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ORIGINAL ARTICLE

Integrating transcriptome-wide association study and mRNA expression profiling identifies novel genes associated with bone mineral density

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Abstract

Summary To scan novel candidate genes associated with osteoporosis, a two-stage transcriptome-wide association study (TWAS) of bone mineral density (BMD) was conducted. The BMD-associated genes identified by TWAS were then compared with the gene expression profiling of BMD in bone cells, B cells, and mesenchymal stem cells. We identified multiple candidate genes and gene ontology (GO) terms associated with BMD.

Introduction Osteoporosis (OP) is a metabolic bone disease characterized by decrease in BMD. Our objective is to scan novel candidate genes associated with OP.

Methods A transcriptome-wide association study (TWAS) was performed by integrating the genome-wide association study (GWAS) summary of bone mineral density (BMD) with two pre-computed mRNA expression weights of peripheral blood and muscle skeleton. Then, another independent GWAS data of BMD was used to verify the discovery results. The BMD-associated genes identified between discovery and replicate TWAS were further subjected to gene ontology (GO) analysis implemented by DAVID. Finally, the BMD-associated genes and GO terms were further compared with the mRNA expression profiling results of BMD to detect the common genes and GO terms shared by both DNA-level TWAS and mRNA expression profile analysis.

Results TWAS identified 95 common genes with permutation P value < 0.05 for peripheral blood and muscle skeleton, such as *TMTC4* in muscle skeleton and *DDX17* in peripheral blood. Further comparing the genes detected by discovery-replicate TWAS with the differentially expressed genes identified by mRNA expression profiling of OP patients found 18 overlapped genes, such as *MUL1* in muscle skeleton and *SPTBN1* in peripheral blood. GO analysis of the genes identified by discovery-replicate TWAS detected 12 BMD-associated GO terms, such as negative regulation of cell growth and regulation of glycogen catabolic process. Further comparing the GO results of discovery-replicate TWAS and mRNA expression profiles found 6 overlapped GO terms, such as membrane and cell adhesion.

Conclusion Our study identified multiple candidate genes and GO terms for BMD, providing novel clues for understanding the genetic mechanism of OP.

Keywords Gene expression profiling · Osteoporosis · Transcriptome-wide association study (TWAS)

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Introduction

Osteoporosis (OP) is a common skeletal disease characterized by reduced bone mineral density (BMD) and increased risk of low-trauma fractures [1]. It was estimated that 200 million people suffer from osteoporosis worldwide [2]. In the USA, it has been estimated that the prevalence rate of osteoporosis was about 10.3% in older adults, while low bone mass prevalence rate achieved 43.9% [3]. The main consequence of OP is an increased risk of bone fractures, especially in the elderly and

postmenopausal women [4], which leads to heavy burden on the health care system and society.

BMD is one of the major diagnostic indexes of osteoporosis (OP), which has a strong genetic component. The estimated heritability of OP achieved more than 50% [5]. During the past decades, extensive efforts have been paid to explore the biological mechanism of OP [6]. Multiple large-scale genetic studies of OP have been conducted, providing important clues for clarifying the biological mechanism of OP. For instance, Kemp JP et al. conducted a genome-wide association study (GWAS) in 142,487 individuals from the UK Biobank and identified 153 new loci associated with heel BMD [7]. Karol Estrada et al. highlighted the polygenic nature of BMD and the crucial roles of several biological pathways influencing the risks of OP or fractures [8]. Additionally, functional studies identified multiple important molecules implicated in the development of OP. For example, transforming growth factor beta 1 is abundant in bone tissue and plays a key role in the biological function of both osteoblast and osteoclast [9]. Hypoxia-inducible factor 1-alpha is essential for osteoclast activation and represents a potential therapeutic target to prevent osteoclast activation and bone loss in postmenopausal OP patients [10]. Although extensive studies of OP have been conducted, the biological mechanism of OP still remains elusive now [11].

GWAS mainly focus on the association signals of genetic variants within or close to known genes. However, there are several issues and limitations to these prevailing types of genetic association studies [12]. GWAS signals often span many putative target genes, which may affect gene expression through regulation [13]. It has been demonstrated that genetic loci identified by GWAS were enriched in the non-coding regulatory regions [14], such as expression quantitative traits loci (eQTLs) and methylation quantitative traits loci (meQTL) [15]. These causal genetic variants within non-coding regulatory chromosomal regions are difficult to be distinguished from neighboring markers in previous GWAS. Recently, a powerful strategy called transcriptome-wide association study (TWAS) was proposed, which can integrate tissue-related gene expression measurements with GWAS summary data. Actually, recent studies have demonstrated the good power of TWAS for gaining insights into the genetic basis of complex traits and diseases [16, 17]. For instance, Gusev A et al. identified 157 TWAS-significant genes, of which 35 did not overlap a known GWAS locus [16]. In addition, a TWAS of 229,000 women identified new susceptibility genes for breast cancer [17].

In this study, using large GWAS discovery and replicate data of BMD and reference mRNA expression profiles of peripheral blood and muscle skeleton, we firstly conducted two TWAS to scan novel candidate genetic loci for BMD. Subsequently, the significant common genes identified by discovery-replicate TWAS were subjected to GO analysis. The TWAS results of significant genes and GO terms were further compared with the mRNA expression profile analysis results of OP (including bone cells, B cells, and mesenchymal stem cells (MSC)) to detect common genes and GO terms.

Materials and methods

Discovery GWAS data of BMD

The GWAS summary data of BMD was driven from the Genetic Factors for Osteoporosis (GEFOS) Consortium (http://www.gefos.org/). Briefly, the GEFOS project used meta-analysis of whole genome sequencing, whole exome sequencing, and deep imputation of genotype data to identify candidate variants associated with the risk of osteoporosis using 52,236 individuals of European ancestry. ALSPAC and TwinsUK cohorts were sequenced at an average read depth of 6.7× through the UK10K program (http://www.UK10K.org) using the Illumina HiSeq platform and aligned to the GRCh37 human reference using BWA [18]. The AOGC, FHS, RS-I, ESP, and ERF cohorts were whole exome sequenced as described in the Supplementary Information in the published study. All remaining discovery cohorts were genome-wide genotyped and imputed to the UK10K/1000 Genomes reference panel. Lead single-nucleotide variants were selected for replication genotyping, which was performed at LGC Genomics, Erasmus MC, and deCODE genetics using KASP genotyping. Single variants with a minor allele frequency (MAF) > 0.5% were tested for an additive effect on femoral neck BMD (FN-BMD), lumbar spine BMD (LS-BMD), and forearm BMD (FA-BMD), adjusting for sex, age, age^2, and weight as covariates. Detailed information of the subjects, genotyping, imputation, and quality control could be found in the published study [19].

Replication GWAS data of BMD

Another GWAS data of BMD estimated from quantitative heel ultrasounds (eBMD) was used as the replication data of this study. Briefly, they performed a GWAS of eBMD in 142,487 individuals from the UK Biobank (https://www.ukbiobank.ac.uk/) [20]. Imputation was conducted by the IMPUTE2 against the 1000 genome and UK10K reference panels. Association analysis was performed using the linear mixed non-infinitesimal model of BOLT-LMM [21]. Genotyping array, age, and sex were included as covariates in all models. Detailed information of the subjects, genotyping, imputation, and quality control could be found in the published study [7].

Gene expression profile of B cells

The OP differently expressed genes identified in B cells for postmenopausal osteoporosis were obtained from the published study [22]. In brief, researchers recruited 20 unrelated postmenopausal white women, 54–60 years of age, including

10 with low and 10 with high BMD. Affymetrix HG-U133A GeneChip arrays were used to identify genes differentially expressed in B cells between 10 low and 10 high BMD postmenopausal women. The significance of gene expression difference was determined by *t* test and adjusted for multiple testing with the Benjamini and Hochberg (BH) procedure. The differently expressed genes were identified at BH-adjusted *P* values ≤ 0.05. Detailed description of sample characteristics, experimental design, statistical analysis, and quality control can be found in the previous study [22].

Gene expression profile of mesenchymal stem cell

The OP differently expressed genes identified in mesenchymal stem cell (MSC) were driven from the published study [23]. In this study, MSCs of patients suffering from osteoporosis were isolated from femoral heads after low-energy fracture of the femoral neck. Additional criteria for confirming primary osteoporosis in these donors were vertebrae fractures and advanced age. The MSCs of 5 elderly patients (79–94 years old) suffering from OP (hMSC-OP) and 5 age-matched controls were subjected to mRNA expression profile analysis, implemented by Affymetrix GeneChips Human Genome U133_Plus_2.0. Significance analysis of microarray software was used to analyze the gene expression profile data [24]. The differently expressed genes were identified at fold changes ≥ 2.0, and false discovery rates < 10%. Detailed description of sample characteristics, experiment design, and statistical analysis of this data is available in the published study [23].

Gene expression profile of bone

The OP gene expression profile data of bone tissue was obtained from the Gene Expression Omnibus database (access number: GSE8406). Briefly, the OP samples were obtained from 10 female patients (aged from 74 to 87 years), which underwent hip arthroplasty caused by femoral neck fracture. A Compugen Human 19K-oligo library spotted onto glass slides by the Adelaide Microarray facility was used. The slides were interrogated by competitive hybridization of Cy3- and Cy5-labeled pairs of 9 OP patients and 9 controls, matched for age and sex. The print-tip Lowess normalization and the Bayesian statistical analyses of LIMMA package were used for mRNA expression profile analysis [25–27]. The differently expressed genes between OP patients and controls were identified at fold change > 1.5 and adjusted *P* value < 0.05. Detailed description of sample characteristics, experimental design, statistical analysis, and quality control can be found in the previous study [28].

TWAS of BMD

The TWAS of BMD was conducted using the FUSION software (http://gusevlab.org/projects/fusion/) in this study [29]. Using pre-computed gene expression weights together with GWAS summary data, FUSION is capable of evaluating the gene expression associations between every gene and target diseases [29]. Briefly, the FUSION software calculated gene expression weights using a small set of individuals with both SNP genotype and mRNA expression data. For a given gene, the SNP-mRNA expression weights in the 1-Mb cis locus were firstly computed with the Bayesian linear mixed model. Let w denoted the weights, Z denoted the Z-scores of BMD, and L denoted the SNP correlation matrix. The associations between imputed gene expression levels and BMD values were estimated as $Z_{\mathrm{TWAS}} = w' Z \ (w'Lw)^{1/2}$ [29]. The imputed gene expression data can be viewed as a linear model of genotypes with weights based on the correlation between SNPs and gene expression in the training data, while accounting for linkage disequilibrium among the SNPs [30]. Specific for this study, the pre-computed gene expression weights of muscle skeleton and peripheral blood were downloaded from the FUSION website (http://gusevlab.org/projects/fusion/). The gene expression weights of muscle skeleton were driven from the Genotype-Tissue Expression Project (version 7; $n = 361$) [31]. The gene expression weights of peripheral blood were calculated from 1245 unrelated control individuals from the Netherlands Twin Registry study [32, 33]. The permutation tests of FUSION were implemented to control the potential impact of multiple testing problem. Two thousand permutations were conducted in this study. Significant genes were identified at permutated *P* values < 0.05.

GO analysis

The BMD-associated genes identified by the two-stage TWAS were further analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) tool for GO enrichment analysis [34, 35]. For mRNA expression profiles, the GO analysis of differently expressed genes identified in bone cells, B cells, and MSC was also performed by DAVID. Finally, the DAVID analysis results of TWAS were compared with those of mRNA expression profiles to detect the common GO terms shared by TWAS and mRNA expression profiles. The default GO annotation database of the DAVID tool was used here, including biological processes, cellular components, and molecular function.

Result

TWAS results of BMD

The top 10 genes identified by both discovery and replication TWAS for muscle skeleton and peripheral blood are shown in Tables 1 and 2, respectively. For FA-BMD, the two-stage TWAS identified 19 genes for muscle skeleton and 7 genes for peripheral blood, such as MFGE8 (discovery $P_{\mathrm{permu}} =$

Table 1 List of top 10 genes identified by both discovery and replication TWAS for muscle skeleton

Gene	Discovery TWAS		Replication TWAS P_{permu}
	P_{permu}	Skeletal site	
ATL2	0.002	Forearm	0.039
MUL1	0.002	Femoral neck	0.031
ZFAT	0.002	Femoral neck	0.040
HOMER1	0.004	Femoral neck	0.018
MFSD1	0.004	Femoral neck	0.044
TMOD3	0.004	Lumbar spine	0.042
DDX59	0.005	Forearm	0.049
	0.037	Lumbar spine	
ZNF835	0.005	Lumbar spine	0.002
AC005682.8	0.005	Femoral neck	0.044
TMBIM1	0.007	Femoral neck	0.036

P_{permu} denotes the permutated P value calculated by the FUSION software

0.044 and replication $P_{permu} = 0.001$) in muscle skeleton and TMEM66 (discovery $P_{permu} = 0.001$ and replication $P_{permu} = 0.024$) in peripheral blood (Supplementary Tables S1 and S2). For FN-BMD, the two-stage TWAS identified 25 genes for muscle skeleton and 16 genes for peripheral blood, such as MUL1 (discovery $P_{permu} = 0.002$ and replication $P_{permu} = 0.031$) in muscle skeleton and TMEM9B (discovery $P_{permu} = 0.003$ and replication $P_{permu} = 0.029$) in peripheral blood (Supplementary Tables S3 and S4). For LS-BMD, the two-stage TWAS identified 34 genes for muscle skeleton and 16 genes for peripheral blood, such as TMOD3 (discovery $P_{permu} = 0.004$ and replication $P_{permu} = 0.042$) in muscle skeleton and RERE (discovery $P_{permu} = 0.006$ and replication $P_{permu} = 0.002$) in peripheral blood (Supplementary Tables S5 and S6).

Table 2 List of top 10 genes identified by both discovery and replication TWAS for peripheral blood

Gene	Discovery TWAS		Replication TWAS P_{permu}
	P_{permu}	Skeletal site	
TMEM66	0.001	Forearm	0.024
FCHO2	0.001	Lumbar spine	0.025
TMEM9B	0.003	Femoral neck	0.029
GMPPB	0.006	Femoral neck	0.033
	0.025	Lumbar spine	
	0.039	Forearm	
RERE	0.006	Lumbar spine	0.002
	0.018	Femoral neck	
C11orf10	0.008	Lumbar spine	0.021
	0.026	Forearm	
CCR1	0.010	Lumbar spine	0.004
C14orf118	0.010	Femoral neck	0.029
ZNF318	0.010	Forearm	0.005
CORO1C	0.012	Femoral neck	0.027
	0.026	Lumbar spine	

P_{permu} denotes the permutated P value calculated by the FUSION software

Common genes shared by TWAS and mRNA expression profiles

We further compared the genes identified by the two-stage TWAS with the differently expressed genes identified by mRNA expression profiles. We detected 18 common genes shared by TWAS and MSC, such as TMEM66 (fold change $(FC)_{mRNA} = 0.48$), TMTC4 ($FC_{mRNA} = 2.19$), and RERE ($FC_{mRNA} = 5.26$) (Table 3). After querying the GWAS Catalog database, 2 (SPTBN1 and RERE genes) of the 18 genes have been reported by previous studies of osteoporosis.

GO enrichment analysis results

GO analysis of BMD-associated genes identified by both discovery and replication TWAS detected 12 GO terms, such as negative regulation of cell growth ($P_{TWAS} = 0.002$) and regulation of glycogen catabolic process ($P_{TWAS} = 0.016$) (Table 4). Further comparing the GO analysis results of TWAS and mRNA expression profiles detected 6 common GO terms, such as membrane ($P_{TWAS} = 0.002$, $P_{mRNA} = 5.46 \times 10^{-27}$) and cell adhesion ($P_{TWAS} = 0.011$, $P_{mRNA} = 7.70 \times 10^{-7}$) (Table 5).

Discussion

In this study, we conducted an integrative analysis of discovery-replicate TWAS and mRNA expression profiles of OP considering multiple tissues or cells. We identified multiple BMD-associated genes and GO terms detected at both DNA-level TWAS and mRNA expression profile analysis, such as SPTBN1 and RERE.

SPTBN1, which encodes spectrin beta chain, brain 1, was commonly observed for FN-peripheral blood TWAS, replicate TWAS, and mRNA expression profile study of MSC in our study. Calabrese GM et al. found that SPTBN1 was potentially responsible for the effects of BMD GWAS loci on chromosome 2p16.2 [36]. It has also been illustrated that SPTBN1 is a molecular scaffolding protein that links the actin cytoskeleton to the plasma membrane [37]. By this way, SPTBN1 has been implicated in the regulation of cell shape, adhesion, and transforming growth factor β (TGF-β) signaling [37]. It has a strong relationship with genes involved in alpha-actinin binding and cell adhesion in the bone co-expression network, suggesting that it plays the same role in bone [36].

Table 3 List of common genes detected by the two-stage TWAS and mRNA expression profiles of mesenchymal stem cell

Gene	TWAS		mRNA expression fold change
	Discovery P_{permu}	Replication P_{permu}	
TMEM66	0.001	0.024	0.48
MUL1	0.002	0.031	2.27
RERE	0.006	0.002	5.26
C14orf118	0.010	0.029	0.45
DDX17	0.015	0.039	2.31
ZNF281	0.016	0.014	0.30
SPTBN1	0.018	0.005	3.61
PIP4K2B	0.019	0.004	2.44
GRINA	0.020	0.035	6.11
FXYD5	0.026	0.008	2.92
PPP1R3B	0.030	0.034	5.06
TMTC4	0.031	0.028	2.19
TMEM87B	0.032	0.043	0.47
SORT1	0.036	0.030	8.68
COPA	0.037	0.042	4.22
SPATA20	0.039	0.027	2.36
NCSTN	0.041	0.040	2.26
ZDHHC20	0.049	0.001	2.14

P_{permu} denotes the permutated P value calculated by the FUSION software

Furthermore, the variants in SPTBN1 were also identified for one susceptibility genetic locus for osteoporotic fracture in postmenopausal Chinese women [38].

RERE gene encodes a member of the atrophin family of arginine-glutamic acid (RE) dipeptide repeat-containing proteins. It was commonly observed for FN-peripheral blood TWAS, replicate TWAS, and mRNA expression profile study of MSC in our study. The encoded protein co-localizes with a transcription factor in the nucleus, and its overexpression triggers apoptosis [39]. In addition, RERE has been identified as a new positional candidate gene for regulation of BMD in a group of families of Caucasian origin [40]. Furthermore, a published genome-wide association study in the southern Han Chinese population suggested that RERE is one of the promising candidate genes related to BMD [41].

Table 4 Gene ontology analysis results of the common genes identified by both discovery and replication TWAS

Gene ontology	Name	*P* value*
GO:0030308	Negative regulation of cell growth	0.002
GO:0016020	Membrane	0.002
GO:0007155	Cell adhesion	0.011
GO:0005981	Regulation of glycogen catabolic process	0.016
GO:1903561	Extracellular vesicle	0.017
GO:0005794	Golgi apparatus	0.020
GO:0005765	Lysosomal membrane	0.023
GO:0042587	Glycogen granule	0.023
GO:0032868	Response to insulin	0.031
GO:0005979	Regulation of glycogen biosynthetic process	0.032
GO:0051260	Protein homooligomerization	0.036
GO:0010008	Endosome membrane	0.037

**P* value calculated by the DAVID tool

We identified 6 common GO terms shared by TWAS and mRNA expression analysis, such as cell adhesion GO term (GO:0007155) and membrane GO term (GO:0016020). Carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) is a major human adhesion molecule. Ma C et al. illustrated the concentration of serum CEACAM1 was positively correlated with BMD in postmenopausal women [42]. Previous studies also demonstrated the role of cell adhesion molecules in the regulation of bone cell differentiation and biological function. For example, Michigami T et al. demonstrated that cell-cell contact between marrow stromal cells and myeloma cells via vascular cell adhesion molecule-1 improves the production of osteoclast-stimulating activity [43]. Another interesting GO term is membrane, which was detected by both TWAS and mRNA expression profiling of MSC. Previous studies suggested that membrane proteins were involved in the development of multiple human diseases, including osteoporosis. For instance, Zeng Y et al. identified 36 membrane proteins that were differentially expressed between high and low BMD groups [44].

In contrast with Mendelian randomization approach, TWAS method has less strict goal of identifying significant associations and can operate on summary GWAS data [19]. TWAS does not require individual-level GWAS data and is applicable directly to large-sample GWAS summary data, thus increasing discovery power. To the best of our knowledge, this is the first TWAS for OP. In addition, we further integrated data from mRNA expression profiles to validate the TWAS results, enhancing the reliability and persuasiveness of our study.

There are also two limitations of this study that should be noted. First, although TWAS is not confounded by reverse causality (disease→expression independent of SNP), instances of pleiotropy (in which a SNP or linked SNPs influence osteoporosis and expression independently) are statistically indistinguishable from truly causal susceptibility genes. Second, all study subjects of BMD GWAS were European in this study. Due to different genetic background, it should be careful to apply our study results to other populations. Further studies are warranted to confirm our findings and clarify the potential mechanism of identified genes and GO terms involved in the development of OP.

In conclusion, we conducted a discovery-replicate TWAS analysis by integrating the summary GWAS data with two pre-computed gene expression weights and then compared

Table 5 List of common gene ontology terms shared by TWAS and mRNA expression profiling of mesenchymal stem cell

Gene ontology	Name	TWAS *P* value*	MSC *P* value*
GO:0016020	Membrane	0.002	5.46×10^{-27}
GO:0007155	Cell adhesion	0.011	7.73×10^{-7}
GO:0005794	Golgi apparatus	0.020	2.50×10^{-7}
GO:0005765	Lysosomal membrane	0.023	7.56×10^{-5}
GO:0051260	Protein homooligomerization	0.036	0.040
GO:0010008	Endosome membrane	0.037	0.041

MSC mesenchymal stem cells

**P* value calculated by the DAVID tool

with the gene expression profiling data in bone cells, B cells, and mesenchymal stem cells (MSC). We identified a group of common genes and GO terms associated with OP. We hope that our study results can provide novel clues for the pathophysiology and therapeutic studies of osteoporosis.

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Compliance with ethical standards

Conflicts of interest None.

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