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ORIGINAL ARTICLE

Common variants in *FLNB*/*CRTAP*, not *ARHGEF3* at 3p, are associated with osteoporosis in southern Chinese women

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Abstract
Summary We performed an association study of five candidate genes within chromosome 3p14-25 in 1,080 Chinese female subjects. Polymorphisms in *FLNB/CRTAP* are associated with bone mineral density (BMD) in Chinese.
Introduction Chromosomal region 3p14-25 has shown strong evidence of linkage to BMD in genome-wide linkage scans. The variants responsible for this linkage signal, nonetheless, remain obscure.
Methods Thirty SNPs in five positional and functional candidate genes within 3p14-25 (*PPARG*, *CRTAP*, *TDGF1*, *PTHR1*, and *FLNB*) and rs7646054 in the *ARHGEF3* gene were genotyped in a case-control cohort of 1,080 Chinese females. Allelic and haplotypic association were tested using logistic regression analysis implemented in PLINK software. Potential transcription factor binding sites were predicted with MatInspector.
Results Multiple SNPs and haplotypes in *FLNB* were significantly associated with BMDs, with the strongest association between lumbar spine BMD and rs9828717 (p=0.005). SNP rs7623768 and the haplotype G-C of rs4076086-rs7623768 in *CRTAP* were associated with femoral neck BMD (p=0.009 and p=0.003, respectively). *PTHR1* showed haplotypic associations with lumbar spine and femoral neck BMD (p=0.02 and p=0.044, respectively). Nevertheless, the association between rs7646054 in *ARHGEF3* and BMD observed in Caucasians was not replicated in our samples. Comparative genomics analysis indicated that rs9828717 is located within a highly conserved region. The minor T allele at rs9828717 may lead to loss of binding site for nuclear factor of activated T cells which binds and triggers the transcriptional program of osteoblasts.
Conclusions Our data suggest that variants in *FLNB* and *CRTAP* at 3p are involved in BMD regulation in southern Chinese.

Keywords Association · BMD · *CRTAP* · *FLNB* · Osteoporosis

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Introduction

Osteoporosis is a complex disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk [1]. Assessment of bone mineral density (BMD) is a common approach to evaluate the risk of osteoporosis. BMD is under strong genetic control with heritability ranging from 0.63 to 0.75 at the femoral neck, 0.61 to 0.83 at the lumbar spine, and 0.66 to 0.79 at total hip [2–4]. Recently published genome-wide association studies have revealed a few well-known candidate genes, such as low-density lipoprotein receptor-related protein 5, receptor activator of nuclear factor kappa B ligand (*RANKL*), osteoprotegerin, estrogen receptor 1, and sclerostin as the causal genes that contribute to BMD variation [5–7]. Since these genes are thought to account for only a small proportion of the total variation in spine and hip BMD, further identification of additional variants remains vital to understand the pathogenesis of osteoporosis.

Chromosomal region 3p14-25 is a susceptible quantitative trait locus (QTL) for BMD regulation that has been identified by four independent linkage studies [8–11] and genome scan meta-analyses [12, 13]. The meta-analysis of published linkage scores in 12,685 individuals from 3,097 families suggested that the summed rank of 3p22.2-p14.1 (bin 3.3) is significantly higher than expected (p=0.012) [12]. Our recent meta-analysis of genome-wide linkage data, which included 11,842 subjects from 3,045 families, showed that 3p25.3-p22.1 (bin 3.2) had a statistically significant high average rank for lumbar spine BMD in both the whole-sample and female-specific analysis [13].

Mullin et al. [14] recently genotyped 17 SNPs in Rho guanine nucleotide exchange factor 3 (*ARHGEF3*) and observed the strongest association for rs7646054, which was associated with BMD Z-score at spine (p=0.006) and femoral neck (p=0.0007) in postmenopausal Caucasian women. The Rho guanine nucleotide exchange factor 3 specifically activates two members of the RhoGTPase family: RHOA which has been implicated in osteoblast differentiation and RHOB which has a role in cartilage biology [14]. It is unclear whether rs7646054 exerts the same effect in Chinese women who have a different genetic background and lower osteoporosis prevalence compared with Caucasian women [15].

To identify the causal genes contributing to BMD regulation in 3p14-25, a gene-wide and tag SNP-based association study was conducted in 1,080 case-control subjects using both single marker and haplotype approaches on five candidate genes: peroxisome proliferator-activated receptor gamma (*PPARG*), cartilage-associated protein (*CRTAP*), teratocarcinoma-derived growth factor 1 (*TDGF1*), parathyroid hormone receptor type 1 (*PTHR1*), and filamin B, beta (*FLNB*). The bone-related traits and phenotypes in knockout mice of these five genes are summarized in Table 1. A SNP rs7646054 in novel *ARHGEF3* gene, which was recently reported to be associated with BMD regulation in Caucasians [14], was also examined in our population.

Materials and methods

Subjects

This study included 1,080 southern Chinese female subjects selected from an expanding database of the Hong Kong Osteoporosis Study. Participants were ambulatory subjects recruited at road shows and health talks on osteoporosis since 1998. Women with a history of diseases known to affect bone mass including vitamin D deficiency, hypercalcaemia, primary and secondary hyperparathyroidism, hyper- and hypothyroidism, metabolic and congenital bone diseases, and use of medications that would affect bone metabolism were excluded. A detailed description of subject ascertainment, inclusion, and exclusion criteria has been described previously [4]. BMD was measured by dual energy X-ray absorptiometry (Hologic QDR 4500 plus, Waltham, MA, USA). The in vivo precision of the machine for lumbar spine, femoral neck, and total hip region was 1.2%, 1.5%, and 1.5%, respectively. Subjects with extreme BMD Z-scores at either lumbar spine L1–4 or femoral neck were included in the current study. Subjects with BMD Z-score≤−1.28 (lowest tenth percentile of the population) were defined as cases, while those with BMD Z-score≥+1 (highest 15th percentile of the population) were defined as controls. All participants gave informed consent, and the study was approved by the Ethics Committee of the University of Hong Kong and conducted according to the Declaration of Helsinki.

There were 457 cases and 254 controls for lumbar spine, 399 cases and 283 controls for femoral neck, and 356 cases and 260 controls for total hip. The Student's t test was applied to compare the characteristics and phenotypes of the cases and controls. Age, height, and weight are potential confounding factors influencing BMD variation. According to our previous heritability estimates for BMD, the proportion of variation explained by age, age^2, height, and weight was around 0.3 in women [4]. Factors with significant difference in the cases and controls were employed as covariates in the subsequent analysis.

SNP selection and genotyping

Twenty-seven tag SNPs (tSNPs) from five candidate genes (*PPARG*, *CRTAP*, *TDGF1, PTHR1*, and *FLNB*) in the chromosomal region 3p14-25 were selected for genotyping based on the genotype data obtained from the Han Chinese panel of the phase II HapMap data [39]. The criterion for tagging was set at r^2>0.8 and minor allele frequency (MAF)>0.2. The 27 tag SNPs captured 82.4% of common variants in five genes. SNPs rs709157, rs2177153, and rs1131356 showed significant association with BMD in previous studies and are thus, examined in this study. A total of 30 SNPs were genotyped using high-throughput massArray technology. In the genotyping process, 5% of samples were duplicated for quality check, and the reproducibility rate exceeded 99.8%.

Mullin et al. recently reported strong associations between rs7646054 in *ARHGEF3* and BMD Z-scores at the spine and femoral neck in postmenopausal women [14]. We, thus, also genotyped rs7646054 using the TaqMan Genotyping Assay C__29978110_10 (Applied Biosystems, CA, USA) in our case-control samples. Each reaction contained template DNA and a final concentration of 1x TaqMan PCR Master Mix, unlabeled forward and reverse

Table 1 The gene–disease/trait association and bone-related phenotypes of the gene-deficient mice of the five candidate genes

Gene	Gene–disease/trait association in humans	Bone-related phenotypes of target-gene-deficient mice
FLNB	Boomerang dysplasia [16] Larson syndrome [17, 18] Spondylocarpotarsal synostosis [18, 19]	Smaller body size, reduced body weight, "hourglass" body shape, restricted upper body movement, and aberrant mineralization in the neural arches leading to fusion of individual vertebrae [22]
	Atelosteogenesis I and III [18, 20] BMD [21]	Kyphotic and scoliotic malformations of the vertebral column, reduced body weight, shorter bones, reduced BMD in the middiaphyseal area of tibiae, reduced cortical thickness, reduction in hyaline cartilage in the ribs, metacarpal bones, phalanges, and tarsal bones [23]
PPARG	Serum osteoprotegerin level [24, 25] Total body BMD [26]	*PPARG*$^{+/-}$ mice exhibit high bone mass compared with wild-type mice. The difference in bone volume between the mice of two genotypes becomes more prominent at 52 weeks [27]
PTHR1	Femoral neck BMD [28–31] Eiken syndrome [32] Blomstrand chondrodysplasia [32, 33]	No abnormal bone-related phenotypes were reported in *PTHR1*-deficient mice
CRTAP	Osteogenesis imperfecta [34–37]	Shortening of long bone segments (particularly the proximal segment of the limb), decreased bone volume/tissue volume ratio, decreased trabecular thickness, decreased trabecular number, increased trabecular separation, reduced bone formation rate due to a reduction in the mineral apposition rate, and decreased mineralization lag time [35]
TDGF1	Ranked first in the prediction of osteoporosis candidate genes within the 3p14-25 [38]	No abnormal bone-related phenotypes were reported in *TDGF1*-deficient mice

primers, VIC, and 6FAM dye-minor groove binder labeled probe for detection of the two alleles. The polymerase chain reaction program was set at 50°C incubation for 2 min followed by 10 min at 92°C. A two-step reaction was repeated with 40 cycles, with denaturation at 92°C for 15 s and annealing and extension at 50°C for 1 min. Subsequent endpoint reading was performed on the PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA). The reproducibility and the call rate of the TaqMan assay were 100% and 98.7%, respectively.

Statistical analysis

PLINK, an open source tool set designed for analysis of large data sets in a computationally efficient manner [40], was utilized in quality control filtering, single- and multiple-marker association tests. SNPs missing greater than10%, MAF of less than 1%, or violating the Hardy–Weinberg equilibrium (HWE) ($p<0.001$) were excluded from further analysis. Logistic regression for the additive model, with adjustment for covariates, was applied to test the single-marker genotypic association with BMD at different skeletal sites. The Fisher's exact test was employed to execute the basic allelic association test. The variable-size sliding window approach was adopted in haplotype analysis as it includes the SNPs that may fall outside predefined linkage disequilibrium (LD) block and thus, enables the full information on genetic variability to be utilized in haplotype analysis [41, 42]. Another advantage of the variable-size sliding window approach is its greater detection power compared with other association-mapping strategies that employ haplotype block or single-SNP locus [41]. With adjustment of covariates, global omnibus test was conducted on a set of SNPs: in H haplotypes with a frequency more than1%, an H-1 degree of freedom test was performed to compare the alternate model (each haplotype having a unique effect) with the null (no haplotypes having any different effect). When the omnibus test was deemed significant, haplotype-specific test was performed. A conditional haplotype test that controlled for a particular haplotype among a set of haplotypes was also conducted to determine if that particular haplotype alone leads to the significant omnibus association result. Haploview 4.1 [43] was adopted to generate the haplotype block structure for the genotyped markers that passed the quality control requirements. LD is not calculated if markers are greater than 500 kb apart. Statistical power was estimated by the "Case-Control for threshold-selected quantitative traits" module of the web-based Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/qcc.html) [44].

Bioinformatics analysis

A comparative genomics approach was adopted to determine potential functional elements in the candidate region associated with BMD variation. The chromosomal position of the region was submitted to the VISTA Genome browser. Pre-computed whole-genome alignment among large vertebrates, which had a high sensitivity in covering more than 90% of known exons, was available on the browser with timely update upon the release of new genome assemblies [45].

The sequence encompassing the significantly associated SNP was scanned against the weight matrices for vertebrates that were publicly available on MatInspector [46]. The optimized matrix threshold of a weight matrix was defined as the threshold that allowed a maximum of three matches in 10 kb of non-regulatory test sequences. The matrix similarity was calculated on-the-run by scanning the imported sequence against the relative frequency of each nucleotide at a particular position in the matrix. Only potential binding sites with: (1) matrix similarity exceeding the optimized threshold; and (2) matrix similarity greater than 0.85 were considered good matches.

Results

Subject characteristics

The characteristics of the subjects are outlined in Table 2. Student's *t* test was used to compare the mean age, height, weight, and BMD in the case- and control-group, without assuming equal variances. The covariates that showed significant differences between cases and controls were potential confounding factors for BMD variation. These were adjusted in the subsequent analysis as indicated in Table 2.

Quality control

The genomic position, MAF, HWE test statistic, and call rate for each tSNPs that satisfied quality control criteria are listed in Table 3. Two tSNPs (rs4684846 and rs4135280) had call rates less than 90%. One SNP (rs1805192) was monomorphic in our study population. These three SNPs, all located within *PPARG*, were excluded from further analysis. A SNP in *CRTAP* (rs4678478) violated the HWE with a $p<0.001$ in both the case- and control-group and was also discarded from association analysis.

Single-marker association

The association of each SNP with BMDs at the lumbar spine, femoral neck, and total hip was evaluated using the additive and allelic model. SNPs with p value≤ 0.05 in the single-marker association test are shown in Table 4. Multiple SNPs (rs9828717, rs1718454, and rs1718456) in *FLNB* showed significant genotypic association with lumbar spine BMD ($p=0.03$–0.005). For femoral neck BMD, significant genotypic association was detected for rs7623768 in *CRTAP* ($p=0.009$) and rs1718456 in *FLNB* ($p=0.027$). Significant association with total hip BMD was only observed for multiple SNPs in *FLNB*: rs9828717, rs1718454, and rs9822918 ($p=0.016$–0.048).

Haplotype analysis

SNPs with MAF of less than 0.1 were removed from the haplotype analysis to reduce the type I error rate. Table 5 lists the combinations of SNPs most significantly associated with BMD in each gene resulting in $p<0.05$ in the covariate-adjusted omnibus test. Only haplotypes with frequency of greater than 0.05 are shown in the table.

The global omnibus test revealed that a region rs724448–rs2242116 within the *PTHR1* gene, which was in strong LD ($r^2=0.96$), was significantly associated with lumbar spine and femoral neck BMD after adjustment of height and weight ($p=0.02$ and $p=0.044$, respectively).

FLNB showed regional associations with BMDs at all three measured skeletal sites. The region rs9828717–rs1718456–rs1718481–rs1718454 was significantly associated with lumbar spine BMD ($p=0.003$). However, none of the common haplotypes are associated with lumbar spine BMD. The region rs1718456–rs1718481–rs1718454 was significantly associated with femoral neck BMD ($p=0.03$). The T–A–C haplotype was associated with lower BMD status ($p=0.013$, odds ratio (OR)=1.52) while the C–A–C haplotype was associated with higher BMD status ($p=0.0145$, OR=0.41). The global omnibus test was no longer significant after controlling for either of the haplotypes, indicating their potential role in regulation of femoral neck BMD. The region rs1718454–rs9822918 was significantly associated with total hip BMD ($p=0.027$). The C–T and T–G haplotype were correspondingly associated with the increased ($p=0.006$, OR=1.69) and reduced risk of low BMD ($p=0.025$, OR=0.66).

The global omnibus test demonstrated that the region rs4076086–rs7623768 in *CRTAP* was significantly associated with femoral neck ($p=0.028$) and total hip BMD ($p=0.015$). According to the haplotype-specific and conditional haplotype test, G–C was potentially the haplotype that conferred a protective effect on femoral neck ($p=0.003$, OR=0.43) and total hip ($p=0.007$, OR=0.44) BMD.

rs7646054 in *ARHGEF3* and BMD

Mullin et al. [14] recently reported a significant association between rs7646054 and BMD Z-score in postmenopausal

Table 2 Characteristics and BMD measurements of the 1,080 subjects and the constituent 533 postmenopausal women

	Whole study population			Postmenopausal women		
	Cases	Controls	*p* value (*t* test)	Cases	Controls	*p* value (*t* test)
Skeletal site: lumbar spine						
Number	457	254	–	314	107	–
Age (year)	51.71±13.78	49.56±14.35	0.05	59.92±5.90	63.55±8.16	<0.01*
Height (m)	1.53±0.06	1.576±0.06	<0.01*	1.52±0.057	1.55±0.05	<0.01*
Weight (kg)	49.98±7.22	60.34±9.76	<0.01*	51.03±7.43	62.45±9.79	<0.01*
BMD (g/cm^2)	0.71±0.09	1.12±0.10	<0.01	0.66±0.07	1.06±0.10	<0.01
BMD Z-score	−1.73±0.40	1.53±0.63	<0.01	−1.8±0.43	1.68±0.71	<0.01
Skeletal site: femoral neck						
Number	399	283	–	186	98	–
Age (year)	45.89±15.27	45.56±14.32	0.77	60.60±6.09	61.05±8.26	0.63
Height (m)	1.54±0.06	1.46±1.087	<0.01*	1.51±0.06	1.54±0.06	<0.01*
Weight (kg)	48.44±6.40	61.11±12.31	<0.01*	49.64±7.07	63.41±9.17	<0.01*
BMD (g/cm^2)	0.56±0.07	0.90±0.10	<0.01	0.51±0.05	0.83±0.06	<0.01
BMD Z-score	−1.68±0.34	1.58±0.53	<0.01	−1.7±0.36	1.48±0.38	<0.01
Skeletal site: total hip						
Number	356	260	–	194	86	–
Age (year)	48.44±14.70	45.51±13.76	0.01*	60.52±6.02	60.97±7.59	0.63
Height (m)	1.54±0.06	1.54±0.66	0.99	1.52±0.06	1.55±0.057	<0.01*
Weight (kg)	48.62±6.37	62.42±10.88	<0.01*	49.57±6.78	64.38±9.00	<0.01*
BMD (g/cm^2)	0.63±0.07	0.99±0.07	<0.01	0.59±0.06	0.93±0.06	<0.01
BMD Z-score	−1.83±0.44	1.67±0.54	<0.01	−1.89±0.49	1.60±0.45	<0.01

*$p<0.05$, the parameters with * are adjusted as covariates in subsequent analysis

women: subjects homozygous for the G allele had lower BMD than subjects heterozygous or homozygous for the A allele. The same model (AA+AG vs GG) was, therefore, adopted in the analysis of this SNP using logistic regression implemented in SPSS. No association was observed between rs7646054 and BMD Z-score at the lumbar spine, femoral neck, or total hip in the whole study population, nor in the 533 postmenopausal case-controls (results not shown).

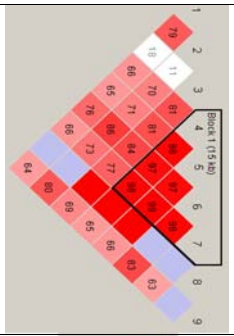
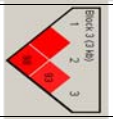
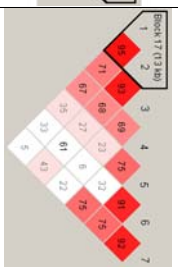
Bioinformatics analysis

Since four of the five SNPs genotyped within intron 1 of *FLNB* showed significant associations with BMD in the single-marker test, the chromosomal position of intron 1 (Chr3:57,969,624-58,037,812) was submitted to VISTA genome browser to determine the presence of any potential conserved elements. RankVISTA for multiple alignment shows that intron 1 of *FLNB* in humans is a conserved noncoding sequence among five other species, including rhesus, dog, horse, mouse, and rat (Fig. 1). It is worth noting that rs9828717 is located within a highly conserved region with an alignment *p* value of 2.4×10^{-16}. Prediction of potential transcription factor binding sites with MatInspector revealed that the minor T allele at rs9828717 may lead to the loss of binding site for nuclear factor of activated T cells (NFAT). The similarity score for the major C allele with NFAT matrix was 0.96.

Discussion

In the present study, we tested associations between common variants in five candidate genes in 3p14-25 (*FLNB*, *PPARG*, *TDGF1*, *CRTAP*, and *PTHR1*) and BMD in 1,080 southern Chinese women. Among these candidate genes, *FLNB* showed the strongest and most consistent association with BMD in both single-marker and haplotype analysis. At the SNP level, rs9828717, rs1718456, rs1718454, and rs9822918 were significantly associated with lumbar spine, femoral neck, or total hip BMD ($p=0.005$–0.029). At the haplotype level, the strongest association was observed with total hip BMD for the haplotype C–T of rs1718454–rs9822918 ($p=0.006$, OR=1.69). Additionally, SNP rs7623768 and the haplotype G–C of rs4076086–rs7623768 in *CRTAP* is associated with

Table 3 The genomic position, minor allele frequency (MAF), Hardy–Weinberg equilibrium (HWE) test statistic, linkage disequilibrium (LD) plot, and call rate for each of the SNPs

Gene	LD (*D′*) plot	SNP ID	Genomic position (bp)	Genic position	Alleles (major/minor)	MAF	HWE *p*	Call rate
PPARG 3p25		rs17036188	12315925	Intron 1	C/T	0.388	0.112	0.997
		rs2028760	12347882	Intron 1	A/G	0.382	0.326	1.000
		rs10510418	12363563	Intron 1	A/C	0.162	0.344	0.987
		rs2938392	12409608	Intron 4	C/T	0.448	0.070	0.925
		rs1875796	12418657	Intron 4	C/T	0.447	0.154	0.994
		rs4135275	12418844	Intron 4	A/G	0.456	0.521	1.000
		rs1822825	12424963	Intron 5	C/T	0.448	0.160	0.998
		rs709157	12437024	Intron 6	A/G	0.011	0.1126	0.999
		rs3856806	12450557	Exon 7	T/C	0.275	1.000	0.998
CRTAP *3P22.3*		rs11129545	33134270	Intron 1	A/G	0.456	0.797	0.999
		rs4076086	33136902	Exon 2	A/G	0.463	0.600	0.998
		rs7623768	33157056	Intron 6	A/C	0.270	1.000	1.000
		rs12635415	33162086	3′	A/G	0.264	0.678	0.979
TDGF1 3p21.31		rs11130097	46595618	Exon 2	C/T	0.390	1.000	0.995
		rs2280413	46596797	Intron 5	C/T	0.129	0.317	0.997
		rs1049667	46598694	3′	A/C	0.168	0.049	0.983
PTHR1 3p22-p21.1		rs6442037	46904550	Intron 3	A/G	0.306	1.000	0.996
		rs724448	46910738	Intron 4	G/T	0.422	0.005	0.999
		rs2242116	46916120	Intron 8	C/T	0.425	0.002	0.994
FLNB 3p14.3		rs9828717	57974026	Intron 1	C/T	0.499	0.481	0.997
		rs1718456	57987388	Intron 1	C/T	0.448	0.438	0.999
		rs1718481	58000943	Intron 1	A/G	0.495	0.160	1.000
		rs1718454	58025988	Intron 1	C/T	0.362	0.213	1.000
		rs9822918	58032724	Intron 1	G/T	0.325	0.827	0.994
		rs2177153	58067386	Intron 11	A/G	0.022	0.376	0.999
		rs1131356	58084202	Exon 21	A/G	0.077	1.000	1.000

femoral neck BMD (p=0.009 and p=0.003, respectively). *PTHR1* showed haplotypic associations with lumbar spine and femoral neck BMD (p=0.02 and p=0.044, respectively).

Mutations in *FLNB* have been observed in a number of human skeletal disorders, including boomerang dysplasia [16], Larson syndrome [17, 18], spondylocarpotarsal synostosis [18, 19], and atelosteogenesis I and III [18, 20]. Together with the intense and uniform *FLNB* expression detected throughout the growth plate in normal mouse embryos in resting, proliferating, and prehypertrophic and hypertrophic chondrocytes, it is thought that *FLNB* plays a central role in skeletogenesis and joint formation [18]. Interestingly, a number of mutations that lead to the broad phenotypic spectrum are located within the actin-binding domain of *FLNB*. A functional actin cytoskeleton may be important for many normal morphogenetic processes, including skeletogenesis [16].

The phenotypes of *FLNB*-deficient mice also revealed the importance of the gene in skeletogenesis. *FLNB*$^{-/-}$ mice have vertebral fusions and abnormalities and decreased hyaline cartilage in the vertebral, carpal, and tarsal bones (Table 1) similar to the human clinical malformations seen in vertebral segmentation, joint formation, and skeletogenesis in the syndromes of spondylocarpotarsal syndrome [22, 23], atelosteogenesis I and III [23], Larsen syndrome [23], and boomerang dysplasia [23]. Scoliotic and kyphotic abnormalities of the vertebral column in *FLNB*$^{-/-}$ mice resemble those observed in human boomerang dysplasia [23].

In addition to these monogenic bone diseases, *FLNB* is also associated with human BMD measured at various sites. SNPs rs9822918 and rs2177153 were associated with age-corrected BMD at both the femoral neck (p=0.02–0.0002) and total hip (p=0.02–0.0006) in 771 women from the

Table 4 SNPs significantly associated with BMD in additive model

SNP	Gene	Lumbar spine BMD (adjusted with height and weight)		Femoral neck BMD (adjusted with height and weight)		Total hip BMD (adjusted with age and weight)	
		p value	Odds ratio	*p* value	Odds ratio	*p* value	Odds ratio
rs7623768	*CRTAP*	0.33	0.87 (0.65–1.15)	0.009*	0.66 (0.48–0.90)	0.099	0.75 (0.53–1.06)
rs9828717	*FLNB*	0.005*	1.51 (1.13–2.00)	0.09	1.32 (0.96–1.82)	0.048*	1.43 (1.00–2.04)
rs1718456	*FLNB*	0.029*	1.37 (1.03–1.83)	0.027*	1.44 (1.04–1.99)	0.14	1.30 (0.92–1.85)
rs1718454	*FLNB*	0.029*	0.73 (0.55–0.97)	0.08	0.76 (0.56–1.03)	0.019*	0.66 (0.47–0.93)
rs9822918	*FLNB*	0.27	1.19 (0.88–1.61)	0.105	1.31 (0.95–1.81)	0.017*	1.55 (1.08–2.23)

*$p<0.05$

Table 5 Result of haplotype association tests

Gene	Markers	Omnibus test (adjusted *p* value)	Haplotype-specific test				Omnibus test controlling for haplotype (adjusted *p* value)
			Haplotype	Frequency	Adjusted *p* value	Odds Ratio	
Phenotype: lumbar spine BMD[a]							
PTHR1	rs724448	0.02*	GC	0.40	0.01*	0.59	NA[c]
	rs2242116		TT	0.59	0.02*	1.60	NA[c]
FLNB	rs9828717	0.003*	TCGT	0.29	0.04*	0.72	0.009*
	rs1718456		CCGC	0.05	0.203	1.59	0.003*
	rs1718481		TCGC	0.14	0.479	1.16	0.002*
	rs1718454		CTAC	0.39	0.083	1.30	0.005*
			TCAC	0.05	0.144	0.59	0.004*
Phenotype: femoral neck BMD[a]							
CRTAP	rs4076086	0.028*	GC	0.21	0.003*	0.43	0.959
	rs7623768		AC	0.06	0.886	0.93	0.011*
			GA	0.26	0.273	1.34	0.019*
			AA	0.47	0.094	1.49	0.042*
PTHR1	rs724448	0.044*	GC	0.40	0.031*	0.62	NA[c]
	rs2242116		TT	0.59	0.044*	1.55	NA[c]
FLNB	rs1718456	0.031*	CGT	0.29	0.121	0.77	0.042*
	rs1718481		CGC	0.19	0.571	1.12	0.018*
	rs1718454		TAC	0.40	0.013*	1.52	0.194
			CAC	0.05	0.015*	0.41	0.177
Phenotype: total hip BMD[b]							
CRTAP	rs4076086	0.015*	GC	0.21	0.007*	0.44	0.217
	rs7623768		AC	0.06	0.264	1.93	0.010*
			GA	0.27	0.127	1.57	0.017*
			AA	0.47	0.622	1.14	0.006*
FLNB	rs1718454	0.027*	CT	0.30	0.006*	1.69	0.478
	rs9822918		TG	0.33	0.025*	0.66	0.127
			CG	0.34	0.781	0.95	0.011*

NA not applicable

[a] Adjusted for height and weight as covariates

[b] Adjusted for age and weight as covariates

[c] Only haplotypes GC and TT were observed for rs724448 and rs2242116. Both alleles of rs724448 were never observed on the same haplotypic background. In this case, the null model is identical to the alternate model, and hence, the control effect cannot be tested

*$p<0.05$

GENOS sib-pairs study [21]. Such association was replicated in a population-based cohort of 1,192 unrelated Caucasian women from the CAIFOS (CAlcium Intake Fracture Outcome Study)/CARES (Caring for Adults Recovering from the Effects of Stroke) study [21]. Both rs9822918 and rs2177153 were included in our present study. In our cohort, rs9822918 was also significantly associated with total hip BMD (p=0.017, OR=1.55). No association was nevertheless observed for rs2177153 (p>0.05). The large discrepancy between the MAF of rs2177153 in Caucasian (MAF=0.292 from HapMap) and southern Chinese women (MAF=0.02 from the present study) may explain the association difference.

Kiel et al. [47] used the Affymetrix 100K SNP GeneChip marker set in the Framingham Heart Study to examine genetic associations with BMD. Two SNPs in *FLNB* were included in the 100K marker set. According to the results available at http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v3.p2, rs1658397 was significantly associated with lumbar spine BMD using the additive generalized estimating equation model (p=0.0005) while rs6445945 demonstrated only a modest association (p=0.03) in all the 1,141 phenotyped individuals. Both rs1658397 and rs6445945 are located within the BMD-associated rs9828717–rs1718456–rs1718481–rs1718454–rs9822918 locus. Nevertheless, HapMap phase II data revealed a large discrepancy in the MAF of these two markers between different ethnic groups. The frequency of the minor allele C of rs1658397 is 0.325 and 0.044 in Europeans and Han Chinese, respectively. With a MAF of 0.4 in the European population, rs6445945 is monomorphic in the Han Chinese. Thus, other variants within the locus may affect BMD regulation in the southern Chinese population. In our study, association was more significant at haplotype level than single-marker level, presumably implying that the real causal variant is located within this locus but was not tagged. Another possibility is that overall variation in this locus may influence BMD regulation. We have recently demonstrated that multiple genes at 1p36 contribute to osteoporosis susceptibility in Chinese [48]. Resequencing and genotyping with higher marker density in the *FLNB* gene may provide more evidence of a regional association with BMD.

The strongest association was observed for rs9828717 with lumbar spine BMD. Comparative genomics analyses indicated that the rs9828717 is located within a conserved noncoding sequence. Prediction of potential transcription factor binding sites shows that the minor T allele at rs9828717 may abolish the binding site of NFAT that the major C allele possesses. NFAT is a family of transcription factors with activity inhibited by calcineurin inhibitors. Bone loss has been observed in both humans [49] and rats [50] treated with calcineurin inhibitors. Such bone loss is attributable to the suppressive effects of calcineurin inhibitors on osteoblast differentiation and osteoblastic bone formation [51]. This has outweighed its inhibition of osteoclastogenesis by suppressing *NFAT* induction by *RANKL* [52]. In addition, NFATc2 knockout mice suffered from a reduction of trabecular bone volume caused by the downregulation of markers for osteoblastic bone formation [51]. The regulatory role of *NFAT* in osteoblastogenesis is in line with our association result that the minor T allele increases the risk of low BMD, as NFAT fails to bind and trigger the transcriptional program of osteoblasts.

CRTAP is expressed in both osteoblasts and osteoclasts. *CRTAP* shares homology with a family of putative prolyl 3-hydroxylases and can form a complex with cyclophilin B and prolyl 3-hydroxylase 1 which is crucial for bone development and collagen helix formation [53]. Loss of *CRTAP* in mice causes osteochondrodysplasia which is characterized by severe osteoporosis due to deficient bone formation [35]. Loss of *CRTAP* in humans is associated with recessive osteogenesis imperfecta [35]. In the current study, rs7623768 in *CRTAP* is significantly associated with femoral neck BMD (p=0.009), and the haplotype G–C of rs4076086–rs7623768 is consistently associated with femoral neck BMD (p=0.003) and total hip BMD (p=0.007). We recently demonstrated that variants of the sclerostin gene that cause sclerosteosis and van Buchem disease are also associated with osteoporosis [54]. Association of *CRTAP* polymorphisms with femoral neck BMD further supports previous observations that genes associated with monogenic bone diseases also contribute to BMD variation and osteoporosis risk in the general population.

PTHR1 is a member of the superfamily of G-protein-coupled receptors. The gain-of-function mutations in the *PTHR1* gene cause Jansen's metaphyseal chondrodysplasia that is characterized by growth plate abnormalities and increased bone resorption, while loss-of-function mutations in *PTHR1* cause Blomstrand chondrodysplasia which is characterized by advanced endochondral bone maturation and increased BMD. In the current study, *PTHR1* showed haplotypic association with lumbar spine and femoral neck BMD (p=0.02 and p=0.044, respectively), although no association was observed between BMD and individual SNP in *PTHR1*. It is worth noting that two previous studies also reported the association of BMD with haplotypes but not single SNPs in this region of *PTHR1* [29, 31]. It is likely that untyped common variant or multiple rare variants are responsible for the observed association. Because SNPs in this region of *PTHR1* are in strong LD, it is difficult to clearly define the primary associated variant(s) by population genetics approaches. Functional assessment of the variants via computational methods, laboratory assays, or model systems will be required to determine variant(s) responsible and the mechanism of the observed association.

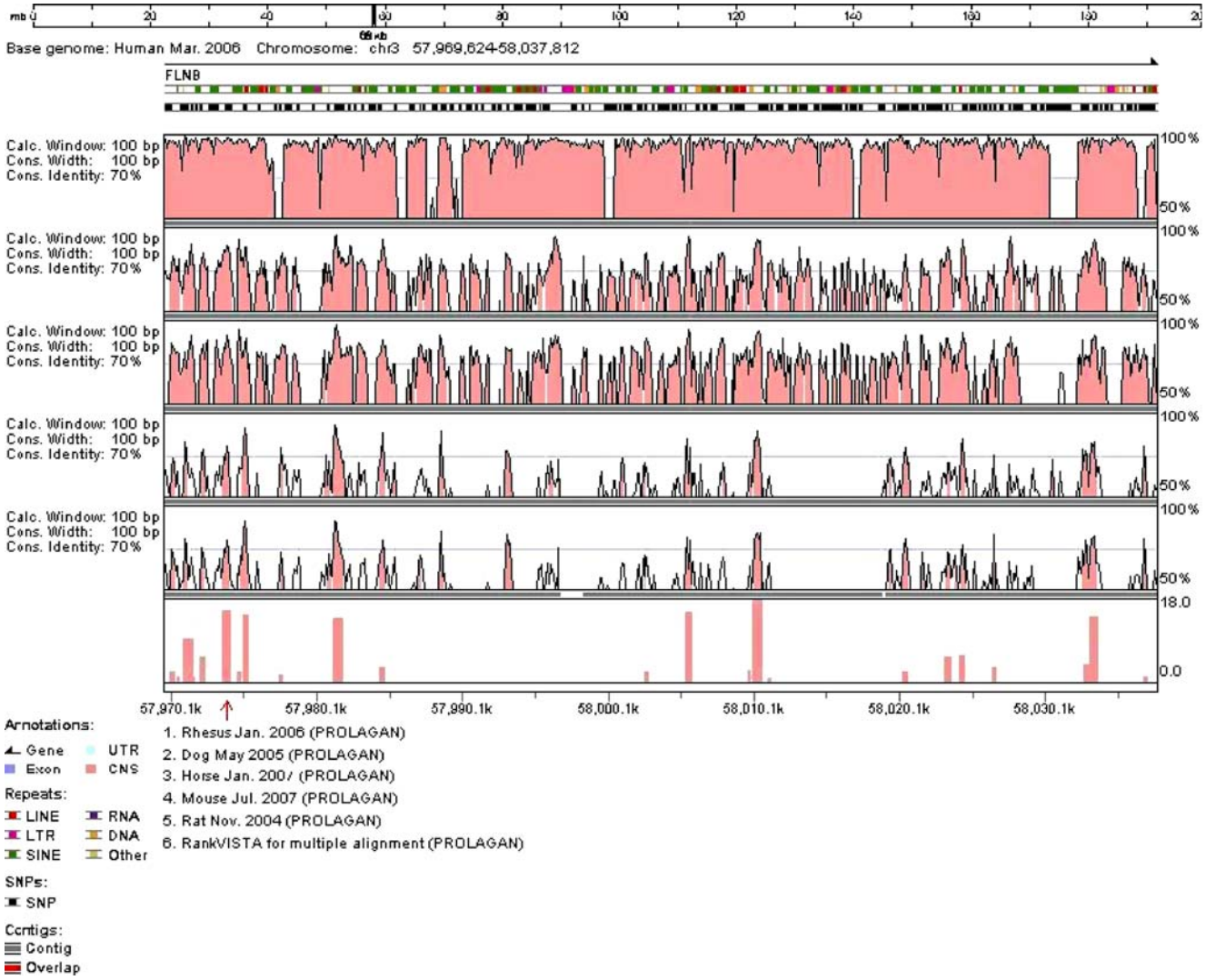


Fig. 1 VISTA browser plot of the comparative analysis for intron 1 in *FLNB* (Chr3:57,969,624-58,037,812 on the human March 2006 genome). The position of rs9828717 was indicated by the *red arrow*

The strength of our study is that the selected sampling strategy can substantially increase power over random sampling for detection of allelic association [55]. Assuming a marker is in complete LD ($D'=1$) with a QTL or the causal allele accounting for 1% of BMD variation and the MAFs of the marker and QTL are both 0.1, more than 98% power can be achieved to detect the additive genetic effects of the marker at a significance level of $\alpha=0.05$ in the whole study population. Making the same assumptions with use of the same parameters, the power was 87%, 77%, and 73% for lumbar spine, femoral neck, and total hip BMD, respectively, in the postmenopausal women subgroup. Based on the power calculation, our study should have sufficient power to detect any association between a marker and BMD. Nonetheless, this study failed to replicate the association between rs7646054 in *ARFGEH3* and BMD in postmenopausal women recently observed by Mullin et al. [14]. The limitation of this study was that some susceptibility genes/variants may have been missed in this well-replicated region, because a candidate gene approach was used. Recent genome-wide association studies demonstrated that many associations implicate non-protein-coding regions [5–7]. Another limitation of this study was no correction for multiple testing. Although smaller *p* values generally provide greater support for a true association, it is the consistency and strength of the association across one or more replication studies, rather than the strength of the *p* value in a single study, that is critical to exclude false-positive association. Thus, we mainly evaluated the significance of our association in relation to previous replication. Since our design and choice of SNPs was based on evidence drawn from previous linkage and functional studies, our success to replicate the

association of some of the SNPs provides evidence that these associations are likely to be valid.

In conclusion, our results suggest that *FLNB* and *CRTAP* are promising susceptibility genes for BMD regulation within 3p14-25 in the southern Chinese women. Further replication and functional studies are required to elucidate their role in bone remodeling.

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Conflicts of interest None.

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