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Multiple analyses indicate the specific association of NR1I3, C6 and TNN with low hip BMD risk

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Supplementary data

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Low hip bone mineral density (BMD) is an important index for osteoporosis and is associated with hip fracture, which leads to more cases of disability and mortality than all other kinds of fractures (Kanis et al., 2007). BMD's heritability is more than 60% (Arden et al., 1996). A number of candidate loci for BMD have been previously identified by Genome Wide Association Studies (GWAS) (Xiong et al., 2009; Karasik et al., 2010; Zhang et al., 2013). Nevertheless, many significant signals based on GWAS are difficult to interpret because common single nucleotide polymorphisms (SNPs) selected for GWAS have small effects and explain less than 10% of BMD variation (Takai, 2005). Some low frequency SNPs and rare SNPs with larger effects may be missed by GWAS (Takai, 2005).

Sequencing can provide detailed genetic variant information to identify low frequency variants that GWAS may have missed. Several sequencing studies have been conducted on osteoporosis. One study based on whole genome sequencing (WGS) found that a rare mutation in the leucine rich-repeat-containing G-protein-coupled receptor 4 (*LGR4*) gene is strongly associated with low BMD in Icelandic subjects (Styrkarsdottir et al., 2013). Another WGS study suggested that *COL1A2* is associated with both low hip and spine BMD in Icelanders (Styrkarsdottir et al., 2015). A recent WGS indicated that EN1 is a determinant of bone density and fracture in Europeans (Zheng et al., 2015). All of these studies focused mainly on Western populations; so far, no sequencing study has been conducted for osteoporosis in the Asian population. As established by The International HapMap Consortium and others (Wang et al., 2013), different ethnic populations may have distinct allele frequencies and linkage disequilibrium patterns for specific potential functional variants. The results from one ethnic group may not hold true for other ethnic groups. Meta-analyses across different ethnic groups may have some advantages, because analyzing samples with diverse ancestries will increase statistical power to identify those responsible loci shared by different ethnic populations (Do et al., 2012).

In this study, we performed whole exome sequencing (WES) with high sequencing depth (more than 40×) in 101 Chinese individuals, carried out a meta-analysis for hip BMD by combining WES and WGS (sequencing depth more than 50×) analysis in 44 Caucasian individuals, and compared the genotypes of extremely high- (top 20%) and low-hip BMD (bottom 20%) individuals. Moreover, other data were used to test the causality of variants in the meta-analysis, which include 1) GWAS with imputed genotype of 11,140 individuals in seven individual studies, 2) gene expression data with 40 extremely high-hip BMD (top 20%) *versus* 40 extremely low-hip BMD (bottom 20%) in Caucasian individuals, and 3) functional interaction analysis of significant genes expressed with high correlation. The combination of multiple datasets and independent studies may help to identify the functional variants for hip BMD. The workflow of the study design is shown in Fig. S1.

WES generated 332,894 SNPs, and WGS yielded 10,871,465 SNPs. About 14,000 SNPs were common between these two independent studies. Based on the meta-analysis of these two studies, 262 SNPs achieved nominal significant levels with P meta values less than 0.01 (call rate more than 90% and minor allele frequency (MAF) more than 1%). The 262 SNPs were examined by GWAS of seven studies that included Asian, Caucasian, African American, and Hispanic subjects ($P \leq 0.05$). Eleven SNPs were detected to be significantly associated with hip BMD (Fig. 1A). These 11 SNPs are located in 10 genes, including *NCF4*

(rs2075939), *NR1I3* (rs2307424), *DOCK8* (rs10814836), *TNN* (rs2072031), *TNFRSF1A* (rs1800692), *MLPH* (rs3817362), *WNT16* (rs2707466), *SCGB1A1* (rs11549442), *C6* (rs4957374 and rs10075985), and *SLC4A2* (rs12703112). The *C6* gene has two nominally significant SNPs (P meta ≤ 0.01, and P GWAS ≤ 0.05), which are close to each other in physical distance (about 4500 bp). The SNP rs12703112 in the osteo-related gene *SLC4A2* was a low-frequency SNP (MAF < 5%). The MAF of the 11 significant SNPs are displayed in Table S1.

Peripheral blood mononuclear cells (PBMC) have been reported to be related to the function of osteoclasts (Lei et al., 2011). Based on previous microarray results (Hammer et al., 2012), the differential expression of these 10 significant genes between high- and low-hip BMD individuals was analyzed, and mRNA levels of five genes were identified to be significantly different between high- and low-hip BMD individuals (P < 0.05) (Fig. 1A and Table S2). These genes include *NR1I3*, *TNN*, *C6*, *SCGB1A1* and *SLC4A2*.

As the most significant gene, *NR1I3* encodes a hormone receptor and was previously reported to be related to BMD regulation (Swindle et al., 2001). The correlation between the mRNA level of *NR1I3* and the other significant genes (*NCF4*, *DOCK8*, *TNN*, *TNFRSF1A*, *MLPH*, *WNT16*, *SCGB1A1*, *C6*, *and SLC4A2*) was evaluated within both high-hip BMD individuals and low-hip BMD individuals (Fig. 1A and Table S3). The mRNA levels of *C6* (P = 0.050) and *TNN* (P = 0.083) were highly correlated with mRNA levels of *NR1I3* in 40 low-hip BMD individuals (Fig. 1A and Table S3), but not significant in high-hip BMD individuals.

According to gene expression correlation analysis, the transcription of *TNN* and *C6* was correlated with the expression level of *NR1I3*. Since NR1I3 is a steroid hormone receptor, we performed promoter prediction analyses to search for hormone-related *cis*-elements within 5-kb upstream from the translation start sites of *C6* and *TNN*. *BRCA1-ERE*s were found in the promoters of both *C6* and *TNN*. They are located at −1673 to −1644 of *C6* and −1918 to −1889 of *TNN* (Fig. S2A).

We verified the interaction of the NR1I3 protein with *BRCA1-ERE* using the yeast one-hybrid system. Based on the alignment (Fig. S2A), a consensus probe was designed as the bait according to the following rule: using the identical base if the base in the two *BRCA1-EREs*; using the nucleotide that is matched to the original *BRCA1-ERE if not identical*. The counting results indicated that NR1I3 can bind to the *BRCA1-ERE* probe. A higher surviving percentage in screening medium 4 indicated a stronger binding of protein to DNA bait (Figs. 1B and S2B).

The binding of NR1I3 protein to *BRCA1-ERE* was also confirmed by electrophoretic mobility shift assay (EMSA) (Fig. 1C). The end-labeled probe for *BRCA1-ERE* was retarded remarkably by NR1I3 protein, and the retardation was inhibited by the excess amount of cold probes (Figs. 1C and S2C).

WGS and WES can reveal some functional loci that may not be identified by GWAS (Hendrickx et al., 2014). Considering that most apparent functional loci are located within the coding region, WES is an efficient, cost-effective tool to capture susceptibility loci

responsible for human disease (Smith et al., 2009). Several WGS osteoporosis studies using European samples have identified some variants responsible for the risk of low BMD. In this study, 11 SNPs in 10 genes were identified to be associated with the risk of low-hip BMD in a Chinese cohort by a meta-analysis of WES and WGS. Further, we used the evidence of GWAS replication, gene expression and protein-DNA interaction to verify the association analysis.

NR1I3, *TNN*, *C6*, *SCGB1A1*, *and SCL4A2* were differentially transcribed in PBMCs between high- and low-hip BMD subjects, which indicated that these five genes may play roles in the differentiation of osteoclasts (Lu et al., 2009). Because PBMCs are associated with the function of osteoclasts rather than osteoblasts (Osabe and Negishi, 2011), the other five genes (*NCF4*, *DOCK8*, *TNFRSFIA*, *WNT16*, *MLPH*) that are not differentially expressed may also be involved in BMD regulation. For example, WNT16 was mainly functional in differentiation of osteoblasts (Styrkarsdottir et al., 2015).

The expression correlation of *TNN* and *C6* with *NR1I3* in low-hip BMD individuals suggested the specific association of these three genes with low-hip BMD risk. The binding ability of NR1I3 to upstream *BRCA1-ERE*s of *TNN* and *C6* indicated possible regulation of NR1I3 for *TNN* and *C6*. Both *TNN* and *C6* have the epidermal growth factor (EGF)-like region. Some EGF-like factors are involved in bone metabolic metastasis. EGF-like proteins may be regulated by NR1I3. Nevertheless, research has shown that EGF can inactivate NR1I3 by activating ERK1/2 proteins. There may be a negative feedback regulation between NR1I3 and EGF or EGF-like protein. This kind of feedback regulation can benefit the homeostasis of bone metabolism and other biological processes. Expression levels of several other EGF-related genes are also significantly correlated with mRNA levels of *NR1I3*, which further confirms the functional relevance of EGF-like proteins and NR1I3 (Table S5). Further studies may be carried out to investigate the mutual regulation of NR1I3 with EGF-related proteins and the role of these regulations on BMD remolding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

SNP ID	Chr.	Position	Gene	Role	P (WES) [a]	P (WGS) [b]	Meta [c]	P (GWA-META) [d]
rs 2075939	Chr 22	37271882	NCF4 [e]	Missense	0.018	2.25×10^{-4}	5.37×10^{-5}	0.138
rs 2307424	Chr 1	161202605	NR1I3△*	Synonymous	0.048	0.003	0.002	0.007
rs 10814836	Chr 9	418003	DOCK8	Intron	0.002	0.132	0.002	0.048
rs 2072031	Chr 1	175052828	TNN△*	Intron	6.16×10^{-4}	0.504	0.003	0.034
rs 1800692	Chr 12	6442346	TNFRSF1A	Intron	0.014	0.036	0.004	0.049
rs 3817362	Chr 2	238449007	MLPH	Missense	0.005	0.117	0.005	0.018
rs 2707466	Chr 7	120979089	WNT16	Missense	0.035	0.017	0.005	1.77×10^{-6}
rs 4957374	Chr 5	41154150	C6△*	Intron	0.211	0.004	0.006	0.044
rs 10075985	Chr 5	41158671	C6△*	Intron	0.263	0.004	0.008	0.034
rs 11549442	Chr 11	62186542	SCGB1A1*	Utr-5	0.003	0.406	0.008	0.036
rs 12703112#	Chr 7	150765236	SLC4A2 *	Intron	0.113	0.007	0.006	0.022

B

Constructs	Mediam 1	Mediam 2	Mediam 3		Mediam 4	
	Number	Number	Number	Percentage*	Number	Percentage*
PGADT7-Rec-NR1I3	808	320	90	28.13%	0	0
PHIS2-ERE	228	136	25	18.38%	0	0
PGADT7-Rec-NR1I3/PHIS2-ERE	840	776	632	81.44%	156	20.10%
Negative control	580	458	35	7.642%	0	0
Positive control	1228	856	828	96.73%	117	13.67%

C

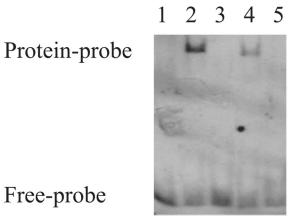

Fig. 1.

The significant genes associated with hip BMD and the interaction of NR1I3 with *BRCA1-ERE*. **A:** The significant loci identified by the meta-analysis of whole exome sequencing (WES) and whole genome sequencing (WGS). Superscript a indicates Fisher's exact test of WES in Chinese cohort (sample size is 101). Superscript b indicates Fisher's exact test of WGS in Caucasian cohort (sample size is 44). Superscript c indicates the meta-analysis of WES and WGS. Superscript d indicates Genome Wide Association (GWA) meta-analysis of imputed genotypes in seven studies. Superscript e indicates the most significant marginal SNP in GWA meta-analysis. # indicates the significant SNP with low frequency (2%). * indicates genes that expressed significantly differential between high-hip BMD individuals and low-hip BMD individuals. △ indicates genes significantly correlated with NR1I3 ($P < 0.05$). **B:** Results of yeast one-hybrid of NR1I3 and *BRCA1-ERE*. * indicates that the number of colonies in medium 2 is used as a divisor. **C:** The EMSA assay of NR1I3 and *BRCA1-ERE*. Lane 1: labeled probe for *BRCA1-ERE*; lane 2: NR1I3 protein + labeled probe for *BRCA1-ERE*; lane 3: NR1I3 protein + labeled probe for *BRCA1-ERE* + 130-fold

unlabeled probe for *BRCA1-ERE*; lane 4: positive control; lane 5: negative control, NR1I3 with one random sequence probe.