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Transcriptomic Data Identified Key Transcription Factors for Osteoporosis in Caucasian Women

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Abstract

Osteoporosis is a prevalent bone metabolic disease, mainly caused by excessive bone resorption (by osteoclasts) over bone formation (by osteoblasts). Identifying the key transcription factors and understanding the regulatory network influencing osteoclastogenesis will be helpful to explore the potential biological mechanism for osteoporosis. In our study, peripheral blood monocyte (PBM) was used as a cell model for bone mineral density (BMD) research. PBMs serve as progenitors of osteoclasts and produce important cytokines for osteoclastogenesis. In our study, via exon arrays, gene expression profiles of PBMs were analyzed between high versus low hip BMD groups. Transcription factors for differentially expressed genes were then predicted based on the enrichment analysis. We found that 591 genes were differentially expressed between the two BMD groups (nominally significant, raw p value < 0.05). For high BMD subjects, 482 genes were up-regulated and 109 genes were down-regulated. We then found 29 potential transcription factors for up-regulated genes and nine transcription factors for down-regulated genes. Among these transcription factors, HMGA1 and NFKB2 were differentially expressed between high versus low BMD groups. In addition, their regulation types with their target genes were consistent with the information from public databases. Our findings of key transcription factors and their target genes for osteoporosis were further validated by GWAS analysis. Overall, we predicted important transcription factors for osteoporosis. We were also able to infer the regulatory mechanism that exists between transcription factors and target genes in bone metabolism.

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Compliance with Ethical Standards

Conflict of interest Yu Zhou, Wei Zhu, Lan Zhang, Yong Zeng, Chao Xu, Qing Tian, and Hong-Wen Deng declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All the research methods employed in this study were conducted in accordance with the rules and guidelines of the Institutional Review Boards of University of Missouri Kansas City and Tulane University. The Institutional Review Boards of University of Missouri Kansas City and Tulane University approved the study. Written informed consent was obtained from all participants before inclusion in this study.

Keywords

Bone mineral density; Gene expression; Transcription factors

Introduction

Osteoporosis is the most prevalent metabolic bone disease, characterized by a decrease in bone mineral density (BMD). A key pathophysiological mechanism of this disease is the excessive bone resorption (by osteoclasts) over bone formation (by osteoblasts).

To investigate the transcriptome features relevant to the risk of osteoporosis, peripheral blood monocyte (PBM) is selected as a cell model in our study [1] with the following reasons. First, as a progenitor of osteoclast, PBMs are able to differentiate into osteoclasts [2–5]. Notably, PBMs are the sole source of osteoclast precursors for adult peripheral skeleton [6]. Second, PBMs can secrete various cytokines that are important for osteoclast differentiation, activation, and apoptosis [7–10]. Third, PBMs constitute 3 ~ 8% of human leukocytes in the blood and can be easily harvested with a high degree of purity that is important for transcriptome studies.

Transcription factors (TFs) are proteins involved in the process of transcription from DNA to messenger RNA, by binding to a specific DNA sequence. Accumulating evidence suggests that TFs play significant roles in mediating osteoclast differentiation, survival, and resorption activity [11]. For example, transcription factor PU.1 is essential for the differentiation of osteoclast precursors [12]. PU.1 expression level increased with the osteoclastogenesis induced by either 1,25-dihydroxyvitamin D3 or dexamethasone. The development of osteoclasts was arrested in PU.1-deficient mice [12]. c-Jun is a TF activated by RANKL. If the c-Jun is blocked, the RANKL-induced osteoclast differentiation will be significantly inhibited in vitro [13]. A study also showed that nuclear factor kappa B (NFkB) knocked-out mice developed osteopetrosis because of a defect in osteoclast differentiation [14]. Further investigation on the regulation network of TFs and their target genes in PBMs may provide novel therapeutic strategies for osteoporosis caused by abnormal osteoclast differentiation and function.

In our current study, we used exon-array data from 73 Caucasian females. Differentially expressed genes (DEGs) were detected between high versus low BMD groups. We also screened the potential TFs regulating DEGs. Then, gene ontology (GO) term enrichment analysis was performed for target DEGs to predict the function of TFs in osteoporosis. In addition, we highlighted the TF and its target gene if they were both differentially expressed between high versus low BMD groups. These pairs of TF and target gene were further validated using the largest osteoporosis GWAS meta-analysis dataset from the Genetic Factors for Osteoporosis Consortium (GEFOS).

Materials and Methods

Subjects' Characteristics and Sample Preparation

Seventy-three unrelated Caucasian females with extremely high versus low hip BMD were recruited in this study [15]. (High BMD group: $Z_{\text{BMD}} > +0.84$, $n = 42$ vs. Low BMD group: $Z_{\text{BMD}} < -0.52$, $n = 31$). Via strict exclusion criteria, individuals with diseases that might affect bone metabolism were excluded. The hip BMD (g/cm^2) of each subject was measured using a Hologic dual-energy X-ray absorptiometer (DXA) scanner (Hologic Corp., Waltham, MA). The Z score was defined as the number of standard deviations a subject's BMD differed from the mean BMD of their age-, gender-, and ethnicity-matched population. It represented the location of a subject in the BMD value distribution in our study population. Here, high versus low BMD is defined as belonging to top versus bottom 30% of BMD values in our population. The detailed characteristics of subjects are shown in Table 1 and Ref. [15].

Sixty milliliter of peripheral blood was obtained from each subject and PBMs were immediately isolated using monocyte-negative isolation kit (Miltenyi Biotec Inc, Auburn, CA) following the manufacturer's recommendation. Then, total RNA from PBMs was extracted using Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA) and mRNA expression levels were determined by the GeneChip Human Exon 1.0 ST Array (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. The raw microarray data for this cohort have been submitted to Gene Expression Omni-bus (GEO) under the accession number GSE56814.

Differentially Expressed Genes Detection

The pipeline of this study is shown in Fig. 1. For the microarray data analysis, the Affy package in R was used to import and process all raw CEL files. Then, the robust multiarray average method [16] was applied to normalize the array signals [17] and differential expression analysis was performed using Student's t -test through the Bioconductor's linear models for microarray data (LIMMA) package [18, 19]. If multiple probe IDs were mapped to the same gene symbol, the probe ID with the lowest p value among these probe IDs was selected to represent the gene. Because the sample size was limited (although still among the largest of such studies in the field), the adjusted p values were deemed to be too large after multiple testing control. We used raw p value < 0.05 as threshold for nominally significant differential expression.

Potential Transcription Factors Detection

TFactS [20] (<http://www.tfacts.org/>) was used to predict TFs for DEGs generated from microarray data. TFactS contains a comprehensive TF database. It integrates the TF regulatory data from curated TRED [21], TRRD [22], PAZAR [23], and NFIREgulomeDB [24] databases, and the regulation type ('up' or 'down') was based on the original publications. The up- and down-regulated DEGs were uploaded and screened separately. For each TF, if at least one of its annotated target genes is included in the DEG lists, we compare its annotated target genes with the DEG lists and use Fisher's test to estimate the enrichment

of the DEGs for this TF. The threshold for the significance of predicted TF was false discovery rate control (FDRc) < 0.05 [25].

Gene Enrichment Analysis for TFs Target Genes

To analyze the potential pathways that TFs regulated in osteoporosis, we loaded all DEGs regulated by the predicted TFs into the Database for Annotation, Visualization and Integrated Discovery (DAVID). The function of TFs was then predicted by analyzing their target genes on gene ontology (GO) terms and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The threshold for significant enrichment was $FDR < 0.05$.

Correlation Between TFs and Their Target Genes

If the TF and its target genes were both included in the DEGs list, we considered this TF to be more important for osteoporosis because expression level change of this TF may affect BMD variation directly. Pearson correlation was performed between differentially expressed TF and its target genes. The threshold for significant association was nominal p values < 0.05 .

GWAS Data Validation

To investigate if the key candidate TFs and their target genes were associated with BMD in larger human populations, we also leveraged the results from the meta-analysis for the Genetic Factors for Osteoporosis (GEFOS) Consortium (GEFOS-seq, meta-analysis results were downloaded from <http://www.gefos.org/>). It is the largest meta-analysis based on next-generation sequencing technologies and associated imputation to date for BMD study, including nine sub-cohorts and 32,965 Caucasians in the discovery cohort, both males and females [26]. We first selected the single-nucleotide polymorphisms (SNPs) which were located in the candidate TFs or their target genes regions. The SNPs in the region from 5-kb upstream to 5-kb downstream of each gene were assigned to the gene. Then, we obtained the meta-analysis p values of those SNPs for forearm BMD (FA BMD), lumbar spine BMD (LS BMD), and femoral neck BMD (FN BMD) traits in women. The most significant SNP for a given gene was chosen as a gene-level p value. The threshold for significant association between genes and BMD traits was nominal p values < 0.05 .

Results

DEGs Detection

In total, 22,011 genes (core set genes) were detected through microarray data processing. 591 genes were considered as differentially expressed genes (nominally significant, raw p value < 0.05) between high versus low BMD groups. In high BMD group, 482 genes were up-regulated and 109 genes were down-regulated.

Potential TFs Detection

Twenty-nine TFs were predicted to regulate the genes in up-regulated DEGs with $FDRc < 0.05$. Table 2 summarizes the regulatory network including 49 interactions between 29 predicted TFs and 21 target genes in up-regulated DEGs. Several genes were regulated by

multiple TFs. For down-regulated DEGs, the regulatory network consisted of 11 interactions between nine predicted TFs and six target genes (Table 3).

Gene Enrichment Analysis

Table 4 shows the results of GO term analysis for 27 target genes. Prediction terms with a $FDR < 0.05$ were selected and ranked by FDR. The most significant terms were *GO:0010745 ~ negative regulation of macrophage derived foam cell differentiation* (biological process aspect) and *GO:0015485 ~ cholesterol binding* (molecular function aspect). As expected, genes were also significantly enriched in terms related to *regulation of transcription from RNA polymerase II promoter* (biological process aspect). To analyze the pathway affected by predicted TFs, we performed a kegg pathway analysis for target genes (Table 4). *hsa04380: Osteoclast differentiation*, *hsa04064: NF-kappa B signaling pathway*, and *hsa04668: TNF signaling pathway* were found to be the three most enriched pathways.

Important Candidate TFs Selection

To further explore the TFs directly associated with BMD variation, we screened the predicted TFs in DEGs list and detected three differentially expressed TFs: HMG-box transcription factor 1 (HBP1), high mobility group AT-hook 1 (HMGA1), and nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFKB2). Then, Pearson correlation was conducted to examine the relationship between these three TFs and their target genes. JUNB (jun B proto-oncogene) was not associated with HBP1 ($r = -0.201$, $p = 0.088$) and the relationship was different from the known regulation type in the datasets (“up”). For the other two TFs, cyclin B2 (CCNB2) and HMGA1 were associated with each other ($r = 0.441$, $p < 0.001$). Interleukin 8 (IL8) was associated with NFKB2 ($r = 0.401$, $p < 0.001$). Moreover, they were positively associated with their target genes, consistent with the known regulation type in the datasets (“up”).

Validation by GWAS Studies

Table 5 showed the results for GEFOS-seq meta-analysis approach and gene-level p values for FA BMD, FN BMD, and LS BMD, represented by the most significant SNP/ marker in the gene. We used nominal p value < 0.05 as criteria for significance. HMGA1 was associated with FA BMD, FN BMD, and LS BMD, whereas its target gene CCNB2 was not. NFKB2 and IL8 were associated with FA BMD. We also tested our most significant predicted TF, HMGA2. HMGA2 was associated with FA BMD, FN BMD, and LS BMD.

Discussion

In this study, we screened the DEGs in PBMs between high versus low hip BMD via Human Affymetrix Exon 1.0 ST Array. Since the transcription process of genes was regulated by TFs, we used DEGs as candidate target genes to detect potential TFs that might affect skeletal metabolism. Gene enrichment and pathway analysis were conducted to further investigate the roles of TFs in osteoclastogenesis and bone density regulation process. Finally, we selected the TFs included in DEGs as the candidate TFs directly affecting BMD variation. We found that two TFs, HMGA1 and NFKB2, were significantly associated with their target genes and regulation types were consistent with evidenced information from the

dataset. To validate the importance of key predicted TFs and their target genes for BMD, we screened the SNPs located in these TFs or genes in the largest GWAS database (GEFOS-seq) for bone study.

Transcription factors have been reported to be associated with bone development and related diseases [27]. In our study, we took the advantage of the TFactS [20] tool to predict the candidate TFs. As a comprehensive database, it contains TFs and their target genes information from four TF datasets and public articles. Using Fisher's exact test, we calculated the enrichment of target genes in DEGs for each TFs and found the key TFs for osteoporosis. A total of 33 unique TFs were found to be potentially involved in the regulatory network for BMD variation. Many of these TFs were also found in previous bone-related studies. For example, High Mobility Group AT-Hook 2 (HMGA2), the most significant TFs predicted in our study, was also suggested to be associated with BMD in a genotyping study [28]. And in our validation study, we also found that this TF was associated FA BMD, FN BMD, and LS BMD in the largest GWAS database for bone study (data not shown here). Via conditional gene deletion cells, Smad family was indicated to be important for osteoclast differentiation [29]. In the animal model, SMAD4 conditional knockout mice showed significantly reduced bone mass and elevated osteoclast formation relative to controls [29]. Signal transducer and activator of transcription (STAT) family, predicted in our study, was another well-known TF group modulating osteoclast differentiation [30, 31].

To find the biological mechanism for candidate TFs affecting BMD variation, we performed the pathway analysis for target genes. The most significant GO term was "negative regulation of macrophage derived foam cell differentiation" (Table 4). Macrophage is another cell type differentiated from PBMs besides osteoclasts [32]. This suggested that our predicted TFs might modulate the differentiation process of PBMs. A similar result was also found by performing a KEGG pathway analysis. The target genes were most enriched in "Osteoclast differentiation" pathway (Table 4). It is demonstrated that candidate TFs might affect osteoclastogenesis in vivo.

TFs regulate gene expression via a complex set of mechanisms, including structure change and amount change. Therefore, we focused on the pair of TF and target gene both contained in the DEGs list. We found three pairs in DEGs list and two of them have the same regulation type as the known TF databases of TFactS including TRED, TRRD, PAZAR, and NFIREgulomeDB. In the three pairs, JUNB and HBP1 were not associated with each other in our results. For the other two TFs, Yao et al. demonstrated that the NFKB2 (p100) played as a negative regulator of osteoclastogenesis [33]. The mice with deletion of both NFKB1 and NFKB2 had severe osteopetrosis [14]. The other TF, HMGA1, was reported as a novel osteoclast-related gene in a proteomic analysis study using quantitative mass spectrometry strategy [34]. A large number of HMGA1 target genes were found to be associated with osteoclastogenesis [35]. However, the currently existing bone-related studies are still lacking regarding both of these TFs. So, we screened these two TFs and their target genes in GEFOS-seq database for SNP analysis. HMGA1, NFKB2, IL8 were associated with at least one BMD trait (Table 5).

There were several limitations of this study. First, PBMs are not equal to osteoclasts. Our findings could show the potential mechanism of osteoporosis, but direct validation is needed. Second, the cell and RNA samples were not left in this study. So, we could not conduct functional experiment to fully illustrate our findings in the regulation of BMD.

In summary, through a gene expression array analysis, we predicted 34 unique TFs which might be important for osteoporosis. Through GO term and pathway analyses, the main process that these TFs were involved in was suggested to be the differentiation from PBMs to osteoclasts. Two predicted TFs, HMGA1 and NFKB2, were differentially expressed (nominally) between high versus low BMD subjects and showed the same regulation type with their target genes as the known information from public databases. These findings may provide novel insights into the regulatory network of bone-related metabolism.

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Fig. 1.
The pipeline of transcription factor analysis

Table 1

Basic characteristics of subjects for monocyte microarray analyses

Menopausal status	High BMD		Low BMD	
	N	Age	Hip BMD Z score	Hip BMD Z score
Premenopausal	16	51.0 (1.8)	1.54 (0.52)	− 0.93 (0.36)
Postmenopausal	26	54.0 (1.8)	1.28 (0.46)	− 1.17 (0.60)
Total	42	52.9 (2.3)	1.38 (0.49)	− 1.05 (0.51)

Age and hip BMD Z score are shown as mean (standard deviation)

Table 2

Regulation type between predicted transcription factors and target genes in up-regulated DEGs

Gene name	Transcription factor	Regulation type	FDRc
SCAP	AR	Up	1.21E-02
PGC	AR	Up	1.21E-02
NFKBIA	CTNNB1	Down	2.58E-02
NR4A1	CTNNB1	Up	2.58E-02
IL8	CTNNB1	Up	2.58E-02
BIK	CTNNB1	Up	2.58E-02
DKK1	CTNNB1	Up	2.58E-02
CCNB2	FOXO1	Down	4.09E-02
CNR1	FOXO1	Down	4.09E-02
IL8	FOXO1	Down	4.09E-02
DKK1	FOXO1	Up	4.09E-02
IL8	FOXO3	Down	3.64E-02
DKK1	FOXO3	Up	3.64E-02
RASSF8	GLI1	Up	2.88E-02
JUNB	GLI2	Down	3.94E-02
JUNB	HBP1	Up	1.67E-02
NR4A1	HIF1A	Up	2.12E-02
CCNB2	HMGA1	Up	3.03E-03
CCNB2	HMGA2	Up	1.52E-03
IL8	IRF9	Down	4.39E-02
SMAD7	JUN	Up	1.97E-02
IL8	JUN	Up	1.97E-02
RPS18	MYC	Up	2.42E-02
PPIF	MYC	Up	2.42E-02
HMG2	MYC	Up	2.42E-02
IL8	NFKB2	Up	4.55E-03
RELB	NOTCH1	Up	9.09E-03
NFKB2	NOTCH1	Up	9.09E-03
RELB	RBPJ	Up	1.06E-02
NFKB2	RBPJ	Up	1.06E-02
NFKBIA	RBPJ	Up	1.06E-02
IL8	RELA	Up	7.58E-03
MMP3	RELA	Up	7.58E-03
SMAD7	SMAD2	Up	1.82E-02
SMAD7	SMAD3	Up	3.49E-02
SMAD7	SMAD4	Up	2.73E-02
PIM1	SP1	Up	1.36E-02
CETP	SP1	Up	1.36E-02
SMAD7	SP1	Up	1.36E-02

Gene name	Transcription factor	Regulation type	FDRc
NFKBIA	SP1	Up	1.36E-02
CETP	SREBF1	Up	3.18E-02
CETP	SREBF2	Up	6.06E-03
IL8	STAT1	Down	5.00E-02
IL8	STAT2	Down	4.24E-02
RNF149	STAT3	Up	3.33E-02
DKK1	TCF7L2	Up	1.52E-02
IL8	TCF7L2	Up	1.52E-02
DKK1	TP53	Up	3.79E-02
CETP	YY1	Up	3.03E-02

Table 3

Regulation type between predicted transcription factors and target genes in down-regulated DEGs

Gene name	Transcription factor	Regulation type	FDRc
TOP1	E2F1	Up	1.36E-02
HBP1	FOXO3	Up	6.06E-03
GATM	GLI1	Down	3.94E-02
ABCA1	GLI2	Down	1.21E-02
PPAT	MYC	Up	1.52E-02
ABCA1	NR1H2	Up	3.03E-03
ABCG1	NR1H2	Up	3.03E-03
ABCG1	NR1H3	Up	1.52E-03
ABCA1	NR1H3	Up	1.52E-03
ABCA1	SP3	Down	3.79E-02
ABCA1	SREBF2	Down	3.64E-02

Table 4

Gene enrichment analysis for target genes of predicted transcription factors

Category	Term	Count	FDR
GOTERM_BP	GO:0010745 ~ negative regulation of macrophage-derived foam cell differentiation	4	9.81E-07
GOTERM_MF	GO:0015485 ~ cholesterol binding	4	2.87E-05
GOTERM_BP	GO:0055091 ~ phospholipid homeostasis	3	9.45E-05
GOTERM_MF	GO:0005548 ~ phospholipid transporter activity	3	9.57E-05
GOTERM_BP	GO:0008203 ~ cholesterol metabolic process	4	1.63E-04
GOTERM_MF	GO:0017127 ~ cholesterol transporter activity	3	2.22E-04
GOTERM_BP	GO:0043691 ~ reverse cholesterol transport	3	3.98E-04
GOTERM_BP	GO:0042157 ~ lipoprotein metabolic process	3	1.78E-03
GOTERM_BP	GO:0034097 ~ response to cytokine	3	3.30E-03
GOTERM_BP	GO:0042632 ~ cholesterol homeostasis	3	4.93E-03
GOTERM_BP	GO:0045944 ~ positive regulation of transcription from RNA polymerase II promoter	6	1.42E-02
GOTERM_BP	GO:0045944 ~ positive regulation of transcription from RNA polymerase II promoter	6	1.42E-02
GOTERM_BP	GO:0000122 ~ negative regulation of transcription from RNA polymerase II promoter	5	2.17E-02
KEGG_PATHWAY	hsa04380: Osteoclast differentiation	4	3.11E-03
KEGG_PATHWAY	hsa04064: NF-kappa B signaling pathway	3	1.57E-02
KEGG_PATHWAY	hsa04668: TNF signaling pathway	3	2.22E-02

Table 5

Validation in GEFOS-seq dataset

Gene	SNP ID	Effect allele	Other allele	Effect allele frequency	p Value
Association with forearm BMD					
HMGAI	rs75195065	G	A	0.006	1.37E-02
CCNB2	rs142078254	C	A	0.005	5.25E-02
NFKB2	rs36226954	C	T	0.023	3.39E-02
IL8	rs2227543	T	C	0.369	1.60E-02
Association with lumbar spine BMD					
HMGAI	rs4713761	A	T	0.044	3.53E-02
CCNB2	rs188208891	A	T	0.000	1.27E-01
NFKB2	rs1572532	T	C	0.995	1.37E-01
IL8	rs142715747	T	C	0.005	4.66E-01
Association with femoral neck BMD					
HMGAI	rs4713761	A	T	0.044	9.66E-03
CCNB2	rs28383510	C	G	0.014	1.03E-01
NFKB2	rs3740418	G	C	0.321	4.82E-01
IL8	rs1126647	T	A	0.364	7.86E-02