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Identification of novel variants associated with osteoporosis, type 2 diabetes and potentially pleiotropic loci using pleiotropic cFDR method

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Abstract

Aims: Clinical and epidemiological findings point to an association between type 2 diabetes (T2D) and osteoporosis. Genome-wide association studies (GWASs) have been fruitful in identifying some loci potentially associated with osteoporosis and T2D respectively. However, the total genetic variance for each of these two diseases and the shared genetic determination between them are largely unknown. The aim of this study was to identify novel genetic variants for osteoporosis and/or T2D.

Methods: First, using a pleiotropic conditional false discovery rate (cFDR) method, we analyzed two GWAS summary data of femoral neck bone mineral density (FN_BMD, $n = 53,236$) and T2D ($n = 159,208$) to identify novel shared genetic loci. FN_BMD is an important risk factor for osteoporosis. Next, to explore the potential functions of the identified potential pleiotropic genes, differential expression analysis was performed for them in monocytes and peripheral blood mononuclear cells (PBMCs) as these cells are relevant to the etiology of osteoporosis and/or T2D. Further, weighted gene co-expression analysis (WGCNA) was conducted to identify functional connections between novel pleiotropic genes and known osteoporosis/T2D susceptibility genes by using transcriptomic expression datasets in bone biopsies (E-MEXP-1618) and pancreatic islets (GSE50397). Finally, multi-trait fine mapping for the detected pleiotropic risk loci were conducted to identify the SNPs that have the highest probability of being causal for both FN_BMD and T2D.

Results: We identified 27 significant SNPs with $cFDR < 0.05$ for FN_BMD and 61 SNPs for T2D respectively. Four loci, rs7068487 (PLEKHA1), rs10885421 (TCF7L2), rs944082 (GNG12-AS1 (WLS)) and rs2065929 (PIFO||PGCP1), were found to be potentially pleiotropic and shared

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Author contributions

H.W.D conceived and initiated this project, oversaw the implementation of the statistical method, contributed suggestions for manuscript revision, and approved final version of manuscript. Y.H. perform data analysis and write the manuscript. L.J.T., X.D.C., J.G., H.W.D. provided interpretation for results. L.J.T. and H.W.D. provided advice and suggestions for manuscript revision.

Disclosures

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between FN_BMD and T2D (ccFDR < 0.05). PLEKHA1 was found differentially expressed in circulating monocytes between high and low BMD subjects, and PBMCs between diabetic and non-diabetic conditions. WGCNA showed that PLEKHA1 and TCF7L2 were interconnected with multiple osteoporosis and T2D associated genes in bone biopsy and pancreatic islets, such as JAG, EN1 and CPE. Fine mapping showed that rs11200594 was a potentially causal variant in the locus of PLEKHA1. rs11200594 is also an eQTL of PLEKHA1 in multiple tissue (e.g. peripheral blood cells, adipose and ovary) and is in strong LD with a number of functional variants.

Conclusions: Four potential pleiotropic loci were identified for shared genetic determination of osteoporosis and T2D. Our study highlights PLEKHA1 as an important potentially pleiotropic gene. The findings may help us gain a better understanding of the shared genetic determination between these two important disorders.

Keywords

Osteoporosis; Type 2 diabetes; Pleiotropy; cFDR; WGCNA

1. Introduction

Hundreds of genome-wide association studies (GWASs) have been conducted to study the genetic determinant of complex human traits, including osteoporosis and type 2 diabetes (T2D). Results of GWASs only explain a small portion of genetic contributions to complex traits/diseases [1]. The phenomenon is referred as the “missing heritability” which suggests that complex diseases are affected by many genetic variants with small or moderate effects (but important) that are difficult to be identified with current sample size and analytical strategies [1]. There is a need to apply novel statistical methods to leverage available information of existing GWASs datasets without extensive additional subject recruitment. Previous studies have suggested that many genetic loci may influence several related traits. Recently, it is estimated that about 5% of SNPs and 17% of genes have pleiotropic effects [2]. Therefore, integrative analysis of GWAS data of multiple genetically related traits/phenotypes could be a promising research strategy and direction.

Recently, a novel genetic pleiotropy-informed conditional false discovery rate (cFDR) method, proposed by Andreassen et al. [3], leverages pleiotropy to detect pleiotropic genetic variants for two related traits by using existing GWAS summary statistics data. In the method, there is a higher probability for a variant being detected in multiple independent studies with significant effects on two correlated traits. This method incorporates the summary statistics from two independent GWAS to test variants for association with the principal trait conditional on different strengths of association with the second one. The cFDR method has been implemented and successfully discovered novel pleiotropic loci that are associated with two related traits, including those studies from our own team [3–10].

Osteoporosis is a metabolic bone disease and characterized by reduced bone mineral density (BMD) and quality of bone, deficiencies in the structure of bone tissue, damage of bone microstructure and increased risk of low trauma fractures [11]. BMD is the most common measurement for predicting the risk of osteoporosis and is a highly heritable trait with heritability as high as 0.85 [12]. To date, approximately 200 genes have been identified

associated with osteoporosis-related traits through previous GWASs [13–18], which however, only explained about 10% of the total genetic variance [14].

T2D is a chronic metabolic disease that is characterized by insulin resistance and finally deficiencies in beta-cell function [19]. The individual risk of T2D is strongly influenced by genetic factors and the heritability was estimated at ~72% [20]. To date, previous GWASs have identified about 200 genome-wide significant genes associated with T2D [21–24]. Although the genetic contribution to T2D is well recognized, the result captures at best 10% of the heritability of the disease [24]. The increasing prevalence of T2D and limitations in therapy highlight the need for a more complete understanding of T2D pathogenesis.

Clinical and epidemiological evidences showed that osteoporosis and T2D were intercorrelated. Most of studies showed that T2D was always accompanied by high BMD and fracture [25,26]. During the past two decades, accumulated epidemiological studies suggests that type 2 diabetes is associated with increased risk of hip and other nonvertebral fractures [27–30]. Increased bone loss, higher cortical porosity, and deficits in bone material properties contribute to the diabetic skeletal fragility and fracture [31–35]. Insulin, a hormone secreted by pancreatic β -cell, participates in regulating the metabolism of carbohydrates and controlling glucose level [36] and has direct effects on bone cells, especially on osteoblast [37]. In addition, the accumulation of advanced glycation end products (AGEs), formed from increased glycose exposure in T2D, cause type I collagen non-enzymatic crosslinks within and across collagen fibers [38] and affect osteoblast-induced bone formation [39] as well as osteoclast-induced bone resorption [40]. Osteocalcin has been viewed as a common link between bone and glucose metabolism [41]. Undercarboxylated osteocalcin, a hormone secreted by osteoblast and underwent posttranslational γ -carboxylation in a milieu with acidic pH, can increase β -cell proliferation as well as insulin secretion and sensitivity, and osteoblasts are in turn recognized as insulin targets causing osteocalcin activity enhancement [42]. These findings point to some mechanistic explanation for the epidemiological observations of co-occurrence of T2D and BMD/osteoporosis.

More importantly, some genetic overlap for osteoporosis and T2D were also found in previous meta-analysis. For example, *KLHDC5* and *CDKAL1* were identified not only to be associated with femoral neck BMD (FN_BMD) (p-value = $1.87\text{E-}12$ for *KLHDC5* and p-value = $2.70\text{E}13$ for *CDKAL1*) [16] but also associated with T2D (p-value = $6.1\text{E-}10$ for *KLHDC5* and p-value = $6.00\text{E-}36$ for *CDKAL1*) [22,23]. Billings et al. identified Integrin, Alpha 1 (*ITGA1*) as a new pleiotropic locus candidate, capable of influencing both fasting glucose and BMD [43]. Despite these isolated findings of potential pleiotropic genes between osteoporosis and T2D, there is no systematic search for shared genetic loci influencing these two phenotypes/diseases.

BMD, as assessed by dual-energy X-ray absorptiometry (DXA), is currently the best clinical indicator of fracture risk [44]. Furthermore, patients with T2D may have higher BMD at the hip and radius and suffer from an increased risk of hip fractures [25,26]. Since hip fracture is the most severe type of osteoporotic fractures and directly associated with high morbidity and mortality, FN_BMD is an important risk trait used for studying osteoporosis risk. In our

study, we applied the cFDR method for two GWAS summary statistics data (FN_BMD and T2D) to identify novel variants with pleiotropic effects on FN_BMD and T2D. We found that 4 pleiotropic variants reached the significant association which may implicate some of the shared genetic mechanism of FN_BMD and T2D. The results of this study make it possible for us to better characterize the genetic mechanisms that affect FN_BMD and T2D and understand the potential mechanistic relationships of osteoporosis and T2D.

2. Materials and methods

2.1. GWAS datasets

Two datasets used in our study were taken from available online database. The data contained summary statistics, including the p-values for association and direction of effect for each variant, and the association results of meta-analysis were corrected by genomic control to ensure that the variance estimates for each SNP are not inflated due to potential population structure [14,45].

The summary statistics dataset that contains the results for association with FN_BMD was downloaded from the Genetic Factors for Osteoporosis (GEFOS) Consortium (<http://www.gefos.org/?q=content/data-release-2015>) [14]. The GWAS meta-analysis, including 53,236 Caucasian subjects, used combination of whole-genome sequence, whole-exome sequence and deep imputation of genotype data of previous GWAS analyses to identify variants association with three BMD traits: FN_BMD, Lumbar Spine BMD, and Forearm BMD. This study is currently the largest GWAS meta-analysis for BMD measured by DXA in Caucasians. > 10 million SNPs were analyzed for associations for these three traits.

The dataset with the results for association with T2D was taken from a GWAS meta-analysis of 26,676 cases and 132,532 controls (European descent) performed by DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium (<http://diagram-consortium.org/downloads.html>) [45], which is currently the largest T2D GWAS meta-analyses in individuals of European ancestry. This meta-analysis was to detect the association between T2D and > 10 million genotyped or imputed SNPs.

2.2. Data preparation

Several data preparation steps and the cFDR/ccFDR for each variant were performed following the steps detailed in the study of Andreassen et al. [3]. Briefly, first, the SNPs that passed Bonferroni-corrected significance threshold in the original GWAS were removed and the common SNPs of the two GWAS meta-analysis were annotated. Next, we started at a window of 50 SNPs where LD was calculated between each pair by using the HapMap III genotypes to remove variants with large correlations between each pair. The SNP with the smaller minor allele frequency (MAF) of the pair that has an r^2 value > 0.2 was removed. Following the initial removal of SNPs, the window shifted 5 SNPs forward and repeated the process until there were no pairs of SNPs with $r^2 > 0.2$. At last, 132,735 variants for FN_BMD and T2D were used for conditional FDR analysis.

2.3. Stratified QQ plots for pleiotropic enrichment

To assess the enrichment of association with principal phenotype, conditional QQ plots were plotted based on varying levels of significance for the conditional phenotype. The nominal $-\log_{10}(p)$ values for association with principal phenotype of the subset of variants that are below each significance threshold in the conditional phenotype are plotted on the y-axis and the quantiles of the nominal p-values are plotted on the x-axis. To determine whether the cFDR method results in the enrichment of associated SNPs, we successively restricted the subset of SNPs based on the level of significance for the association of each variant with the conditional trait, using the following criteria for $P_j < p_j$; $P_j < 1$ (all SNPs), $P_j < 0.1$, $P_j < 0.01$, $P_j < 0.001$, $P_j < 0.0001$ (P_j : nominal p values for the association with the conditional phenotype across all SNPs; p_j : the association for a particular variant with the conditional phenotype). Pleiotropic enrichment can be assessed by the degree of leftward shift from the expected null line [3].

2.4. Calculation of conditional FDR

The uFDR (unconditional false discovery rate) is characterized as the probability of a random SNP is not associated with the single phenotype [46]. The cFDR extends it to the two phenotypes, where cFDR is defined as the probability of a random SNP with a false positive association with the principal phenotype when the observed p-values for association with both the principal and conditional phenotypes are smaller than pre-defined significance thresholds [3].

The cFDR can be expressed as $cFDR(p_i|p_j)$. The p_i and p_j represent the association for a particular variant with the principal phenotype and the conditional phenotype respectively. The term $H_0^{(i)}$ stands for the null hypothesis that there is no association between the particular SNP and the principal trait.

$$cFDR(p_i|p_j) = Pr(H_0^{(i)} | P_i \leq p_i, P_j \leq p_j)$$

The cFDR for each variant was computed when FN_BMD is the principal phenotype conditioned on the association with T2D (FN_BMD|T2D) and vice versa (T2D|FN_BMD). SNPs with a cFDR value smaller than 0.05 were deemed to be significantly associated with the principal phenotype.

2.5. Calculation of conjunction FDR

Next, we computed the conjunction cFDR (ccFDR) value, the probability that a given SNP is false positive associated with both principal and conditional phenotypes, to identify pleiotropic loci association with both phenotypes. The ccFDR of all SNPs, the maximum cFDR value of the two phenotypes, were calculated and the variant with ccFDR value smaller than 0.05 was considered to be significantly associated with both phenotypes.

2.6. Manhattan plots

To show all the SNPs analyzed in our study and visualize their chromosomal location in the genome, we present a conjunction Manhattan plot based on the ranking of ccFDR. Any variant with a $-\log_{10}(\text{ccFDR})$ value > 1.3 (corresponding to $\text{ccFDR} = 0.05$) was determined to be pleiotropic.

2.7. Differential gene expression analysis

To explore the functions of the putative pleiotropic genes in relationship to the related phenotypes, differential gene expression analysis for the potentially pleiotropic genes were conducted by *t*-test in osteoporosis and T2D related transcriptomic data, respectively, where the genes with p -values < 0.05 were deemed as potentially and nominally differential expression genes. Dataset GSE7158, a transcriptomic expression dataset of circulating monocytes, was designed to identify genes associated with bone using a design of high vs low BMD [47]. Circulating monocytes, the precursor of the osteoclast, produce a wide variety of factors involved in bone metabolism, such as interleukin-1, interleukin-6 and tumor necrosis factor- α (TNF- α) [47]. Experiment of GSE9006, which contains 12 patients with T2D and 24 healthy controls, was designed to detect potential biomarkers of T2D through peripheral blood mononuclear cells (PBMCs) [48]. PBMCs can be used to characterize the mRNA/miRNA expression profiling for the different diabetes manifestations [49]. Moreover, metabolic derangements associated with diabetes potentially affect all cells of the body such as bone and muscle and the resulting changes of gene expression may be sampled in PBMCs suggesting PBMCs can be used as reporter cells to explore the mechanism of T2D [48]. Differential gene expression analysis was also performed in the transcriptomic data of bone biopsies (E-MEXP-1618)/pancreas (GSE50397).

2.8. Weighted gene co-expression network analysis (WGCNA)

To identify functional connections between novel potentially pleiotropic genes and known osteoporosis/T2D susceptibility genes, WGCNA was conducted in transcriptomic data of bone biopsies/pancreas. Firstly, we searched osteoporosis or T2D GWAS references with keywords “BMD”/“osteoporosis”/“T2D” and “GWAS” in Pubmed, and selected genes with p -value of variants $< 5.0 \times 10^{-8}$. A total of 203 osteoporosis and 234 T2D genes were selected. Next, combining osteoporosis/T2D GWAS genes and cFDR detected genes, the probes representing 150 osteoporosis (11 novel and 139 known genes) and 180 T2D related genes (33 novel and 147 known genes) in gene expression datasets E-MEXP-1618 (<http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-1618/>) and GSE50397 were applied to generate networks, respectively. Expression dataset E-MEXP-1618 was generated from 84 postmenopausal white women trans-iliacal bone biopsies (50 to 86 years) with low versus high BMD value (41 low FN_BMD vs 43 normal or high FN_BMD women), where most cells are osteocytes, with very small proportions of osteoclasts and osteoblasts. The women were healthy or had a primary osteopenic or osteoporotic status with no osteoarthritis or other primary or secondary bone diseases. Details of the samples' information can be found in the previously published study [50]. GSE50397 contains data of pancreas on transcriptomic expression of 21 samples with blood glucose (HbA1c) < 6.1 mmol/L and 56

samples $\text{HbA1c} < 6.1 \text{ mmol/L}$, where subject with $\text{HbA1c} \geq 6.1 \text{ mmol/L}$ is considered as diabetic. WGCNA were performed using R package [51]. The visualization of interconnection networks, which include nodes (for genes) and edges (for their potential functional interconnections) with topological overlap measurement (TOM) for pleiotropic genes larger than 0.10, was performed in Cytoscape [52]. Cytoscape is a software for complex networks visualization (<http://www.cytoscape.org/>). TOM is a parameter ranging from 0 to 1 that indicates the interconnection between two genes, where a high TOM is deemed to highly interconnect with the same set of genes [53].

2.9. Fine-mapping of pleiotropic genes

In this part, we performed a new approach, fastPAINTOR [54], which leverages evidence across correlated traits, as well as functional annotation data, to determine the SNPs that have the highest probability of causality for the pleiotropic risk loci detected for FN_BMD and T2D. fastPAINTOR is a recently developed Bayesian fine mapping method for multiple traits and requires only summary GWAS association data for each trait [54]. We define each locus as a 100 kb window of SNPs centered on the pleiotropic SNP identified by cFDR for the associated genetic region (50 kb on each side of the pleiotropic SNP). The SNP with $> 90\%$ posterior probability of being causal in the region denotes the most likely causal variant.

3. Results

3.1. Assessment of pleiotropic enrichment

We used conditional Q-Q plots to graphically assess the pleiotropic enrichment that resulted from successively conditioning the principal phenotype on SNPs with greater strengths of association in the conditional phenotype. Conditional Q-Q plot for FN_BMD given nominal p-values of association with T2D (Fig. 1A) and conditional Q-Q plot for T2D on FN_BMD (Fig. 1B) showed polygenic pleiotropic enrichment across different significance thresholds, as evidenced by the gradually leftward departure from the null line at all conditional thresholds.

3.2. FN_BMD associated gene loci identified with conditional FDR

Conditional on T2D, we identified a total of 27 variants association with FN_BMD with $\text{cFDR} < 0.05$ (Table S1), of which 21 variants have p-values smaller than 1×10^{-5} in the previous GWAS meta-analysis. Almost all of SNPs identified are enriched in the intergenic and intronic genomic regions. Several SNPs are located at or near non-coding RNA, such as *GNG12-AS1* and *MEF2C-AS1* (Table S1). There were three SNPs located at *GNG12-AS1* and one SNP located at *MEF2C-AS1*. It is worth noting that some SNPs identified in our analysis are located at or near the BMD related genes, such as *TNFRSF11B*, *DNM3*, *SOX6* and *GALNT3* [14,16]. In addition, 15 of 34 genes that the 27 identified variants were located at were novel FN_BMD potentially associated genes and not identified by the previous osteoporosis GWAS [14–17], such as *LSMI* and *MSC*, needing for future biological and functional mechanistic experiments to determine its functions in bone metabolism.

3.3. T2D associated gene loci identified with conditional FDR

Using cFDR analysis, there were 61 SNPs associated with T2D conditional on FN_BMD with cFDR < 0.05, which are located at a total of 15 chromosomes (chr1–8,10–13,15,17–20, 22) (Table S2). As same with the FN_BMD association variants, most of the detected SNPs reside in the intronic and intergenic regions, and a small proportion are located at non-coding RNA or un-translated regions (UTR) (Table S2). There were 3 SNPs (rs944082, rs231357 and rs10024198) located at non-coding RNA and 3 variants (rs340839, rs7119 and rs2269920) at UTR. Of the identified SNPs, we found several SNPs located at *KCNQ1*, *TCF7L2*, and *HMG20A*, which were known T2D associated genes in previous meta-analysis [21,45]. More importantly, we identified several novel significant loci, for instance *FAM135B*, *VEGFA* and *PCGF3*, which need for further statistical and experimental validation. Among the 79 annotated genes that these 61 SNPs were located at, 54 were newly detected compared to the previous T2D-related GWAS [21,22].

3.4. Pleiotropic gene loci for FN_BMD and T2D

To identify pleiotropic genetic loci that are associated with FN_BMD and T2D, we adopted the ccFDR analysis, and 4 independent pleiotropic variants reached a significance level of ccFDR < 0.05 for FN_BMD and T2D, which are rs7068487 (*PLEKHA1*), rs10885421 (*TCF7L2*), rs944082 (*GNG12-AS1* (*WLS*)) and rs2065929 (*PIFO*|*PGCPI*) (Table 1 and Fig. 2). All of potentially pleiotropic SNPs of FN BMD and T2D have the same effect direction on both FN BMD and BMI (Table 1). rs7068487 plays an eQTL role for *PLEKHA1* in multiple tissues, including peripheral blood cells, muscle-skeletal cells and pancreas (Blood eQTL browser, <https://genenetwork.nl/bloodeqtlbrowser>; Genotype-Tissue Expression, <https://www.gtexportal.org/home/>). In the three tissues, the less common allele at the variant was positively associated with expression of the *PLEKHA1* gene. rs2065929 was in the peak region of DNase I hypersensitivity track from the ENCODE and its change alter the match of multiple motifs (such as FOXA-family proteins and TCF12).

3.5. Differential gene expression analysis

Differential expression analysis showed that *PLAEKHA1* was a potentially significant gene that differentially expressed in circulating monocyte ($p = 4.50E-02$) between high and low BMD subjects and PBMCs ($p = 1.10E-03$) between non-diabetes and diabetes subjects (Table 2). *PLAEKHA1* mRNA was down-regulated in low BMD subjects compared to high BMD subjects, suggesting that its gene expression level might contribute to BMD. Compared to healthy subjects, *PLAEKHA1* mRNA was up-regulated in T2D subjects PBMCs. The results of differential expression analysis for the 5 potentially pleiotropic genes in the E-MEXP1618 and GSE50397 were almost not significant.

3.6. WGCNA analyses

By constructing gene networks for the 150 putative osteoporosis genes in bone biopsies, 136 genes were parsed into one gene module. There were 4 potentially pleiotropic genes (*TCF7L2*, *WLS*, *PIFO* and *PLEKHA1*) in the turquoise module. In the turquoise module, *PLEKHA1* is a top network hub gene and positively interconnected with 23 osteoporosis related genes with the TOM = 0.10 (Fig. 3B), such as *JAG1*, *EN1* and *FAM3C*. Furthermore,

the putative pleiotropic gene *TCF7L2* interconnected with 9 genes with TOM = 0.10, including *JAG1*, *HOXC6* and *FAM3C* (Fig. 3B). Meanwhile, all the 180 putative T2D genes (including 4 potentially pleiotropic genes) were parsed into one gene module with the WGCNA analyses. *PLEKHA1* and *TCF7L2* interconnected with multiple T2D related genes with the TOM = 0.10 (Fig. 3A). Then, gene ontology (GO) Enrichment analysis (<http://geneontology.org/>) of genes co-expressed with *PLEKHA1* with TOM = 0.10 in bone biopsies showed 7 osteoporosis associated genes (including *PLEKHA1*) are enriched in the GO term “skeletal system development” with p-value = 5.92E-03. Meanwhile, 8 T2D associated genes which interconnected with *PLEKHA1* were enriched in “regulation of hormone levels” (p-value = 1.21E-04), which include “positive regulation of insulin secretion”.

3.7. Fine-mapping of pleiotropic genes

Based on the posterior probability of causality produced by the fastPAINTOR, we presented the SNP of each locus that are most likely to have a causal effect for FN_BMD and T2D (Table 3). Interestingly, there was one SNP rs2065929 (*PIFO|PGCPI*) with about 91% probability of causality. An alternate allele of rs2065929 changes the match of the region (rs2065929 located at) to the *FOXA2*, *TCF12* and *TAL1*. rs11200594 with the highest probability of causality of the locus resides in the intron of the *PLEKHA1*.

4. Discussion

In our study, we assessed the genetic pleiotropy and identify common genetic variants between FN_BMD and T2D by applying the cFDR method that combined the summary statistics from independent GWAS meta-analyses. First, we constructed conditional Q-Q plots for FN_BMD and T2D for different strata of statistical significance with the conditional trait to provide visualization of pleiotropic effect enrichment. Then, we calculated cFDR value and ccFDR of each SNP to identify the variants associated with one trait or with both traits. As a result, we found enrichment of pleiotropic effect between FN_BMD and T2D. In the current study, 27 variants were identified to be associated with FN_BMD, 61 variants were associated with T2D, and 4 variants (rs7068487, rs10885421, rs944082 and rs2065929) may play pleiotropic roles in the genetic mechanisms between FN_BMD and T2D. *WLS* (*GNG12-AS1*), rs944082 located at, was associated with BMD in previous studies [13]. *TCF7L2*, pleiotropic SNPs rs10885421 located at, was known to be related with T2D metabolism [21].

The underlying pleiotropic SNP (rs944082) is located at the gene *WLS*, which has been identified as associated with BMD in previous GWAS [13]. *WLS* gene is critical for osteogenesis and chondrogenesis and *Wls*-knockout mice display early embryonic lethality due to impaired body axis formation [55,56]. We identified a SNP rs2566752 that has 99% probability to be causal for both FN_BMD and T2D located at *WLS*. The evidence suggests that the associations between rs2566752 and BMD are likely mediated by regulatory effects on the *WLS* gene [57]. *WLS* is known to affect the Wnt signaling pathway by influencing the receptors for Wnt signaling proteins and Wnt signaling has an important role in regulating insulin secretion [58], implying it might have an impact on T2D.

There was one pleiotropic SNP (rs10885421) located at *TCF7L2* that is known to increase risk of T2D in genome-wide association studies, and *TCF7L2* plays an important role in the pancreas β -cell proliferation and insulin secretion [21,59]. In addition, *TCF7L2* have an effect on osteoblastogenesis and bone mineralization through the canonical Wnt/ β -catenin signaling pathway [60]. T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family transcription factors (e.g., *TCF7L2*) played an important role in promoting bone-specific gene expression [61], including alkaline phosphatase and *RUNX2*. WGCNA showed that *TCF7L2* co-expression with multiple osteoporosis and T2D associated genes (Fig. 3), suggesting its pleiotropic roles in osteoporosis and T2D. Statistical evidence and reported biological functions suggested that *TCF7L2* may have potentially pleiotropic effects on both bone and T2D through regulating the related cells.

Importantly, the locus on 10q26.13 (rs7068487) is particularly interesting. The SNP rs7068487 is an intron variant located at the *PLEKHA1* and was detected as an eQTL of *PLEKHA1* in peripheral blood cells. Multi-trait fine mapping showed that the potentially causal variant (rs11200594) is also located at the intron of *PLEKHA1*. Variant rs11200594 is located at the region with promotor histone marks (H3K4me3 and H3K9ac) and enhancer histone marks (H3K4me1 and H3K27ac) where changes in it can affect *PLEKHA1* gene expression. PAX-4 and HDAC2 bind differentially to the alleles of rs11200594, where PAX-4 is involved in pancreatic islet development as well as differentiation of insulin-producing beta cells and HDAC2 can regulate osteoclastogenesis [62,63]. Moreover, rs11200594 is an eQTL of *PLEKHA1* and most of the variants in strong LD ($r^2 \geq 0.8$) with rs11200594 within the locus are eQTLs in various tissues including whole blood, adipose, testis and ovary, especially rs71486610 with multiple functions (promotor, DHS (DNase I hypersensitivity sites), eQTL and transcription factor binding site), suggesting that rs11200594 may be a causal variant.

PLEKHA1 (also known as *TAPP1*) encodes a pleckstrin homology domain-containing adapter protein, which is localized to the plasma membrane where it specifically binds phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂). The last 3C-terminal PH domain of TAPP1 are predicted to interact with PDZ domain-containing proteins. To date, TAPP1 was found specifically binding to PtdIns(3,4)P₂ through its C-terminal PH domain. The *TAPP1^{R211L/R211L} TAPP2^{R218L/R218L}* double knock-in mice are viable and displayed significantly enhanced whole body insulin sensitivity in a hyperinsulinaemic–euglycaemic clamp study through PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ [64]. *PLEKHA1* can specifically bind to PtdIns(3,4)P₂ through its C-terminal PH domain to regulate PtdIns(3,4,5)P₃ level. PtdIns(3,4,5)P₃ can up-regulate the phosphorylation of Akt to phosphorylate numerous substrates regulating various cellular processes. *PLEKHA1* is notably associated with the signaling pathway of PI3K/Akt, which plays an important role in osteoporosis [65,66]. Based on a mouse model, deletion of PtdIns (3,4,5)P₃ in the brain results in impaired bone mass and mineralization [67] and down-regulated PtdIns(3,4,5)P₃ levels also affected osteoclast activity and BMD [68]. *PLEKHA1* can regulate PtdIns(3,4)P₂ and PtdIns (3,4,5)P₃ implying that *PLEKHA1* might participate in bone metabolism through regulating PtdIns(3,4,5)P₃.

WGCNA results showed that *PLEKHA1* was one of the top network hub genes in osteoporosis related turquoise module and was found to highly interact with reported osteoporosis associated genes, such as *JAG1* and *ZNF423*. *JAG1* participated in normal trabecular bone formation and inhibited periosteal expansion through affecting osteoprogenitor cells and their progeny [69]. Recently, *Zfp423* was found to act as a hinge regulated coordinating regulation of adipocyte and osteoblast differentiation [70]. Co-expression and positive correlation with *JAG1* and *ZNF423* suggest that *PLEKHA1* might take part in progenitor cell differentiation. Meanwhile, *PLEKHA1* also co-expressed with 18 T2D associated genes (identified by GWAS or cFDR), such as *CPE*. *PLEKHA1* and its co-expressed osteoporosis/T2D genes are enriched in “skeletal system development” or “regulation of hormone levels” indicating that *PLEKHA1* may participate in the development of osteoporosis and T2D through these pathways. *PLEKHA1* was found to differentially express between the two different groups of circulating monocytes (high and low BMD subjects) and PBMCs (diabetes and non-diabetes), suggesting its differential expression may contribute to or result from osteoporosis and T2D. Our analysis and functional research imply that *PLEKHA1* probably have pleiotropic effect on osteoporosis and T2D.

The experiment of GSE9006 was designed on PBMCs from 12 patients with newly diagnosed T2D (age 11.3 ± 4.6) and 24 healthy controls (age 14.0 ± 2.3). Thus, it might be possible to predict pathogenesis etiology of the onset of T2D by detecting changes in gene expression in PBMCs from healthy children with new-onset T2D. The results of GSE9006 might provide a pathophysiological mechanism in one way for T2D. Owing to T2D is a chronic progressive disease viewed as an adult onset disease, the results in children may be not appropriate to explore the pathophysiological mechanism. All osteoclasts in peripheral skeleton and a considerable amount of osteoclasts in the central skeleton come from circulating monocytes [71,72]. A pathophysiological mechanism for osteoporosis is characterized by increased recruitment of circulating monocyte into bone, and enhanced monocyte differentiation into osteoclasts [73]. Circulating monocytes and PBMCs may indeed provide a window for studying the pathophysiological mechanisms for osteoporosis and/or T2D. Almost all the genes were not significant in the two groups of E-MEXP1618 (43 normal or high vs 41 low) and GSE50397 (56 control vs 21 T2D). A possible reason is that bone biopsies were collection of several types of bone cells, potentially leading to the differential expression of these genes confounded by heterogeneous cell proportions (thus with larger noise) of these cells in different subjects. Although β cells accounting for 60% of pancreatic islet, a mass of α cells (with opposite effect on blood glucose) as the confounder probably offset differential expression of these genes in β cells.

The major strength of our work is that cFDR analysis simultaneously analyzes two related traits to detect pleiotropic loci by combining existing GWASs summary results without requiring additional and larger datasets for individual trait. We successfully improved the detection of uncovered FN_BMD and T2D associated loci without additional larger datasets beyond the existing ones and identified several novel pleiotropic SNPs. Despite these novel findings, there may be several limitations of the present study. Firstly, in our study, there are some overlaps in the cohorts (both Framingham Heart Study and Rotterdam Study were in FN_BMD and T2D GWAS meta-analysis) in two summary statistics (FN_BMD and T2D).

The increase of effective sample size with samples overlapping in two summary statistics is not as great as what would be expected in the two independent studies. Additionally, we cannot distinguish the two pleiotropic cases: the locus directly impacts on the two phenotypes ($P1 \leftarrow G \rightarrow P2$, G : the genotype, $P1$: the first phenotype, $P2$: the second phenotype, arrows: the direct effect), or the locus affects only one phenotype and then the change in the one phenotype affects the second one ($G \rightarrow P1 \rightarrow P2$). No causal relationship was detected between T2D and FN_BMD by the method developed by Pickrell al. [74], which determines whether existing causal relationship between two phenotypes, suggesting the identified genes might directly influences osteoporosis and T2D independently as horizontal pleiotropic genes. Furthermore, the cFDR approach just identifies potential novel association loci and cannot be used to identify causal variants. Although we performed multi-trait fine mapping to detect SNPs that are most likely to have a causal effect on two traits, we are unable to associate the genetic findings with clinical outcomes or validate the findings with functional mechanistic experiments. Therefore, those potentially novel pleiotropic genes identified in our analysis should be followed up further with biological and functional mechanistic experiments to determine their clinical and mechanistic roles.

In conclusion, we detected 4 pleiotropic loci and suggested that there is significant pleiotropy between FN_BMD and T2D by performed cFDR with existing individual GWAS summary statistics data. Our study for the first time suggested their (rs7068487, rs10885421, rs944082 and rs2065929) potentially pleiotropic roles. Combined with functional annotation, we suggested that *PLEKHA1* gene was an important pleiotropic gene appearing to co-regulate BMD and T2D. The findings in the present study might provide insight into the shared genetic influences of osteoporosis and T2D and suggest a possible research direction for further biological experiments and clinical replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A.: Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2018.08.020>.

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Fig. 1.

Stratified QQ plots of nominal versus empirical $-\log_{10}$ p-values in principal trait below the standard GWAS threshold of $p < 5.0 \times 10^{-8}$ as a function of significance of the association with conditional trait at the level of $p < 1$, $p < 0.1$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively. A) FN_BMD|T2D (FN_BMD conditional on T2D), B) T2D|FN_BMD. The diagonal indicates the null hypothesis.

Fig. 2.
Conjunction Manhattan plot of conjunction $-\log_{10} \text{ccFDR}$ values for FN_BMD and T2D.
The figure shows the genomic locations of pleiotropic loci.

Fig. 3.

Co-expression network view of identified potentially pleiotropic genes (*PLEKHA1* and *TCF7L2*) with traits associated genes. This network included all edges and their corresponding nodes that co-expressed with potentially pleiotropic genes with TOM ≥ 0.10 . The width of edges presents the value of TOM: the larger TOM, the larger width of the edge. A) Co-expression network of *PLEKHA1* and *TCF7L2* with T2D associated genes. B) Co-expression network of *PLEKHA1* and *TCF7L2* with osteoporosis associated genes.

Table 1

Pleiotropic Gene Loci for T2D and FN_BMD With Conjunction cFDR smaller than 0.05.

RSID	rs7068487	rs10885421	rs944082	rs2065929
ROLE	intronic	intronic	ncRNA_intronic	intergenic
GENE	PLEKHA1	TCF7L2	GNG12-AS1(WLS)	PIFO PGCP1
CHR	chr10	chr10	chr1	chr1
A1	C	G	T	G
A2	T	T	C	A
EFFECT FN_BMD	-	-	-	-
EFFECT T2D	-	-	-	-
P.value FN_BMD	5.61E-04	7.32E-04	1.04E-07	1.62E-04
P.value T2D	8.20E-08	6.00E-07	1.50E-02	3.90E-04
cFDR FN_BMD	1.68E-03	3.66E-03	3.03E-04	3.37E-02
cFDR T2D	1.44E-05	5.82E-05	3.00E-02	3.08E-02
ccFDR	1.68E-03	3.66E-03	3.00E-02	3.37E-02

Note: Independent gene loci ($r^2 < 0.2$) with SNPs that have conjunction cFDR smaller than 0.05 in FN_BMD and T2D. All SNPs are listed with annotation, their nearest gene(s), chromosomal location, effect allele A1, non-effect allele A2, raw p-values for univariate GWAS for each trait, cFDR values for each trait, and ccFDR values. The effect direction of the potentially pleiotropic SNP on FN BMD and T2D was obtained from the original GWAS data. The effect represents the summary of effect directions (“+” indicates positive effect of A1 allele, “-” indicates negative effect of A1 allele).

Table 2
Differential expression analyses for the detected pleiotropic genes in trait-related cell groups.

Sample	S1	S2	S3	S4
Disease Target cells Sample size GSE NO.	osteoporosis Circulating Monocytes 14 high: 12 low GSE7158	Osteoporosis Trans-iliacal bone biopsies 43 normal or high vs 41 low E-MEXP-1618	T2D Peripheral blood mononuclear cells 24 control: 12 T2D GSE9006	T2D pancreatic islets 56 control: 21 T2D GSE50397
Gene symbol	Probe ID	P-value	Probe ID	P-value
PLEKHA1	226247_at	4.50E-02	219024_at	1.10E-03
TCF7L2	212759_s_at	2.31E-01	212761_at	7.80E-02
WLS	221958_s_at	3.99E-01	221958_at	5.80E-02
PIFO	228100_at	9.81E-01	228100_x_at	3.14E-01
				7931081
				7930537
				7901466
				7903959
				3.20E-01
				3.39E-01
				4.13E-01
				5.37E-01

Note: We only listed the most significant expression results of probes within one gene with multiple probes.

The bold is significant with p-value 0.05.

Table 3

FN_BMD and T2D SNPs with high probability of causality in 4 identified loci.

SNP_ccFDR	Gene_ccFDR	SNP_fastPAINTOR	Gene_fastPAINTOR	P.value.FN_BMD	P.value.T2D	Posterior prob
rs7068487	PLEKHA1	rs11200594	PLEKHA1	4.12E-06	4.60E-12	1
rs10885421	TCF7L2	rs11196211	TCF7L2	2.21E-01	3.80E-85	1
rs944082	WLS (GNG12-AS1)	rs2566752	WLS (GNG12-AS1)	3.65E-15	2.10E-03	0.99
rs2065929	P1FO PGCP1	rs2065929	P1FO PGCP1	1.62E-04	3.90E-04	0.91

Note: SNP_fastPAINTOR stands for the SNP detected by fastPAINTOR.
Gene_fastPAINTOR indicates genes that potential causal variants located at or nearby.
Posterior_Prob means posterior probability of causality produced by the fastPAINTOR.