracture is one of the most common OF and is generally considered to be the most severe OF due to its high associated morbidity, mortality [4], and therapeutic cost [5]. An important risk factor for hip fracture is reduced bone strength at the proximal femur [6], which is largely determined by poor femoral neck bone geometry and low BMD [7]. Femoral neck cross-sectional geometric parameters (FNCSGPs) are highly correlated with BMD and play an important role in determining bone strength or OF risk, independent of BMD [8, 9]. FNCSGPs are under strong genetic control, with heritability ranging from 30% to 70% [10, 11]. Although several linkage and association studies have been conducted [10, 12–14], genetic factors responsible for variations in FNCSGPs warrant further investigation.

Copy number variation (CNV) represents a form of structural genomic variation in which the number of copies of a DNA segment, ranging from 1 kb to several Mb, varies in different individuals when compared to a reference genome [15]. According to a study of 270 individuals from four populations with ancestry in Europe, Africa, or Asia, CNVs are widespread, covering approximately 12% (360 Mb) of the human genome [16]. CNVs have been shown to account for nearly 18% of the variation in gene expression, and consequently, it is reasonable to explore the possibility that CNVs play an important role in determining complex traits [17]. Support for this perspective comes in the form of evidence showing that CNVs are associated with certain complex human diseases such as progression of HIV infection, selected autoimmune diseases, and psychiatric disorder autism [18–20]. Recently, a genome-wide CNV association study on hip OF in Chinese suggested the importance of a deletion variant of UGT2B17 gene for pathogenesis of osteoporosis [21].

In the current investigation, we performed a genome-wide association study in Caucasians to identify CNVs that are significantly associated with variations in either BMD or FNCSGPs.

Methods

Research subjects

The study was approved by the necessary Institutional Review Board or Research Administration of involved institutions. Signed informed-consent documents were obtained from all study participants before they enrolled in the study. In total, 1,000 adult US Caucasian subjects (499 men and 501 women) were randomly selected from our established and expanding genetic repertoire, currently containing >6,000 subjects. All 1,000 subjects were self-identified as being of European origin living in Omaha, Nebraska and its surrounding areas. They averaged 50.21 ± 18.28 years in age, 1.71 ± 0.10 m in height and 80.13 ± 17.80 kg in weight.

Subjects with chronic diseases and conditions that might potentially affect bone mass, structure, or metabolism were excluded. These diseases and conditions included chronic disorders involving vital organs (heart, lung, liver, kidney, and brain), serious metabolic diseases (diabetes, hypo- and hyper-parathyroidism, hyperthyroidism, etc.), other skeletal diseases (Paget disease, osteogenesis imperfecta, rheumatoid arthritis, etc.), chronic use of drugs affecting bone metabolism (hormone replacement therapy, corticosteroid therapy, anti-convulsant drugs), malnutrition conditions (such as chronic diarrhea, chronic ulcerative colitis, etc.), etc. In addition, subjects taking anti-bone-resorptive or bone anabolic agents/drugs such as bisphosphonates were also excluded from this study. The purpose of the above exclusion criteria was to minimize the effect of any known environmental and/or therapeutic factors that influence bone phenotypic variation, which should correspondingly provide for augmentation of genetic effects, relative to environmental effects, in the study sample, and consequently increase statistical power for detecting the genetic factors.

BMD measurements

Areal BMD (grams per squares centimeter) at the spine and hip and areal bone size at femoral neck (FN) were measured using Hologic dual energy X-ray absorptiometry (DEXA) machines (Hologic Inc., Bedford, MA, USA). Herein, hip BMD represents a combined BMD from the femoral neck, trochanter, and intertrochanter. The machines were calibrated daily. The coefficient of variation values of the DEXA measurements were 1.98%, 1.87%, 1.87%, and 1.97% for spine BMD, hip BMD, FN BMD, and FN bone size, respectively.

Using DEXA-derived FN BMD and bone size, we estimated three FNCSGPs: cortical thickness (CT), cross-section area (CSA, an indicator of bone axial compression strength), and bulking ratio (BR, an index of cortical instability, indicating the risk of fracture by buckling; the lower the BR, the higher the resistance to buckling). The algorithm and the underlying assumptions regarding FN geometry and structure were communicated earlier [22, 23] and will not be further described herein.

Genome-wide genotyping

Genomic-wide DNA was extracted from peripheral white blood cells using a commercial isolation kit (Gentra systems, Minneapolis, MN, USA). DNA concentration was assessed by a DU530 UV/VIS spectrophotometer (Beckman Coulter, Inc, Fullerton, CA, USA). Before advancing to the genotyping step, the measurements of DNA quantification
were double-checked by pico-green analysis that can detect fluorescent signal enhancement of PicoGreen® dsDNA Quantitation Reagent, which selectively binds to dsDNA. Genotyping with the Affymetrix Mapping 250 k Nsp and Affymetrix Mapping 250 k Sty arrays was performed at the Vanderbilt Microarray Shared Resource at Vanderbilt University Medical Center, Nashville, TN, USA using the standard protocol recommended by the manufacturer. Briefly, for each array, 250 ng of genomic DNA was digested with either Nsp1 or Sty1 and ligated to adapters that recognize the cohesive four base pair overhangs. A generic primer that recognizes the ligated adapter sequence was used to amplify the ligation products in a polymerase chain reaction. The amplified DNA was assayed by agarose gel electrophoresis to verify an average size distribution of 250 to 1,500 bp. The amplified DNA was purified per the manufacturer’s protocol and quantitated by absorbance at 260 and 280 nm. Ninety micrograms of purified DNA was fragmented with DNase I and visualized on a 4% agarose gel. Samples with fragment distributions less than 180 bp were hybridized to the appropriate array (Nsp or Sty). Arrays were stained, washed, and scanned per manufacturer’s protocol using immunopure streptavidin (Pierce, Milwaukee, WI, USA), biotinylated anti-streptavidin antibody (Vector Labs, Burlingame, CA, USA), and R-phycerothrin streptavidin (Invitrogen, Carlsbad, CA, USA). Fluorescence intensities were quantitated using an Affymetrix array scanner 30007G. Data management and analyses were performed using Affymetrix GeneChip Operating System.

CNV and CNVR determination in autosomes

Subjects with genome-wide genotyping call rates <93% were excluded from further analysis. The call rate was assessed using the Bayesian Robust Linear Model with Mahalanobis distance algorithm [24, 25]. Accordingly, a total of 15 Caucasian subjects were excluded, and the resultant sample of 985 subjects was used for subsequent analysis.

Affymetrix GeneChip® Chromosome Copy Number Analysis Tool (CNAT) Software V.4.0.1 was used to identify chromosomal copy number changes from intensity data. CNAT includes a built-in probe-level quantile normalization of signal intensity for test samples, which is based on the intensities of Perfect Match probes across reference samples. The genomic smoothing bandwidth was set as 100 kb. CNAT implements a Hidden Markov Model (HMM) based algorithm for smoothing and segmentation of the copy number (CN) changes. In this HMM model, there are five hidden states representing different copy number variation: homozygous deletion (CN=0), heterozygous deletion (CN=1), normal diploid (CN=2), single copy gain (CN=3), and amplification (CN≥4). In our analyses, CN<2 was categorized as “loss”, CN=2 was defined as “normal”, and CN>2 was categorized as “gain”. Default values in the CNAT program for the Affymetrix GeneChip® Mapping 500K Array were used for the HMM parameters (0.2 for the priors, 0.07 for standard deviation when CN equal to 2, 0.09 for other CN states, and 10 Mb for the transition decay value).

CNAT requires reference data sets to be compared with sample sets for estimating the CN state of each SNP locus. For CNV detection on autosomes, samples on the same plate of 94 starting samples were assigned with equal probabilities into test and reference sets. This process yielded 47±2 subjects in a reference set in various sample plates and screened CN changes for all individuals. A total of 500,446 probes, averaging one SNP per 5.8 kb, were used to detect CN change.

At least two consecutive SNPs were used to define a CNV for each subject. The exact boundaries of CNVs cannot be obtained from data produced by the Affymetrix 500K SNP genotyping platform. However, they can be approximated by physical positions (NCBI Build 36.1, March 2006) of the probe pairs having the maximal distance within a CNV, yielding conservatively shorter defined sizes of CNVs than the actual sizes. Individual CNVs were organized into CNV regions (CNVRs), which are stretches of genomic regions covering overlapping CNVs across subjects. A CNVR started with two overlapping CNVs and was bounded by the two SNPs with maximal distance in the two CNVs. The CNVR would be extended when the next overlapping CNV exceeded the previous boundaries. For association analyses, we divided complex CNVRs, which include CNVs with inconsistent boundaries across the entire samples, into several subregions to ensure that (1) each CNV subregion has the same boundary among subjects and (2) each subject will be either gain or loss at each CNV subregion. CNV subregions with frequencies higher than 1% generated by the above procedure were selected for association analyses. Supplement 1 illustrates the relationship among SNPs, CNVs, CNVRs, and how CNV subregions were assigned in this study.

Genome-wide association analysis between CNV and BMD in the samples

First of all, age and sex were tested for their significance with each phenotype. Due to significant effects of age and sex on BMD and FNCSGPs (p<0.05), both age and sex were used as covariates to adjust the raw phenotypes. Considering significant genetic correlation of weight and height with BMD and FNCSGPs ([26], personal communication), weight and height were not used as
covariates to adjust each phenotype. The adjusted phenotypes, if not following normal distribution, were further subject to a Box–Cox transformation to conform to a normal distribution.

Analyses of variance (ANOVA) were performed to test for association between CNV subregions and adjusted phenotypes using the software package Helixtree (Golden Helix, Bozeman, MT, USA). The independent variable was the CNV subregion, which was divided into three levels according to the CN (gain, CN>2; normal, CN=2; and loss, CN<2). A raw p value<0.05 was considered nominally significant, which was further subjected to a Bonferroni correction to account for multiple testing in this study, where a significance level of 0.05/N (N=243, i.e., the total number of the tested CNV subregions) was used as a significance threshold for a test (p=0.0002). This significance threshold (p = 0.0002) was considered to be reasonable since the six study phenotypes that we studied are correlated to each other, and the Bonferroni correction is very conservative.

For significant CNVs identified, we performed further gender-stratified ANOVA analyses to test whether the significant CNV has a gender-specific effect on the testing phenotypes. The effect of age was adjusted in the analyses.

Assessment of population stratification

The STRUCTURE program version 2.2 [27] was used to detect possible population stratification of our study sample. Two thousand randomly picked unlinked SNPs with an average distance of 1.2 Mb, which were determined to be in Hardy–Weinberg equilibrium, were used to cluster all subjects using a Markov chain Monte Carlo algorithm. The admixture model and correlated allele frequency model were selected. Potential substructure was estimated based on the cluster pattern of all subjects. Existence of substructure is suggested if the subjects were clustered into two or more groups. We further tested our sample for population stratification using the genomic control method [28]. Based on genome-wide SNP information, we estimated the inflation factor (λ), a measure for population stratification. Ideally, for a homogeneous population with no stratification, the value of λ should be equal or near to 1.0.

Results

Basic characteristics of the sample

The basic characteristics of the 985 Caucasian subjects used for association analyses are summarized in Table 1. Use of the STRUCTURE program revealed that all subjects in this Caucasian sample were clustered together and could not be assigned into any subgroups, indicating that there was no significant population stratification within the sample. Further, the genomic control method estimated the λ value to be 1.007, confirming the results achieved through the Structure 2.2 software, indicating that there was essentially no population stratification in this sample. The relative homogeneity of this study sample eliminates potential spurious associations due to population stratification.

Genome-wide CNVs and CNVRs information in the sample

Table 2 summarizes the information for genome-wide CNVs and CNVRs detected in the Caucasian sample. In these 985 Caucasian subjects, we identified 9,196 CNV calls belonging to a total of 1,352 CNVs. These 1,352 CNVs were merged into 593 CNVRs, covering approximately 7.5% (215 Mb) of the 22 autosomes. For these 593 CNVRs, a total of 243 CNV subregions were assigned in the sample and utilized for subsequent genome-wide association analyses. Information for the 243 CNV subregions is presented in Supplement 2.
Genome-wide association analysis of autosomal CNVs in the sample

Figure 1 illustrates the genome-wide association signals for BMD (at spine, hip and FN) and FNCSGPs (CT, CSA, and BR) on all 22 autosomes. The most significant association was detected at CNV_084 (8q22.2), which simultaneously achieved statistical significance for spine BMD, hip BMD, FN BMD, CT, CSA, and BR ($p=0.0001, 0.0002, 0.0001, 0.0001, 0.0028, 0.0174$, respectively; Table 3). Even after applying the stringent Bonferroni correction, associations remained significant for spine BMD, hip BMD, FN BMD, and CT (Table 3).

In addition to the significant association at CNV_084, prominent association signals ($p<0.01$) for BMD and/or FNCSGPs were observed for chromosome regions 1p36.21 (CNV_003), 1q12 (CNV_014), 8q22.2 (CNV_083), 9p11.1 (CNV_099), 9q12 (CNV_103), 14q32.33 (CNV_135), 15q11.2 (CNV_144-147,163), 21p11.2 (CNV_228-229), and 21p11.1 (CNV_230). For a more complete listing, we present all nominal significant associations between CNVs and BMD and/or FNCSGPs ($p<0.05$) detected across the whole genome (Supplement 3); this represents a total of 53 CNV subregions on chromosomes [1, 2, 7–10, 14–17, 21, 22].

**Effect of the significant CNV_084**

For CNV_084, only CNV losses were observed in the study sample; no CNV gains were detected. Of the 985 analyzed subjects, 16 were carriers of a loss (CN=1), representing a minor copy number frequency of 1.62%.

For subjects with one CN vs. two CNs at CNV_084, we compared raw BMD and FNCSGPs (Fig. 2). For the total sample, compared to two copies of CNV_084, one copy is associated with a higher BMD, CT, CSA, and a lower BR. Specifically, compared with those with two CN, individuals with one CN have an average of 14.6%, 12.4%, and 13.6% higher spine BMD, hip BMD, and FN BMD, respectively, 20.0% thicker CT, 10.6% larger CSA, and 12.4% lower

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**Table 2 Information for the CNVs and CNVRs in the Sample**

| Statistics                  | Value            |
|-----------------------------|------------------|
| CNV                         |                  |
| Total number of CNV calls   | 9,196            |
| Total number of CNV calls with gain | 4,748            |
| Total number of CNV calls with loss | 4,448            |
| Total number of CNVs        | 1,352            |
| Mean size of CNVs           | 295 kb           |
| CNVR                        |                  |
| Total number of CNVRs       | 593              |
| Mean size of CNVRs          | 361 kb           |
| Mean number of probes per CNVR | 58              |
| Genome coverage             |                  |
| By CNVs, averaged on subjects | 2.1 Mb           |
| By CNVRs, combined across subjects | 215 Mb |

*CNV* copy number variation, *CNVR* copy number variation region
BR. These consistent findings suggest that a reduced copy number at CNV_084 is associated with stronger bone and a lower risk of OF. As shown in Table 3, with the exception of BR in the female sample, gender-stratified analyses revealed that CNV_084 is associated with all of the study phenotypes in both female and male subgroups ($p<0.05$).

Table 4 shows that CNV_084 covers seven analyzed intron SNPs (rs6468665, rs10098153, rs7818037, rs6991283, rs7459833, rs7842393, rs10808361) of the VPS13B (vacuolar protein sorting 13 homolog B) gene. This CNV extends 79,871 bp, ranging from intron 4 to 16 within the VPS13B gene.

**Discussion**

CNV is a genetic polymorphism recently recognized to be associated with human complex diseases, presumably via a dosage effect on gene expression. Using the Affymetrix Genechip® 500K array, we performed a genome-wide CNV study in a large Caucasian sample for two important bone strength phenotypes—BMD and FNCSGPs. We studied BMD at the spine, hip, and femoral neck. FNCSGPs were studied using three indexes, CT, CSA, and BR, which are all newly studied geometric variables associated with hip fracture [22, 23, 29]. CT and CSA are proportional to FN

| Phenotype | Phenotypic value | p value |
|-----------|------------------|---------|
|           | CNV state = 1    | CNV state = 2 |         |
| SPN BMD   | 1.18 (0.22) [20] | 1.03 (0.16) [965] | **0.0001** | 0.0559 | 0.0003 |
| Hip BMD   | 1.09 (0.20) [22] | 0.97 (0.15) [963] | **0.0002** | 0.0096 | 0.0071 |
| FN BMD    | 0.92 (0.20) [33] | 0.81 (0.14) [952] | **0.0001** | 0.0032 | 0.0103 |
| CT        | 0.18 (0.04) [27] | 0.15 (0.03) [958] | **0.0001** | 0.0029 | 0.0042 |
| CSA       | 3.13 (0.77) [27] | 2.83 (0.64) [958] | 0.0030 | 0.0150 | 0.0510 |
| BR        | 10.71 (2.92) [27] | 12.04 (2.73) [958] | 0.0170 | 0.1140 | 0.0710 |
BMD and are negatively correlated with hip fracture risk [22, 29], whereas BR is an index for bone structural instability [22, 30, 31] that is positively associated with hip fracture. In this study, we found that loss of CNV_084 is associated with higher bone strength. Compared with carriers with a normal copy number for CNV_084, subjects with CNV_084 losses have denser bone mass at the spine, hip, and FN and a more stable bone structure indicated by higher CTs and CSAs and a lower BR. Our findings suggest that loss of copy number CNV_084 is associated with stronger bone, thus reducing the risk of osteoporotic fracture. This association was detected in both women and men, suggesting the gender generality of its effect. The only gene overlapping with CNV_084 is VPS13B, thus implying this gene as a new susceptibility gene for osteoporosis in Caucasians.

The VPS13B gene contains 62 exons and spans ~864 kb on 8q22.2. It is a member of VPS13 gene family, which encodes a potential trans-membrane protein and has VPS13p as its closest yeast homologue [32]. A number of functional motifs have been predicted for the VPS13B gene [33], and it has been proposed that VPS13B might be involved in vesicle-mediated sorting and intracellular protein transport.

The VPS13B gene is more commonly referred to as COH1 since multiple studies have suggested that mutations of this gene cause Cohen syndrome (OMIM 216550) [33–35], an uncommon autosomal recessive disorder characterized by growth delay, slender limbs with narrow hands and feet, short stature, mental retardation, microcephaly, severe myopia, progressive chorioretinal dystrophy, and truncal obesity [34, 35]. Mutations of the COH1 gene are thought to prevent cells from producing a functional COH1 protein. Alaluusua et al. evaluated bone loss from the panoramic radiographs in Cohen syndrome patients and age- and sex-matched controls and found that Cohen syndrome patients were more likely to have alveolar bone loss and more extensive bone loss than controls [36]. Furthermore, Kivitie-Kallio et al. reported that marked kyphosis was relatively common in COH patients [34, 35]. The skeletal abnormalities consistently identified in Cohen syndrome patients suggest a potential role for the VPS13B gene in bone metabolism.

The VPS13B gene has a complicated pattern of alternative splicing, which uses four different termination codons (exons 8, 17, 28, and 62) and three alternative in-frame splicings (exons 28, 28b, and 31) [32]. The VPS13B gene is widely expressed in human tissues [32], with some tissue-specific differences in expression of the various transcript variants. In skeletal muscle and brain, variant 1 (containing exon 28 instead of 28b) is the main transcript, different from that expressed in other tissues where variant 2 (containing exon 28b instead of 28) is the predominant transcript [32]. Due to its complex multi-domain structure, various isoforms of the VPS13B gene product are thought to have different functions [33].

In the current study, we found a strong association between CNV_084 and both BMD and FNCSGPs. The region represented by CNV_084 flanks from intron 4 to intron 16 of the VPS13B gene and CNV_084 fully or partially overlaps with sequences encoding various VPS13B transcripts. To date, at least seven mutations have been identified in this CNVR [34, 37–41]. Interestingly, in an isolated Greek island population, an intragenic COH1 deletion spanning exon 6 to exon 16 was found to be causative for Cohen syndrome [34]. Furthermore, characteristics such as short stature, kyphosis and/or scoliosis, slender limbs with narrow hands and feet, etc. were fairly consistently

### Table 4 Basic information for the SNPs covering CNV_084

| Probe set     | dbSNP RS ID | Chr. region | Physical position | Allele | MAFa (Caucasian) | MAFb (present) | Gene name | Gene region |
|---------------|-------------|-------------|-------------------|--------|-----------------|----------------|-----------|-------------|
| SNP_A-2312363 | rs6468665   | 8q22.2      | 100181088         | G/A    | 0.15            | 0.16           | VPS13B    | Intron 4    |
| SNP_A-2307906 | rs10098153  | 8q22.2      | 100187297         | T/C    | 0.15            | 0.16           | VPS13B    | Intron 5    |
| SNP_A-2296088 | rs7818037   | 8q22.2      | 10018363          | G/C    | 0.20            | 0.21           | VPS13B    | Intron 5    |
| SNP_A-2086716 | rs6991283   | 8q22.2      | 100189139         | C/T    | 0.14            | 0.16           | VPS13B    | Intron 5    |
| SNP_A-4282840 | rs7459833   | 8q22.2      | 100205307         | C/T    | 0.19            | 0.21           | VPS13B    | Intron 8    |
| SNP_A-1879552 | rs7842393   | 8q22.2      | 100207232         | G/A    | 0.15            | 0.16           | VPS13B    | Intron 8    |
| SNP_A-2024722 | rs10808361  | 8q22.2      | 100260959         | G/C    | 0.21            | 0.21           | VPS13B    | Intron 16    |

a The second allele is the minor allele in the sample
b The frequency in SNP annotation database
c The frequency in the studied sample

SNPs within CNV’s usually are not in Hardy–Weinberg equilibrium; thus, association between these SNPs and the study phenotypes were not evaluated. CNV_084 was located within variation_3743 (chr8:100,098,024…100,265,857) archived in Database of Genomic Variants (http://projects.tcag.ca/variation/)

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observed in patients with this deletion. Consequently, it appears reasonable to speculate that CNV_084 may play an important role in regulation of bone development and metabolism. However, the molecular mechanism, by which BMD and bone geometry structure is influenced by CNV_084, must be delineated by further focused functional studies. It should be noted that only heterozygous loss of CNV_084 was detected in our sample, and this loss did not lead to Cohen syndrome presumably because Cohen syndrome is inherited in an autosomal recessive manner.

In summary, this is the first genome-wide CNV association study for BMD and FNGCGPs in Caucasians. Our data, supported by previous associations between VPS13B mutations and skeletal abnormalities, strongly suggest that VPS13B is a novel candidate gene for osteoporosis.

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Conflicts of interest None.

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