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Meta analysis identifies a novel susceptibility locus associated with heel bone strength in the Korean population☆

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ABSTRACT

Introduction: Calcaneal quantitative ultrasound has been recognized as a non-invasive method for evaluation of bone strength and prediction of osteoporotic fracture.
Methods: To extend a thorough genetic catalog for osteoporotic bone properties, we performed a genome-wide association study (rural cohort I, n = 1895) of speed of sound (SOS) using the 1000 genome-based imputation in the discovery stage and then carried out *in silico* lookups (rural cohort II and III, n = 2,967) and *de novo* genotyping (rural cohort IV, n = 4,296) in the replication stage.
Results: In the combined meta-analysis (n = 9,158), we identified a novel variant associated with SOS (rs2445771 in the *GLDN* gene, $P = 2.27 \times 10^{-9}$) reaching genome-wide significance in the Korean population. We further demonstrated that allele-specific regulatory modifications found to be associated with functional enrichments by ENCODE annotations. Conclusion: Our findings could provide additional insights into understanding of genetic and epigenetic regulations on bone metabolism.

1. Introduction

Osteoporotic fracture (OF) is the most severe aspect of osteoporosis characterized by compromised bone quality and content. Although bone mineral density (BMD) has been regarded as the gold standard for diagnosis, a large percentage of people who suffer a fracture do not have osteoporotic bone density [1]. Quantitative ultrasound (QUS) measurements such as broadband ultrasound attenuation (BUA) and speed of sound (SOS) have been recognized as a clinically useful trait for assessment of bone strength or elasticity. Calcaneal SOS is effective for prediction of osteoporotic fracture risk [2–4].

Genomic association studies for various skeletal phenotypes have provided valuable insights into the genetic architecture in the regulation of bone metabolism [5–7]. However, the majority of genome-wide association studies (GWAS) have mainly focused on BMD assessed by central dual-energy X-ray absorptiometry (DXA) measurements in European-ancestry populations [8–13]. Recent large-scale meta analyses from the Genetics Factors for Osteoporosis (GEFOS) consortium newly identified novel variants for bone-related traits [14,15]. However, the genetic basis of bone metabolism has not been fully explored in non-European populations. Given genetic and physiologic heterogeneity among different ethnic groups, multi-stage GWAS of SOS in homogeneous cohort populations of non-European ancestries may increase the chance to discover additional novel loci on heel bone properties.

2. Results

To increase genomic coverage and resolution, we performed a three-stage study comprising a discovery GWAS (stage 1) using imputed SNPs from the 1000 Genome Project data, follow-up *in silico* replication (stage 2) and *de novo* genotyping validation (stage 3) in the Korean population. SNPs showing the deviation between the distributions of the observed and expected *P*-values were observed on the quantile-quantile plot (Supplementary Fig. 1). The estimated value of the genomic control inflation factor ($\lambda_{sos} = 1.020$) indicated limited evidence of population stratification in Rural study samples and supported the validity of ignoring stratification in our cohort subjects. Participant characteristics are listed in Table 1.

Stage 1 analysis in Rural I (n = 1,895) revealed signals showing strong evidence for SOS associations (Supplementary Figure 2). Those signals likely represent new SOS loci that require the validation in additional investigation. In the recapitulation of 59 loci underlying multiple diverse effects on bone properties from two previous GWAS studies, 10 SNPs (rs3754032, rs10496734, rs2099082, rs9294466, rs2214681, rs4869739, rs3020331, rs2908007, rs597319 and rs9533090) in *WDR77*, *ZRANB3*, *ESM1*, *MAP3K7*, *CNTNAP2*, *CCDC170*, *ESR1*, *WNT16*, *TMEM135* and *AKAP11* genes were significantly associated with SOS in

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Table 1
Descriptive characteristics of cohort samples.

	Stage 1	Stage 2		Stage 3
	Rural I	Rural II	Rural III	Rural IV
N	1895	1678	1289	4296
M/F	746/1149	786/872	489/800	1706/2590
Age	62.8 ± 8.5	60.9 ± 6.6	58.3 ± 10.6	54.7 ± 7.7
Weight	60.5 ± 10.3	61.8 ± 10.1	60.5 ± 9.7	61.5 ± 9.8
Height	157.4 ± 8.4	158.2 ± 8.5	158.3 ± 8.3	158.5 ± 8.4
SOS	1533.6 ± 34.1	1591.1 ± 45.3	1538.8 ± 29.1	1599.6 ± 40.4

our stage 1 (Supplementary Table 1). For follow-up *in silico* replication, we selected 6 independent signals (pair-wise linkage disequilibrium (LD) statistics $r^2 < 0.2$ and minor allele frequency (MAF) ≥ 0.01 within a 500 kb window of the genomic region) from the stage 1 analysis based on our arbitrary inclusion threshold ($P < 5 \times 10^{-6}$) (Supplementary Table 2). Of these lead SNPs, the stage 2 replication analysis in Rural II and III cohorts (n = 2,967) showed a statistically significant association (rs2446422 in the *GLDN* gene) in the same direction of association as in the stage 1 analysis results. To consolidate genetic associations of the promising SNP (rs2446422), we confirmed its consistent association with SOS using *de novo* genotyping in Rural IV cohort (n = 4,296). An overall meta-analysis of the total samples (4 studies, up to 9,158) identified a novel locus in *GLDN* reaching genome-wide significance (rs2446422, $P_{overall} = 2.27 \times 10^{-9}$) (Table 2 and Fig. 1).

We next explored the potential impact of sequence variation in the context of ENCODE annotations. We found differential alterations in 4 regulatory motifs (HDAC2, NRSF, RXRA, and Zfx) from the lymphoblastoid cell line (GM12878). In addition, we observed allele-specific signals of histone modifications (H3K4me1 and H3K4me3) as a regulatory enhancer mark. This specific region is also associated with DNase I hypersensitivity in fibroblast (FibroP), hepatocellular carcinoma (Huh-7.5) and epidermal melanocytes (Melano) cell types. To test epigenetic effects underlying regulatory elements, we performed a *cis-me*QTL analysis from adipose tissues collected from 856 healthy female twins of the MuTHER resource. We observed a significant association between rs2445771 and the methylation status of a CpG island within GLDN (probe ID: cg19558802). Again, the significant *me*QTL association with minor allele [G] was correlated with decreased methylation in the *GLDN* (beta = − 0.039, $P_{meQTL} = 4.46 \times 10^{-6}$) (Supplementary Figure 3). A network ontology analysis highlighted biological connectivity among a newly identified gene and previously established SOS genes based on the regulation of bone metabolism (Supplementary Figure 4 and Supplementary Table 1).

3. Discussion

Clinical diagnostics have predominantly focused on standard bone densiometry, however, BMD measures alone are not sufficient for osteoporotic fracture risk prediction. Quantitative ultrasound has recognized as an alternative measure for assessment of the 10-year fracture probability [16] and for screening purposes in population-based cohort studies [3].

Table 2
A novel genetic variant associated with SOS at genome-wide significance in the Korean population.

Study	Effect allele	Other allele	Effect allele Freq	EFFECT ± SEM	*P*
Stage 1				0.16 ± 0.034	3.10E-06
Stage 2	T	G	0.67	0.098 ± 0.028	5.02E-04
Stage 3				0.064 ± 0.024	8.30E-03
Combined				0.096 ± 0.016	2.27E-09

To date, osteoporosis-related genetic loci have been identified by large-scale genome-wide association analyses [8,15,17], however, there still remains genetic determinants to be clarified for better understanding of osteogenesis in bone metabolism. More recently, QUS loci newly identified from the GEFOS consortium were derived from populations of European ancestry with a few East-Asians (~4%, China and Korea) as a small portion of the total subjects [14]. In addition, each group performed genotype imputation using the HapMap Phase II as reference panels in GWAS discovery and replication meta-analysis. This GWA meta-analysis revealed genetic and phenotypic heterogeneity for genetic determinants of heel bone properties at different skeletal sites among diverse ethnic populations. A validation study demonstrated that the most SNPs associated with BUA and SOS in the Framingham GWAS on bone ultrasound phenotypes [18] were not replicated in an independent sample of unrelated European men (n = 2,377). They suggested that lack of replication could be due to population stratification, low genomic coverage and limited tagging property [19].

Recent the 1000 Genomes-based imputation studies have been shown to identify novel variants on a genome-wide scale and refine association signals at a regional level [20–22]. To detect the genetic susceptibility to SOS, we conducted a GWAS screen with imputation using up-to-date reference panel from the 1000 Genomes Project. In multistage GWA meta-analysis, we identified a novel heel SOS locus (rs2446422 in the *GLDN* gene) at genome-wide significance. Despite our GWAS meta-analysis demonstrating straightforward enrichment of associations, larger sample size at the initial stage will be required to detect lower-frequency or higher-penetrance variants. Compared to ASN frequency (0.26) from the 1000 Genomes Phase 1 frequencies, the new locus (rs2446422) has very relatively low minor allele frequencies (MAF) in AFR (0.01), AMR (0.08) and EUR (0.06). Considering substantial differences in MAF and LD between different ethnic groups, genetic variants with low frequency are hardly detected in the genetic association study [23]. In addition, previous 59 loci [14,19] reported from Eurocentric-GWAS meta-analyses were recapitulated in our discovery set. Of these loci, 10 SNPs were found to be associated with SOS in the same direction of effects. The identification of these loci as shared common SNPs between Europeans and East-Asians may increase our understanding of the genetic architecture underlying osteoporosis [24]. Further trans-ethnic fine mapping will be needed to clarify whether a new variant detected in this study are specific to East Asians [25]. To determine pleiotropic effects on bone-related traits, we tested lookup analyses using our previous GWAS sets for osteoporotic fracture and osteoarthritis [26,27]. We did not find significant multiple associations between the SNP rs2446422, osteoporotic fracture and osteoarthritis in the expected direction of effects (data not shown). Although a previous prospective study demonstrated that ultrasound heel measurement predict the risk of hip fracture in elderly women [3], however, supporting evidence for heel SOS effects on skeletal site-specific fracture or OA risk has not been fully understood yet [2,28].

The SNP rs2446422 signal on chromosome 15q15 is located in an intron of the *GLDN* gene (gliomedin), expressed by myelinating Schwann cells. Several functional studies have showed that GLDN plays a functional role in the formation of peripheral nodes of Ranvier along myelinated axons [29–31]. A morphological study reported that two types of ossification (endochondral and intramembranous) and Ranvier's groove were involved in developing human calcaneus and talus [32]. Dysfunction and disruption of Ranvier's nodes have recognized as major contributors to the pathophysiology of human neurodegenerative diseases [33]. The *OLFM3* (olfactomedin 3) known as an important paralog of *GLDN* gene has been recently suggested to be associated with Parkinson's disease *via* recent GWAS [34]. Previous candidate gene-based studies reported that axon guidance pathway genes were associated with osteoporosis-related traits such as bone mineral density and osteoporotic fracture risk [35,36] as well as bone remodeling such as bone resorption and formation [37–39].