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Enhanced Identification of Potential Pleiotropic Genetic Variants for Bone Mineral Density and Breast Cancer

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Abstract

Epidemiological and clinical evidences have shown that bone mineral density (BMD) has a close relationship with breast cancer (BC). They might potentially have a shared genetic basis. By incorporating information about these pleiotropic effects we may be able to explore more of the traits' total heritability. We applied a recently developed conditional false discovery rate (cFDR) method to the summary statistics from two independent GWASs to identify the potential pleiotropic genetic variants for BMD and BC. By jointly analyzing two large independent GWASs

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Contributors:

Hong-Wen Deng conceived and initiated the development of this study, he is responsible for general development and design of the study and contributed to critical revisions and finalization of the manuscript, he is guarantor. Cheng Peng contributed to the acquisition and analysis of the data and drafted the manuscript. Xu lin, Chun-Ping Zeng and Kuan-Jui Su contributed to data analysis. Ji-Rong Long contributed to the data acquisition. Lan Zhang, Jonathan Greenbaum, Yu-Mei Li and Wei-Feng Deng contributed to critical revisions. Feng Liu, Hui-Ling Lou and Jie Shen contributed to the general study design and development. All authors have given approval to the final version of the manuscript. All authors agree to be accountable for the work and ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Conflicts of Interest:

Cheng Peng, Hui-Ling Lou, Feng Liu, Jie Shen, Xu Lin, Chun-Ping Zeng, Jirong Long, Kuan-Jui Su, Lan Zhang, Jonathan Greenbaum, Wei-Feng Deng, Yu-Mei Li, Hong-Wen Deng declare that they have no conflicts of interest.

of BMD and BC, we found strong pleiotropic enrichment between them and identified 102 single nucleotide polymorphisms (SNPs) in BMD and 192 SNPs in BC with cFDR < 0.05, including 230 SNPs that might have been overlooked by the standard GWAS analysis. cFDR-significant genes were enriched in GO terms and KEGG pathways which were crucial to bone metabolism and/or BC pathology (adjP<0.05). Some cFDR significant genes were partially validated in the gene expressional validation assay. Strong interactions were found between proteins produced by cFDR-significant genes in the context of biological mechanism of bone metabolism and/or BC etiology. Totally we identified 7 pleiotropic SNPs that were associated with both BMD and BC (conjunction cFDR<0.05), CCDC170, ESR1, RANKL, CPED1 and MEOX1 might play important roles in the pleiotropy of BMD and BC. Our study highlighted the significant pleiotropy between BMD and BC and shed novel insight into trait-specific as well as the potentially shared genetic architecture for both BMD and BC.

Keywords

Bone Mineral Density (BMD); Breast Cancer; Pleiotropy; Genome Wide Association Study (GWAS)

1. Introduction

Bone mineral density (BMD) is a quantitative trait defined as the amount of mineral in bone. It is a hormone-associated phenotype and is believed to be a marker of women's lifetime exposure to estrogen [1]. BMD is highly heritable, with an estimated genetic heritability of 75% at femoral neck (FNK) and 83% at lumbar spine (LS) [2]. Previous genome wide association studies (GWAS) have identified more than 200 BMD-associated loci (<https://www.ebi.ac.uk/gwas>, April 2017), yet only explained a small proportion of the total heritability, leaving much of the heritability missing and awaiting further exploration.

Breast Cancer (BC) is a highly-estrogen-related disease and is the most common malignancy diagnosed among women, with an estimated 1 million new cases and over 400,000 deaths annually worldwide [3]. According to the Center for Disease Control and Prevention, 234,190 women were diagnosed with BC in the USA in 2015 and the number was estimated to reach 252,710 in 2017, which alone would account for 30% of all new cancers in women and 14% of all female cancer death [4]. BC is also a highly heritable disease, as women with a history of BC in a first-degree relative are at approximately two-fold higher risk than women without a family history to develop BC [5]. Several susceptibility genes have been identified to be associated with BC, such as BRCA1/BRCA2, ATM, PALB2 and CHEK2. To date, GWAS has identified more than 90 loci that were associated with BC, but only accounted for about 16 % of the total disease heritability [6]. For better disease control and prevention, more researches on its associated genetic variants are warranted.

Epidemiological and clinical studies have revealed a close relationship between BMD and the risk of BC [7–9]. In a meta-analysis including 70,878 postmenopausal women from 10 studies, higher BMD in the hip and in the spine was found to be associated with a 62% and 82% increased risk of BC respectively [7], and in a prospective study including 9,941 postmenopausal women, hip BMD was found to be able to predict the risk of BC

independently of the Gail score – the tool commonly used to assess BC risk [9]. Also, it is well known that treatments for BC, such as aromatase inhibitor therapy, can induce rapid bone loss and increase the risk of secondary osteoporosis via the suppression of estrogen [10]. Estrogen might be an important bond connecting BMD and BC, since it helps maintain bone mass and promotes BC [1,11]; however, BMD and BC are both complex traits affected by multiple genes, and considering their high missing-heritability mentioned above, the specific mechanisms behind the close relationship still remain largely unresolved, more studies are needed to explore the underlying genetic architecture for each trait as well as for the relationship between them.

The genetic mechanism of the relationship between BMD and BC is not clear; however we expect the presence of pleiotropic effects, meaning one gene or SNP might influence multiple traits. Recently, Andreassen et al. proposed a novel, reliable, and cost effective analysis method - pleiotropy informed conditional false discovery rate (cFDR) method on existing GWAS data and successfully identified potential novel loci for a number of traits and pleiotropic loci for different highly heritable disorders [12]. Using summary statistics from GWAS, cFDR method is able to improve the detection of genetic variants as well as elaborate genetic mechanism associated with multiple phenotypes without the need of larger datasets or new recruitments. Recently, we successfully implemented the cFDR analyses and identified potential novel loci and pleiotropic loci for BMD and/or height [13]. Here, we applied the cFDR method to two independent GWAS data to identify more potential trait-associated single nucleotide polymorphisms (SNPs) and genes as well as potential pleiotropic ones for BMD and BC, aiming to elucidate the trait-specific as well as the potentially shared genetic mechanism of both BMD and BC.

Method and Materials

GWAS datasets

BMD GWAS summary statistics for femoral neck (FNK) BMD and lumbar spine (LS) BMD were obtained from the Genetic Factors for Osteoporosis Consortium (GEFOS) [14]. The dataset contains summary statistics from 35 individual GWAS meta-analysis studies (n=53,236) and is the largest published GWAS data of BMD at present, including 10,586,099 SNPs for FNK and 10,582,866 SNPs for LS. All the SNPs were identified by whole genome sequencing, whole exome sequencing and deep imputation of genotype data [14]. The BC GWAS summary statistics were obtained from Chinese BC Studies in Shanghai (n=5,152), and totally 862,642 SNPs were included [15]. There were no overlapping subjects between the BMD and BC GWAS datasets. The detailed inclusion criteria and phenotype characteristics for the two GWAS were described in the original publications [14,15].

Data processing

First, we performed data pruning on BMD and BC datasets respectively. Plink was used to calculate the linkage disequilibrium (LD) between each pair of SNPs in the datasets. For those SNP pairs with $r^2 > 0.2$, the SNP with the smaller allele frequency (MAF) was removed [16]. The LD threshold of 0.2 is the standard cutoff value used in cFDR method,

and the detailed procedure was described in our earlier work by Greenbaum, et al [13]. After pruning process, there were 369,191 SNPs remained for FNK BMD, 369,514 SNPs for LS BMD and 266,338 SNPs for BC. Next, we combined the datasets, there were 144,380 common SNPs remained for FNK BMD with BC and 144,628 common SNPs remained for LS BMD with BC to be used in the subsequent analysis. Genomic control has already been applied in the two datasets to ensure that the variance estimates for each SNP were not inflated due to population structure [14,15], thus there was no need to reapply this adjustment in our analysis.

Statistical analysis

Pleiotropic enrichment estimation

To evaluate the pleiotropic enrichment between BMD and BC, we used ggplot2 package in R to construct fold enrichment plots of nominal $-\log_{10}(p)$ values for all and subsets of nominally trait-associated SNPs determined by the significance of their association with the conditional trait. The presence of pleiotropy indicates that the proportion of principle trait-associated SNPs at any given significance level varies based on the level of association with the conditional trait, and it can be intuitively observed as an upward deflection in the level of fold-enrichment when the subset is restricted to SNPs having stronger association with the conditional trait. The SNPs reaching the GWAS significance threshold of p value lower than $5E-8$ are of the most interest, which are denoted as $-\log_{10}(p) > 7.3$.

As a complement to fold enrichment plots, Q-Q plot was also conducted by R as a graphical tool to intuitively assess whether the primary phenotype was related to the given conditional phenotype under the null hypothesis. We plotted the QQ curve for the quantile of nominal $-\log_{10}(p)$ values for association of the subset of variants that were below different significance threshold in the conditional trait. The nominal p-values ($-\log_{10}(p)$) were plotted on y-axis and the empirical quantiles ($-\log_{10}(q)$) were plotted on x-axis. As the principal phenotype is successively conditioned on more stringent significance criteria in the conditional phenotype, the pleiotropy enrichment could be assessed by the degree of leftward shift from the expected identity line.

Calculation of cFDR and conjunction cFDR (ccFDR)

The cFDR method is regarded as an extension of the standard FDR framework. GWAS p-values from the pruned datasets are incorporated to calculate the cFDR value, which represents the probability that a random SNP is not associated with the principal phenotype given that the observed p-values for the principal and conditional phenotypes are both less than two pre-defined trait specific significance thresholds [17]. In our study, cFDR for each SNP was computed while BMD (FNK and LS) was the principal phenotype conditioned on the strength of its association with BC (FNK|BC, LS|BC) and reversely (BC|FNK, BC|LS). To assess whether the cFDR method results in the enrichment of specific SNPs, we successively confined the subset of SNPs based on the level of significance for the association of each variant with the conditional trait using the following criteria : $p < 1$ (all SNPs), $p < 0.1$, $p < 0.01$ and $p < 0.001$. A cFDR threshold of 0.05 was used to identify whether the SNP was significantly associated with the principal phenotype. The detailed

procedure was given by Andreassen et al [12]. To visualize the localization of significant SNPs associated with BMD given their association with BC as well as the reverse situation, we constructed cFDR Manhattan plots, which mark the significance of various SNPs and their chromosomal locations. The variant with a $-\log_{10}$ cFDR value more than 1.3 (corresponding to a cFDR value of lower than 0.05) was determined as being significantly associated with the principal phenotype given its association with the conditional phenotype.

To identify pleiotropic SNPs for BMD and BC, we computed ccFDR after the calculation of cFDR. ccFDR refers to the possibility of a given SNP has a false positive association with both first and second traits, and is taken as the maximum cFDR value between both traits. A ccFDR threshold of 0.05 was used to identify whether the SNP was a pleiotropic one and was associated with both BMD and BC. The effect direction of the pleiotropic SNP on BMD was obtained from “effects” in the original BMD GWAS data. The effect represents the Summary of effect directions (‘+’ indicates positive effect of reference allele, ‘-’ indicates negative effect of reference allele, ‘0’ indicates non-significant effect of reference allele). The effect direction of the pleiotropic SNP on BC was determined by the OR (odds ratio) value in the original BC GWAS data. If the OR value of the SNP is lower than 1.00, the effect direction of the SNP on BC is ‘-’, if the OR value is higher than 1.00, then the effect direction is ‘+’.

Gene expressional validation assay

To partially validate our findings, we performed gene expressional analysis with the BMD and BC transcriptomics data respectively. The purpose was to assess if the identified genes by the cFDR analyses would be significant in their differential expression levels regarding the health status of the relevant tissue (cells) under study. First, using gene expression profilings of iliac bone biopsies (largely composed of osteocytes) from 84 postmenopausal women [18], we calculated the Pearson correlation coefficients between BMD and the mRNA transcripts of the cFDR-significant genes for BMD. Then, based on gene expression profilings of epithelium and stroma cells in normal (n=5, normal refers to normal breast tissue from reduction mammoplasty healthy women) and invasive breast cancer tissues from BC patients (n=28) [19], point-biserial correlation coefficients between breast tissues (normal/invasive tissue types) and mRNA transcripts of BC cFDR-significant genes was estimated from the average of the correlation coefficients of 1,000 bootstrap replications. We did not perform the same validation studies on the mRNA transcripts of other bone relevant cells (such as osteoblasts or osteoclasts), and whether all the types of BC were included in the BC gene expression profilings was not clear, so we can only say the analysis is for “partial validation”. This is because while the significant results may lend functional support for the genes under the test here, non-significant results do not negate the potential functional significance of the genes under test as they may well function in other tissues or cells types relevant to BMD/BC but are not tested here. “r” was used to represent the Pearson correlation coefficients in the partial validation analysis with gene expression data for BMD and the point-biserial correlation coefficients in partial validation analysis with gene expression data for BC. The details of the gene expression profiling of iliac bone biopsies and BC biopsies could be found in the original publications [19,18].

Functional term enrichment analysis and protein-protein interaction analysis of identified SNPs and genes

Using online tool SNPinfo Web Server (<https://snpinfom.nih.gov/>), we mapped cFDR-significant SNPs to nearby genes. To explore the functional features of the genes identified by cFDR, we performed functional enrichment analysis including Gene Ontology (GO) term analysis and KEGG canonical pathways analysis by the online tool Web-Based Gene Set Analysis Toolkit [20]. Genes were input into the WebGestalt system and adjp (adjP is the p value adjusted for the multiple testing) less than 0.05 was used as the significant threshold. We pre-filtered GO categories for redundancy by the online tool REVIGO [21]. GO term analysis and KEGG canonical pathway analysis enabled us to assess and partially validate our results by determining associated gene sets that are significantly associated with bone metabolism and/or BC pathology. Protein-protein interaction analysis for cFDR-significant genes was performed by the online tool STRING 10.0 (<http://string-db.org/>), it enabled us better understand the functional roles of the proteins produced by the corresponding genes in the context of biological mechanism of bone metabolism and/or BC etiology.

Results

Pleiotropic enrichment assessment

As shown in fold enrichment plots (Figure 1), strong pleiotropic enrichment could be observed for BMD-associated SNPs (FNK and LS) across different levels of association with BC. As the p-value thresholds for BMD-associated SNPs becoming more stringent, we observed an at least 20-fold increase in the proportion of SNPs associated with FNK BMD at the genome wide significance level of $-\log_{10}(p) > 7.3$ while comparing to the group with all SNPs. And more impressively, a fold increase of at least 35 was found for the proportion of LS SNPs with $-\log_{10}(p) > 7.3$ comparing to the group with all the SNPs. In the Q-Q plots (Supplemental Figure 1), similar patterns of strong enrichment could also be observed for BMD given the nominal p-values of association with BC (FNK | BC and LS | BC) and BC conditioning on BMD (BC | FNK and BC | LS).

BMD SNPs and genes identified by cFDR

We identified 102 BMD SNPs mapping to 18 chromosomes conditioned on BC with a significance threshold of $cFDR < 0.05$ (Supplemental Figure 2). Among these SNPs, 66 were identified for FNK and 58 for LS. There were 22 SNPs overlapped between FNK and LS, the details of the significant SNPs were presented in Supplemental Table 1. Of the 102 cFDR-significant SNPs, 29 have reached genome-wide significance at p-value $5E-8$, there are 73 cFDR-significant SNPs with p-value higher than $5E-8$ (46 for FNK, 37 for LS and 10 overlapped between them) that were overlooked in the original GWAS analysis using standard statistical methods [14]. Totally 11 corresponding genes reaching the detection level of p value < 0.05 were partially validated in our gene expressional assay (bold r value in Supplementary Table 1). We input the corresponding genes of cFDR significant SNPs into WebGestalt system for GO term analysis and KEGG pathway analysis. As shown in Table 1, the most enriched GO terms for BMD associated genes were “ossification” (adjP = $2.68E-07$), “osteoblast differentiation” (adjP = $3.30E-05$) and “skeletal system development” (adjP = $3.30E-05$), which obviously played key roles in bone metabolism. Interestingly,

BMD cFDR significant genes were also enriched in “mammary gland epithelium development” ($\text{adjP} = 2.00\text{E-}04$), which was very important in mammary development. These bone and mammary associated GO term results lend support to our hypothesis of the existence of pleiotropy between BMD and BC. The significantly enriched KEGG pathways were presented in Table 2. “osteoclast differentiation pathway” ($\text{adjP} = 4.52\text{E-}02$) is very crucial to bone resorption. “Wnt signaling pathway” ($\text{adjP} = 4.52\text{E-}02$) plays an very important role in bone metabolism, activation of Wnt signaling pathway increases bone mass while inhibition of it decreases bone mass and promotes osteoporosis in mice [22], and polymorphisms in Wnt pathway have been identified to be associated with altered BMD variation in GWAS [23]. In protein-protein interaction analysis, strong interactions were found between BMD cFDR significant genes (Supplementary Figure 4), especially in terms of bone metabolism. For example, protein produced by LRP5 was the cell-surface coreceptor of Wnt/beta-catenin signaling, which was crucial to bone formation [22], protein produced by TNFRSF11B was decoy receptor for TNFSF11, it inhibited the activation of osteoclasts and promoted osteoclasts apoptosis in vitro.

BC SNPs and genes identified by cFDR

We identified 192 SNPs mapping to 20 chromosomes reaching the significance threshold of $\text{cFDR} < 0.05$ (Supplemental Figure 2). Among the 192 cFDR-significant SNPs, there were 35 SNPs reached genome wide significance at $p\text{-value} = 5\text{E-}8$, totally there were 157 SNPs not discovered in the original BC GWAS analysis by standard GWAS statistical methods. In the gene expression validation assay, 21 BC cFDR significant genes reaching the detection level of $p < 0.05$ were partially validated (bold r value in Supplementary Table 2). We input corresponding genes of all cFDR-significant SNPs into WebGestalt system to conduct the enrichment analysis. Of the most enriched GO terms associated with BC (Table 1), “cell-cell signaling” ($\text{adjP} = 1.34\text{E-}02$) has been reported in previous studies to have close association with pathology of tumors [24], and “ERK1 and ERK2 cascade pathway” ($\text{adjP} = 1.48\text{E-}02$) was involved in the pathology of BC [25]. In KEGG pathway analysis (Table 2), SOS2, VAV3, FGF14, CHRM2, FGF20 were enriched in “regulation of actin cytoskeleton pathway” ($\text{adjP} = 1.28\text{E-}02$), the actin cytoskeleton pathway has been proved to be associated with cancer cell motility and invasion [26]. EFNA5, LRRC4C, NTNG1, ROBO1 were enriched in “Axon guidance Pathway” ($\text{adjP} = 1.28\text{E-}02$), which has been reported to be one of the most important pathways during breast cancer cell’s development [26]. Rich interactions were revealed between BC cFDR-significant genes in protein-protein interaction networks (Supplementary Figure 4), partially validated the functional roles that these genes may have in mammary development. For example, proteins produced by NRG3 was direct ligand for ERBB4 and stimulated the activation of the receptor after binding, and ERBB4 regulated the development of mammary gland as a cell surface receptor for neuregulins and EGF family members.

Potential pleiotropic genetic variants identified by ccFDR

There were 7 pleiotropic SNPs mapping to 5 chromosomes reached the significance threshold of $\text{ccFDR} < 0.05$ (Table 3). Among the 7 pleiotropic SNPs, 3 SNPs (rs1038304, rs7752591, rs865898) were identified in FNK BMD, LS BMD and BC jointly; 4 SNPs (rs7808138, rs865898, rs4793019 and rs9594738) were only identified in LS BMD and BC.

Interestingly, 2 out of 3 pleiotropic SNPs for FNK BMD and BC and 4 out of 7 pleiotropic SNPs for LS BMD and BC have the same effect direction on both BMD and BC (Table 3). Conforming to the positive relationship between BMD and BC found in epidemiological and clinical studies [7,9], the major proportion of the pleiotropic SNPs have the same effect direction on both BMD and BC. We note that individual pleiotropic SNP may have opposite influence on both phenotypes, but the direction of the relationship between the two phenotype is expected to be dominated by the effect direction of the majority of pleiotropic loci and/or other common environmental effects.

Of the 7 pleiotropic SNPs, rs1038304 (CCDC170), rs7752591 (CCDC170 and ESR1), and rs865898 (CCDC170 and ESR1) have been reported in previous GWAS analyses to be associated with BMD [14,27,28] and BC [29,30]. Interestingly, these 3 SNPs were also the ones that identified both in FNK BMD and BC, LS BMD and BC in our analysis. rs9594738 (FABP3P2 and TNFSF11) [31], rs4793019 (MEOX1 and LOC100128016) and rs7808138 (CPED1) [14] have been reported in previous GWASs as well to have significant association with BMD, but no evidence was shown that they have any association with BC. rs1386369 (LOC390844 and LOC100132664) was a GWAS-significant SNP in the original BC GWAS [15], but it was not reported in any BMD studies before. In the GO term enrichment analysis (Table 1), the genes encompassed by pleiotropic SNPs were found to be enriched in biological processes that were important to not only bone metabolism but also BC promotion, such as “ERK1 and ERK2 cascade” ($\text{adjP} = 1.48\text{E-}02$). Esignal-regulated kinase (ERK) was involved in bone morphogenetic protein-2 induced osteoblast differentiation [32], and ERK1 and ERK2 pathway was also associated with the pathology of BC [25]. No significant KEGG canonical pathway was found for the potential pleiotropic genes, presumably due to the small number of pleiotropic loci identified. Three potential pleiotropic genes (CCDC170, FABP3P2, MEOX1) were partially validated in the BMD gene expressional validation analysis (Table 1), and these three genes had strong interactions with other genes in both BMD and BC protein-protein interaction networks (Supplementary Figure 4), further suggesting that they may contribute to the potential common genetic basis for both BMD and BC.

Discussion

By combining the two epidemiologically and clinically related GWAS summary statistics and using cFDR method, we successfully identified 102 SNPs for BMD and 192 SNPs for BC that reached the threshold of $\text{cFDR} < 0.05$ without any further recruitment or new study participants. Of these cFDR significant SNPs, 64 SNPs (29 in BMD and 35 in BC) had reached $p < 5\text{E-}8$ in the original GWAS and were regarded as the successful replication of the previous findings. The other 230 cFDR-significant SNPs (73 in BMD and 157 in BC) with $p\text{-value} > 5\text{E-}8$ would not have been identified in the original samples without using the cFDR method. This result demonstrated the improved power of cFDR in the discovery of trait-associated susceptibility SNPs. Interestingly, some BMD cFDR-significant genes identified in our study were also enriched in mammary development GO term, which partially validated our hypothesis of the potentially shared genetic mechanism between BMD and BC. Totally we identified 7 pleiotropic SNPs, 3 of which have been confirmed in previous GWAS analyses to be associated with both BMD [14,27,28] and BC [29,30], 3

were confirmed to be BMD GWAS-associated SNPs [31,14], but none of them were reported in BC studies. The SNP rs1386369 was a GWAS-significant SNP in the original BC study [15], but it has not been reported in any BMD studies before. Three potential pleiotropic genes were partially validated in gene expressional validation assay. Proteins produced by potential pleiotropic genes were found to have strong interactions with proteins produced by other cFDR significant genes in both BMD and BC protein-protein interaction analysis. These findings further supported the hypothesis of the overlapped genetic basis between BMD and BC. Our study strongly highlighted the cost-effectiveness and high-efficiency of the current cFDR method and its enhanced power in detecting genetic pleiotropy between complex traits and diseases.

The pleiotropy between BMD and BC suggests that some loci related to BMD are also associated with BC. Of the 7 pleiotropic SNPs identified in our study, rs1038304 is located in the intronic region of CCDC170 gene, rs7752591 and rs865898 are located near CCDC170 and ESR1. CCDC170 locates near ESR1, both of them have been reported in previous GWASs to be significantly associated with BMD [14,33], and mRNA level of CCDC170 was partially validated to be associated with BMD in our gene expressional validation assay. In a meta-analysis, genetic variation at CCDC170/ESR1 was found to be associated with BMD after adjustment of gender and age [34]. CCDC170 and ESR1 gene polymorphism was found to be associated with fracture and vertebral fracture risk in Chinese postmenopausal women [35]. As to BC, ESR1 gene is very crucial, it encodes estrogen receptor alpha (ER α), which is the most important therapeutic target in BC treatment [36], and recurrent ESR1-CCDC170 rearrangements have been characterized in an aggressive subset of ER+ breast cancers [37]. CCDC170 was proved to be significantly associated with BC by GWAS in 2010 [38], higher CCDC170 expression was associated with worse clinical outcomes in BC [39]. Taken together the information above, CCDC170 and ESR1 may play important roles not only in bone metabolic process but also in the pathology of BC, and we infer that CCDC170 and ESR1 might be potential pleiotropic genes for both BMD and BC.

Of the 7 pleiotropic SNPs identified in our study, rs9594738 (TNFSF11 and FABP3P2) has been reported in previous GWAS to be significantly associated with LS BMD [31], but it has not been reported in any BC studies before. To date, little is known about the function of FABP3P2, but rs9594738 lies 184kb upstream to TNFSF11 (also known as RANKL), and Interestingly, a previous study revealed that rs9594738 lies in the region where RANKL expression can be regulated by Vitamin D through transcription regulation [31]. RANKL is a BMD GWAS-associated gene [33], and it also plays a very important role in progesterone signaling. RANKL and its receptor RANK contribute to mammary tumorigenesis, inhibition of RANKL substantially curtailed mammary tumorigenesis in Brca1-deficient mouse model [40], and studies revealed that RANKL could control the expansion of Brca1-mutated mouse and human mammary progenitor cells [41]. rs9594738 is a pleiotropic SNP in our cFDR results, considering the potential regulation of rs9594738 might have on RANKL expression and the importance of RANKL in BMD and BC, we infer that rs9594738 might be a potential SNP associated with BC, further replication studies and functional validation experiments could follow up with it. In addition to CCDC170 and ESR1 mentioned above,

RANKL may also play an important role in the potentially shared genetic basis of BMD and BC.

The pleiotropic SNPs rs7808138 (CPED1) and rs4793019 (MEOX1 and LOC100128016) were reported in a large GWAS to be GWAS-associated with forearm BMD and FNK BMD respectively [14]. Loci in CPED1 gene has been reported to be significantly associated with BMD in GWAS [14], but no studies have shown any association between CPED1 and BC. CPED1 locates adjacent to WNT16, which is a very important gene for bone metabolism. WNT16 positively regulates bone mass and structure [42], and it's also functional in the pathology of cancers including BC. Human mammary epithelial cells express WNT16, in most BC cell lines the expression of WNT16 is usually down-regulated [43]. It has been reported in a previous lung development study that the expression of CPED1 can be regulated by SNPs in WNT16 [44], but further studies are still needed to explore the interaction between the two genes. MEOX1 is the nearest gene to the pleiotropic SNP rs4793019 and was one of the genes that were partially validated in our BMD gene expressional validation assay. Mutation in MEOX1 gene was involved in Klippel-Feil syndrome - a skeleton disease with abnormal fusion of two or more cervical vertebrae [45]. In a recent study, MEOX1 was proved to be a key molecular target that regulating breast cancer stem cells and was associated with worse clinical survival in BC patients [46]. Given the information above, we infer that rs7808138 and rs4793019 are not only BMD GWAS-associated SNPs but also potential novel SNPs associated with BC. CPED1 and MEOX1 might also contribute to the pleiotropy between BMD and BC. rs1386369 (LOC390844 and LOC100132664) is a BC GWAS-significant SNP in the original GWAS [15], but it is not reported in any BMD studies before. It lies in chromosome 18, further research is needed to explore its functional role in bone metabolism or tumor promotion.

Although we succeeded in improving the identification of individual trait associated and pleiotropic SNPs for BMD and/or BC, there were some potential limitations of our study. Firstly, the contribution of our findings to the proportion of the trait's variability can not be estimated, since we only analyzed GWAS summary statistics and the raw genotype data were not available. Secondly, cFDR method does not have the power to identify causal variants for the interested trait. The aim of our study is to provide more potential novel genetic variants for BMD and/or BC, thus further replication studies and biological functional validation researches can follow up with them and elucidate the potentially shared genetic mechanism for the two complex traits in the future. Thirdly, the BMD sample size is much larger than the BC sample size. Whether imbalanced sample size will affect the cFDR results is worth further theoretical investigation, but that is not in the scope of the present empirical study. To date, there is no evidence in the original methodology paper or previous cFDR literatures to suggest a sample size difference would result in bias in the cFDR methods [12,13].

In summary, cFDR method greatly increased the effective sample size of existing GWAS data and enhanced power to improve the detection of potential trait-associated genetic variants as well as pleiotropic ones. Our study highlighted the significant pleiotropy between BMD and BC, shed novel insight into trait-specific as well as the potentially shared genetic architecture for both BMD and BC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fold enrichment plots of enrichment versus nominal $-\log_{10}(p)$ values in BMD (FNK and LS) under the GWAS significance threshold of $p = 5E-8$ as a function of the significance of association with BC (FNK|BC, LS|BC) at the level of $-\log_{10}(p_{BC}) = 0$, $-\log_{10}(p_{BC}) = 1$, $-\log_{10}(p_{BC}) = 2$ and $-\log_{10}(p_{BC}) = 3$ (corresponding to all SNPs, $p = 0.1$, $p = 0.01$ and $p = 0.001$) respectively, and reversely BC as a function of significance of the association with BMD (BC|FNK, BC|LS).

Table 1

GO analysis for genes associated with BMD and/or BC (adjP < 0.05)

Trait	GO term	GO ID	C	O	E	R	rawP	adjP
BMD	ossification	GO:0001503	282	11	0.83	13.27	3.74E-10	2.68E-07
	skeletal system development	GO:0001501	387	10	1.14	8.79	1.31E-07	3.30E-05
	osteoblast differentiation	GO:0001649	138	7	0.41	17.26	1.38E-07	3.30E-05
	regulation of developmental process	GO:0050793	1436	16	4.32	3.7	1.98E-06	3.00E-04
	mammary gland epithelium development	GO:0061180	67	5	0.20	25.39	1.46E-06	2.00E-04
	connective tissue development	GO:0061448	199	7	0.58	11.97	1.65E-06	2.00E-04
BC	regulation of B cell activation	GO:0050864	82	6	0.64	9.39	4.24E-05	1.34E-02
	multicellular organismal process	GO:0032501	5644	65	43.98	1.48	4.78E-05	1.34E-02
	cell-cell signaling	GO:0007267	1099	22	8.56	2.57	3.43E-05	1.34E-02
	nervous system development	GO:0007399	1724	29	13.43	2.16	4.11E-05	1.34E-02
	synaptic transmission	GO:0007268	651	17	5.12	3.32	1.26E-05	7.10E-03
	ERK1 and ERK2 cascade	GO:0070371	129	7	1.01	6.96	6.62E-05	1.48E-02
	multicellular organismal homeostasis	GO:0048871	154	7	1.20	5.83	2.00E-04	2.49E-02
	Single-multicellular organism process	GO:0044707	5612	63	43.73	1.44	2.00E-04	2.49E-02
BMD and BC	mammary gland alveolus development	GO:0060749	17	2	0.00	565.57	3.92E-06	3.00E-04
	mammary gland lobule development	GO:0061377	17	2	0.00	565.57	3.92E-06	3.00E-04
	ERK1 and ERK2 cascade	GO:0070371	129	2	0.03	74.53	2.00E-04	2.90E-03
	mammary gland development	GO:0030879	129	2	0.03	74.53	2.00E-04	2.90E-03
	regulation of ERK1 and ERK2 cascade	GO:0070372	113	2	0.02	85.09	2.00E-04	2.90E-03
	positive regulation of RNA metabolic process	GO:0051254	1050	3	0.22	13.74	4.00E-04	3.80E-03
	chromatin binding	GO:0003682	315	2	0.06	31.7	1.3 E-03	1.56 E-02
	sequence-specific DNA binding	GO:0043565	676	2	0.14	14.77	5.90E-03	3.54E-02
	nucleic acid binding transcription factor activity	GO:000107	1004	2	0.2	9.94	1.29E-02	3.98E-02
	sequence-specific DNA binding transcription factor activity	GO:0003700	1002	2	0.2	9.96	1.28E-02	3.98E-02
	receptor binding	GO:0005102	1201	2	0.24	8.31	1.82E-02	4.37E-02

Column definition: GO – gene ontology; BMD – bone mineral density; BC – breast cancer; C – the number of reference genes in the GO term; O – the number of genes in the gene set and also in the GO term; E – expected number in the GO term; R – ratio of enrichment (O/E); rawP – p value from hypergeometric test; adjP – p value adjusted by the multiple test adjustment.

Table 2

KEGG pathway analysis for genes associated with BMD or BC (adjP<0.05)

Trait	Pathway Name	C	O	E	R	rawP	adjP
BMD	Wnt signaling pathway	150	2	0.21	9.75	1.81E-02	4.52E-02
	Osteoclast differentiation	128	2	0.18	11.42	1.34E-02	4.52E-02
BC	B cell receptor signaling pathway	75	4	0.29	13.77	2.00E-04	3.80E-03
	Axon guidance	129	5	0.50	10.01	2.00E-04	3.80E-03
	Fc epsilon RI signaling pathway	79	4	0.31	13.08	3.00E-04	3.80E-03
	Glycosphingolipid biosynthesis-ganglio series	15	2	0.06	34.43	1.50E-03	1.14E-02
	Regulation of actin cytoskeleton	213	5	0.82	6.06	1.50E-03	1.14E-02
	Adherens junction	73	3	0.28	10.61	2.90E-03	1.87E-02
	Rheumatoid arthritis	91	3	0.35	8.51	5.40E-03	2.04E-02
	Neuroactive ligand-receptor interaction	272	5	1.05	4.75	4.30E-03	2.04E-02
	ErbB signaling pathway	87	3	0.34	8.91	4.80E-03	2.04E-02
	Gap junction	90	3	0.35	8.61	5.20E-03	2.04E-02

Column definition: BMD – bone mineral density; BC – breast cancer; C – the number of reference genes in the KEGG pathway; O – the number of genes in the gene set and also in the KEGG pathway; E – expected number in the KEGG pathway; R – ratio of enrichment (O/E); rawP – p value from hypergeometric test; adjP – p value adjusted by the multiple test adjustment.

Table 3

Pleiotropic SNPs for both BMD (FNK and LS) and BC (ccFDR<0.05)

SNP	Chr	p value				ccFDR				effect				Nearby Gene	Gene Expressional Validation	
		FNK	LS	BC	FNK and BC	LS and BC	FNK	LS	BC	FNK	LS	BC	BC		r (BMD)	r (BC)
rs1038 304	6	8.65E-09	1.05E-12	1.75E-06	2.10E-05	8.77E-06	+	+	-	+	+	-	-	CCDC170	-2.90E-01	-2.18E-01
rs7752 591	6	3.44E-07	6.35E-12	6.03E-08	1.20E-05	3.62E-07	+	+	+	+	+	+	+	CCDC170/ESR1	-2.90E-01/- 1.35E-01	-2.18E-01/- 2.81E-01
rs8658 98	6	1.39E-05	1.48E-07	5.20E-07	3.48E-04	4.94E-06	+	+	+	+	+	+	+	CCDC170/ESR1	-2.90E-01/- 1.35E-01	-2.18E-01/- 2.81E-01
rs7808 138	7		9.04E-05	1.25E-04		4.47E-03								CPED1	-2.34E-01	6.65E-02
rs9594 738	13		3.63E-20	3.78E-02		3.78E-02								FABP3P2/TNFSF11	2.75E-01/- 2.53E-03	-3.36E-02/- 1.66E-01
rs4793 019	17		1.55E-05	2.90E-03		3.40E-02								MEOX1/LOC10012 8016	3.01E-01/NA	-1.86E-01/NA
rs1386 369	18		1.24E-02	6.72E-15		2.48E-02				+	+	-	-	LOC390844/LOC10 0132664	NA/NA	NA/NA

Column definition: SNP – single nucleotide polymorphisms; BMD – bone mineral density; BC – breast cancer; Chr – chromosome; Pos – chromosomal position; FNK – femoral neck; LS – lumbar spine; ccFDR – conjunction conditional false discovery rate. r – correlation coefficient (for BMD) or point biserial correlation coefficient (for BC). NA – not applicable (undetected). + indicates positive effect; - indicates negative effect; Bold r values represent nominally significant (p < 0.05). There are 4 SNPs that don't have p values or effect directions for FNK BMD. That is because these 4 SNPs are the pleiotropic SNPs only identified in the cFDR analysis for LS BMD and BC, they are not identified in the cFDR analysis for FNK BMD and BC