Significant pathways detection in Osteoporosis based on the bibliometric network

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Abstract. – BACKGROUND: Osteoporosis is a significant public health issue worldwide. The underlying mechanism of osteoporosis is an imbalance between bone resorption and bone formation. However, the exact pathology is still unclear, and more related genes are on demand.

AIM: Here, we aim to identify the differentially expressed genes in osteoporosis patients and control.

MATERIALS AND METHODS: Biblio-MetReS, a tool to reconstruct gene and protein networks from automated literature analysis, was used for identifying potential interactions among target genes. Relevant signaling pathways were also identified through pathway enrichment analysis.

RESULTS: Our results showed that 56 differentially expressed genes were identified. Of them, STAT1, CXCL10, SOCS3, ADM, THBS1, SOD2, and ERG2 have been demonstrated involving in osteoporosis. Further, a bibliometric network was constructed between DEGs and other genes through the Biblio-MetReS.

CONCLUSIONS: The results showed that STAT1 could interact with CXCL10 through Toll-like receptor signaling pathway and Chemokine signaling pathway. STAT1 interacted with SOCS3 through JAK/STAT pathway.

Key Words:
 Bibliometric network, Osteoporosis, Significant pathways, STAT1, SOCS3.

Introduction

Osteoporosis represents a significant global public health issue for elderly women, affecting approximately 12 million people aged over 50 years in the United States[1]. It is characterized by a loss of bone mineral density (BMD), and often culminates in a fracture of the hip, wrist, and/or vertebrae[2].

The underlying mechanism of osteoporosis is an imbalance between bone resorption and bone formation. Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells. Lack of estrogen increases bone resorption as well as decreasing the deposition of new bone that normally takes place in weight-bearing bones[3]. Estrogen withdrawal is associated with increased production of pro-inflammatory cytokines, including interleukin-1, tumor necrosis factor-α, and interleukin-6, which all have been as primary mediators of the accelerated bone loss at menopause[4]. In addition to estrogen, calcium metabolism plays a significant role in bone turnover, and deficiency of calcium and vitamin D leads to impaired bone deposition. In response to low calcium levels, the parathyroid glands react can secrete parathyroid hormone (PTH), which increases bone resorption to ensure sufficient calcium in the blood. The activation of osteoclasts is regulated by various molecular signals, of which RANKL is one of best studied, a ligand for the receptor activator of NF-κB (RANK) on hematopoietic cells. Osteoblasts also produce and secrete osteoprotegerin (OPG), a decoy receptor that can block RANKL/RANK interactions, and hence suppresses its ability to increase bone resorption.

Recent discoveries also found many transcription factors and signaling pathways are critical for osteoblast differentiation[5]. For example, absence of runt-related transcription factor 2 (Runx2) or a downstream factor, osterix, are critical for osteoblast differentiation[6]. Wnt signaling pathway is also critical for osteoblast differentiation and function. Studies in mice suggest that the increase in bone mass in animals with activating mutations of LRP5 (low density lipoprotein receptor-related protein 5) is due to an increased response to mechanical loading[7]. The fact that fluid shear stress activates β-catenin signaling further supports the concept that Wnt signaling is critical in the response to mechanical loading[8].

However, more transcription factors and signaling pathways are still on demand. Therefore,

in this study, we first identify the differentially expressed genes in osteoporosis patients and control. Further, Biblio-MetReS, a tool to reconstruct gene and protein networks from automated literature analysis[9], could be used for identifying potential interactions among target genes. Although literature-based automated network reconstruction is still far from providing complete reconstructions of molecular networks, its value as an auxiliary tool is high and it will increase as standards for reporting biological entities and relationships become more widely accepted and enforced. Relevant signaling pathways were also identified through pathway enrichment analysis.

Materials and Methods

Microarray Analysis

One transcription profile of GSE7158 was obtained from a public functional genomics data repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) which are based on the Affymetrix Human Genome U133 Plus 2.0 Array. The study was approved by Hunan Normal University, ChangSha, China. All the recruited volunteers signed informed consent form before entering this project[10]. We first recruited 878 healthy Chinese premenopausal females aged 20-45 y with an average of 27.3 y when peak bone mass (PBM) is attainted and maintained. Then, we distributed the total sample according to the hip Z-score of PBM. From the bottom 100 and top 100 subjects of the PBM phenotypic distribution, we selected 12 subjects (Mean Z-score ± SD = −1.72 ± 0.60) and 14 (Mean Z-score ± SD = 1.57 ± 0.57) with extremely low and high PBM for further DNA microarray experiments[10]. Fourteen osteoporosis patients (high PBM) and twelve control (low PBM) chips were applied to identify different expressing genes. The two groups differed dramatically in both hip and spine BMD but not in age, height, or weight. In addition, significant care was taken to exclude individuals with diseases or chronic disorders that might influence the skeleton[11].

For the GSE7158 dataset, the limma method was used to identify different expressed genes (DEGs). The original expression datasets from all conditions were processed into expression estimates using the robust multiarray averaging (RMA) method with the default settings implemented in Bioconductor, and then construct the linear model. The DEGs only with the fold change value larger than 1.5 and *p*-value less than 0.05 were selected.

Bibliometric Network Construction

It is more comparable to use Biblio-MetReS to create networks than other user friendly tools. Furthermore, analysis of full text documents provides more complete reconstructions than those that result from using only the abstract of the document.

Firstly, register the Biblio-MetReS to apply to be able to access the central database. Then, Homo sapiens organism is chosen to work with. There are three types of data sources to choose from: General Engines (Yahoo, ...), Literature Database (Medline, ...) and Journals (Nature, ...). Input the DEGs and choose the Literature Database (Medline, Pubmed and Biomed Central) and the search is started, the tool identifies the documents that contain the gene names provided by the user and their synonyms. Then, it extracts the full text from each document, and analyses for the co-occurrence of any pair of genes. At last, extract the co-occurrence of any pair of DEGs and use the Cytoscape[12] to desplay the bibliometric network.

Pathway Enrichment Analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of online databases dealing with genomes, enzymatic pathways, and biological chemicals. The PATHWAY database records networks of molecular interactions in the cells, and variants of them specific to particular organisms (http://www.genome.jp/kegg/). Database for Annotation, Visualization and Integrated Discovery (DAVIS)[13], a high-throughput and integrated data-mining environment, analyzes gene lists derived from high-throughput genomic experiments. Use the DAVID to identify over-represented pathways using the hypergeometric distribution[14] for the input genes in bibliometric network with the false discovery rate (FDR)[15] < 0.05 and count > 2.

Results

Microarray Analysis and Bibliometric Network Construction

For the datasets of osteoporosis, we obtained publicly available microarray data sets GSE7158 from GEO. After microarray analysis, the differ-

entially expressed genes with the fold change value larger than 1.5 of GSE7158 and *p*-value less than 0.05 were selected. 56 genes were selected as DEGs from GSE7158.

To get the relationships between DEGs and other genes, the bibliometric network was constructed through the Biblio-MetReS. Total 27 DEGs and 510 other genes were displayed in the network, after removing the genes that didn't relate to the DEGs (Figure 1).

In the Figure 1, STAT1, CXCL10, SOCS3, ADM, THBS1, SOD2, and ERG2 were impor-

tant differentially expressed genes, involving in osteoporosis development in the network. Of them, STAT1 could interact with lots of target genes, such as CXCL10, SOCS3, and GBP1.

Pathway Enrichment Analysis

To identify the relevant pathways changed in Osteoporosis, the pathway with the FDR <0.05 and the count >2 was marked as significant pathway using the DAVID.

The pathway enrichment analysis yielded many significant pathways containing Cytokine-
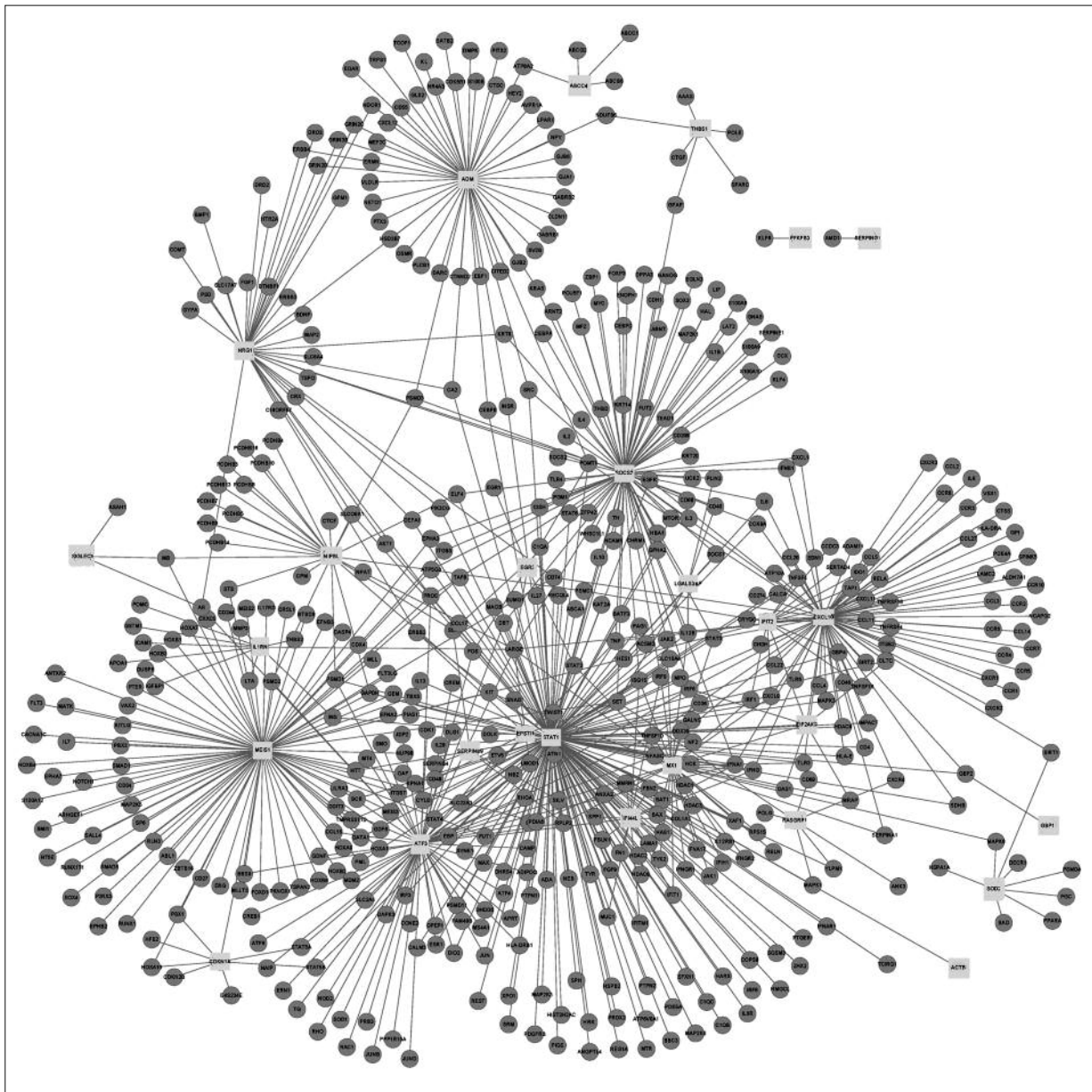
Figure 1. Bibliometric network of Osteoporosis. In the figure, the squares points stand for DEGs and the other points stand for other genes. They were connected by the lines, which mean the two points linked by the edges connected by the same literature.

cytokine receptor interaction (hsa04060), Toll-like receptor signaling pathway (hsa04620) and Chemokine signaling pathway (hsa04062) and so on (Table I). STAT1 (signal transducer and activator of transcription 1) was enriched in the Cytokine-cytokine receptor interaction and Toll-like receptor signaling pathway. CXCL10 was simultaneously enriched in Top 3 significant pathways.

Discussion

Osteoporosis is a very common disease characterized by low bone mineral density, low bone mass and altered bone microstructure which causes bone fragility and fractures[16]. However, the molecular pathological mechanism is still unclear. In this study, we aim to identify the differently expressed genes between osteoporosis patients and control, and further explore genes interaction. Our results indicated STAT1, CXCL10, SOCS3, and ADM were as the hub genes in the bibliometric network. Besides, THBS1, SOD2, and ERG2 were also important differentially expressed genes, involving in osteoporosis development.

There were strong evidences that STAT1 plays an important role in bone metabolism. STAT1 was reported to be up-regulated in femur tissue in osteoporotic mice and human[17]. STAT1 may serve as a primary mediator of interferon (IFN) signaling pathways involving osteoclast differentiation. Through the p38 MAPK (mitogen-activated-protein-kinase) pathway, RANKL stimulates the serine phosphorylation of STAT1 resulting in the migration and adhesion of osteoclast precursors[18]. Besides, STAT1 could interact with Runx2 in its latent form in the cytoplasm, thereby inhibiting the nuclear localization of Runx2, an essential transcription factor for osteoblast differentiation. This function of Stat1 does not require the Tyr 701 that is phosphorylated when Stat1 becomes a transcriptional activator[19]. STAT1 protein degradation was regulated by double-stranded RNA-dependent protein kinase (PKR) in a STAT-interacting LIM protein (SLIM)-dependent pathway[20].

Chemokines are important mediators of chemotaxis, cell adherence, and proliferation and exert specific functions in bone remodeling. Chemokines have potential intriguing role in the regulation of osteoclast functions. For example, IFN-gamma inducible protein-10 (CXCL10), a member of the CXC chemokine family, is expressed in human osteoclasts with changing expression levels during osteoclast differentiation. Recently, IFN-gamma inducible protein-10 (CXCL10), a member of the CXC chemokine family have been suggested contribution to osteoclastogenesis by increasing RANKL expression in CD4+ T cells in an animal model of rheumatoid arthritis. Besides, CXCL1 also could stimulated the expression of tumor necrosis factor-α (TNF-α) in CD4+ T cells, and induced osteoclastogenesis in cocultures of CD4+ T cells and osteoclast precursors, untimately resulting in bone destruction[21]. Besides, our study showed STAT1 could interact with CXCL10, and regulate CXCL10 expression.

Importantly, study showed that osteoblasts secrete IFN-beta in response to viral infection and that endogenous IFN-beta induces both CXCL10 and TLR3 (Toll-like receptor 3) production via an IFN-alpha/beta receptor-STAT1 pathway. PTS2 (peroxisome targeting signal type 2) in-

Table I. Pathway enrichment analysis.

Pathway	Name	Count	p value	FDR
hsa04060	Cytokine-cytokine receptor interaction	67	1.44E-24	1.73E-21
hsa04620	Toll-like receptor signaling pathway	34	3.36E-16	4.00E-13
hsa04062	Chemokine signaling pathway	46	6.21E-16	7.99E-13
hsa04630	Jak-STAT signaling pathway	40	1.34E-14	1.61E-11
hsa05200	Pathways in cancer	58	3.08E-13	3.69E-10
hsa05221	Acute myeloid leukemia	21	1.03E-10	1.24E-07
hsa05020	Prion diseases	16	6.85E-10	8.22E-07
hsa04012	ErbB signaling pathway	21	2.47E-07	2.96E-04
hsa05220	Chronic myeloid leukemia	19	5.04E-07	6.05E-04
hsa04640	Hematopoietic cell lineage	20	9.58E-07	0.001149
hsa04930	Type 2 diabetes mellitus	14	3.68E-06	0.004412
hsa05219	Bladder cancer	13	6.05E-06	0.007256
hsa05215	Prostate cancer	19	7.11E-06	0.008528

hibits IFN-γ-induced IP-10/CXCL10 expression in RAW264.7 cells by targeting the JAK/STAT1 signaling pathway, suggesting that PTS2 could exert anti-inflammatory effects through attenuating the formation of chemokine IP-10/CXCL10. These studies both suggested STAT1 could interact with CXCL10, and regulate CXCL10 expression by Toll-like receptor signaling pathway and Chemokine signaling pathway, which also have been demonstrated in our results.

SOCS3 (suppressors of cytokine signaling) family is cytoplasmic adaptor protein that negatively regulate various cytokine responses in leukocytes. SOCS3 over-expression could augment the TGF-β, TNF-α as well as RANKL-induced osteoclast formation, priming precursors to the osteoclast lineage by suppressing specific anti-osteoclastic JAK/STAT (Janus associated kinase signal transducer and activator of transcription) signals. Besides, Zhang et al[22] also have demonstrated that higher SOCS3 expression level was associated with RANKL-mediated alveolar bone loss and enhanced CD11c+ DC-derived osteoclastogenesis *in vivo* and *in vitro*. More interestingly, reduced expression of functional SOCS3 in CD11c+ DCs results in significantly lower osteoclastogenesis and DDOC development during immune interactions with T cells, based on TRAP expression and bone resorptive activity. Therefore, SOCS3 and STAT1 interaction may play an important role in osteoporosis through JAK/STAT pathway, which has been predicted in our signaling analysis.

Besides, GBP1 gene is also predicted involved in bone metabolism or osteoclast differentiation[10] through a STAT1-dependent manner. The sumoylation-defective STAT1 mutant displayed increased induction of GBP1 and transporters associated with antigen presentation 1 (TAP1) transcription. The mutation in STAT1 gene dramatically reduced the inducibility of the GBP1 and TAP1 genes by IFN[23]. In this study, we found STAT1 and GBP1 may interact with each other indirectly through GBP2.

ADM (Adrenomedullin) is a 52-amino acid peptide first described in a human phaeochromocytoma but since been found to be present in many tissues, including the bone[24]. Systemic administration of ADM could stimulate the proliferation of osteoblasts and promotes bone growth. Treatment with ADM significantly blunted apoptosis of serum-deprived osteoblastic cells, evaluated by caspase-3 activity, DNA fragmentation quantification and annexin V-FITC labeling. This effect could be abolished by CGRP1 (calcitonin gene related peptide) and insulin-like growth factor-I (IGF-1)[25]. The selective inhibitor of MAPK kinase (MEK), PD98059, also abolished the apoptosis protective effect of ADM and prevented ADM activation of ERK1/2 (exogenously-regulated kinase 1/2). These data show that ADM acts as a survival factor in osteoblastic cells via a CGRP1 receptor-MEK-ERK pathway, which provides further understanding on the physiological function of ADM in osteoblasts.

Thrombospondin1 (THBS1) is a glycoprotein present in platelet α-granules and a variety of cell types *in vitro*, including bone cell. THBS1 has been demonstrated to play an important role in osteoclast resorption. However, the detail mechanism is still unclear. THBS1 appeared to promote resorption by a mechanism other than simply increasing adhesion of stromal cells to the dentine slices[26]. Further, the CSVTCG (thrombospondin sequence motif) peptide, which represents the CD36-binding region of THBS1, stimulates resorption in a fashion similar to the intact molecule, while the peptides RGDS, RFYVVMWK, and RFYVVM, representing other cell-binding domains of THBS1, have no effect on resorption. Osteoclastogenesis is cell contact dependent, leading to up-regulation of THBS1 in DCs (dendritic cells). Disruption of CD47-THBS1 interaction by THBS1-blocking antibodies or down-regulation of CD47 on tumor cells by RNA interference abrogates osteoclast formation[27]. In our study, we found THBS1 could interact with SPARC, which have been demonstrated critical regulators of bone remodeling[28].

Study discovered that superoxide dismutase 2 (SOD2) was significantly up-regulated in circulating monocytes (potential osteoclast precursors) at mRNA dn protein level *in vivo* in Chinese with low versus high hip BMD. Besides, eight single-nucleotide polymorphisms (SNPs) at the *SOD2* gene locus were suggestively associated with hip BMD, of them, rs7754103, rs7754295, and rs2053949 were associated with the *SOD2* mRNA expression level, suggesting that they are expression quantitative trait loci (eQTLs) regulating *SOD2* gene expression[29]. In this study, we found SOD2 was differently expressed in osteoporosis patients and control. And it could interact with other genes to regulate osteoporosis development, including PPARA (peroxisome proliferator-activated receptor alpha), MPAK8 (mitogen-activated protein kinase 8), and BAD (Bcl-2 associated death promoter), etc.

EGR2, one of the 4 early growth response genes, is a highly conserved transcription factor implicated in hindbrain development, peripheral nerve myelination, tumor suppression, and monocyte/macrophage cell fate determination. Recent study and our study indicated a novel role for EGR2 in postnatal skeletal metabolism. A low bone mass phenotype (LBM) was present in both the distal femur and the vertebra of EGR2 ± mice, which was attributable to accelerated bone resorption as demonstrated *in vivo* by increased osteoclast number and serum C-terminal telopeptides, a marker for collagen degradation. Furthermore, EGR2 silencing in pre-osteoclasts increased cFms expression and response to macrophage colony-stimulating factor, leading to a cell-autonomous stimulation of cell-cycle progression. In brief, the antimitogenic role of EGR2 in preosteoclasts is the predominant mechanism underlying the LBM phenotype of EGR2-deficient mice. Stimulation of EGR2 expression in pre-osteoclasts may present a viable therapeutic strategy for high-turnover osteoporosis.

Conclusions

In this paper, 27 differentially expressed genes were identified. Of them, STAT1, CXCL10, SOCS3, ADM, THBS1, SOD2, and ERG2 have been demonstrated involving in osteoporosis. Further, a bibliometric network was constructed between DEGs and other genes through the Biblio-MetReS. The results showed that STAT1 could interact with CXCL10 through Toll-like receptor signaling pathway and Chemokine signaling pathway. STAT1 interacted with SOCS3 through JAK/STAT pathway.

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