Original Article
ECM-receptor interaction as a prognostic indicator for clinical outcome of primary osteoporosis

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Abstract: We attempt to identify differentially expressed genes (DEGs) and the potential signaling pathways involved in osteoporosis disease. Expression profiles of osteoporosis (GSE35956) were downloaded from GEO (Gene Expression Omninus), including five mesenchymal stem cells of osteoporosis patients and five control specimens. DEGs between primary osteoporosis and normal samples were identified using Limma package in R language. Meanwhile, the protein-protein interaction (PPI) network was constructed to predict the interactions of the DEGs using STRING (Search Tool for the Retrieval of Interacting Genes) database. Function enrichment analysis was then performed with GSEA (Gene Set Enrichment Analysis) method to reveal the biological process, signaling pathway and transcription factors involved in primary osteoporosis. A total of 406 DEGs were screened out, including 168 down-regulated genes and 238 up-regulated genes in mesenchymal stem cells. Two main functions, nitrin-1 signaling and iron-uptake, were figured by Reactome enrichment analysis. In GO analyses, the up-regulated genes were associated with immune regulation while the down-regulated genes were mainly involved in DNA repairing, processing of nucleic acid and cell cycle. In KEGG enrichment analysis, diverse down-regulated pathways including proteasome, spliceosome and protein export were screened out; while in the up-regulated pathways, focal adhesion, natural killer cell mediated cytotoxicity and ECM-receptor interaction were highly enriched. The discovery of focal adhension, ECM-receptor interaction and iron uptake pathway might contribute to the understanding of the mechanism and effective therapies for osteoporosis. However, further research on the relation between EMC-receptor interaction and osteoporosis were in need.

Keywords: Osteoporosis, protein-protein interaction network, gene set enrichment analysis, signal pathway

Introduction

Osteoporosis is a common age-related disease facing older people of both sexes, resulting in the increase for fracture risk [1]. Among people older than fifty all over the world, half of all women and a quarter of all men may break a bone due to osteoporosis [2, 3]. Besides, the aging society accompanied by the threat of osteoporosis and associated fracture can cause the disability-adjusted life years lost worldwide, resulting in the loss of labor and increase of medical expenses [4, 5]. However, the treatment of osteoporosis is time-consuming and high cost since the lack of prognostic and predictive biomarkers in clinical management. Thus, research on the target genes of osteoporosis disease, investigation and exploration on pathogenesis is urgent and quiet necessary.

As previous studies, bone mass at skeletal maturity and the amount of bone loss in later adulthood are the two main factors that determine whether an individual develops osteoporosis [6]. An inadequate peak bone mass, excessive bone resorption and inadequate formation of new bone during remodeling are the three mechanisms of osteoporosis [7]. In vivo, new bone is synthesized by osteoblasts and resorbed by osteoclasts. Research shows that insufficient bone-formation or excessive bone-resorption can cause the occurrence of osteoporosis [8]. Proliferation and differentiation of osteoblasts and osteoclasts are very important for the pathogenesis of osteoporosis [7].

Signaling pathways, part of a complex system of communication, play a major role in cell development, tissue repairing and immunity ability by perceiving and correctly responding to
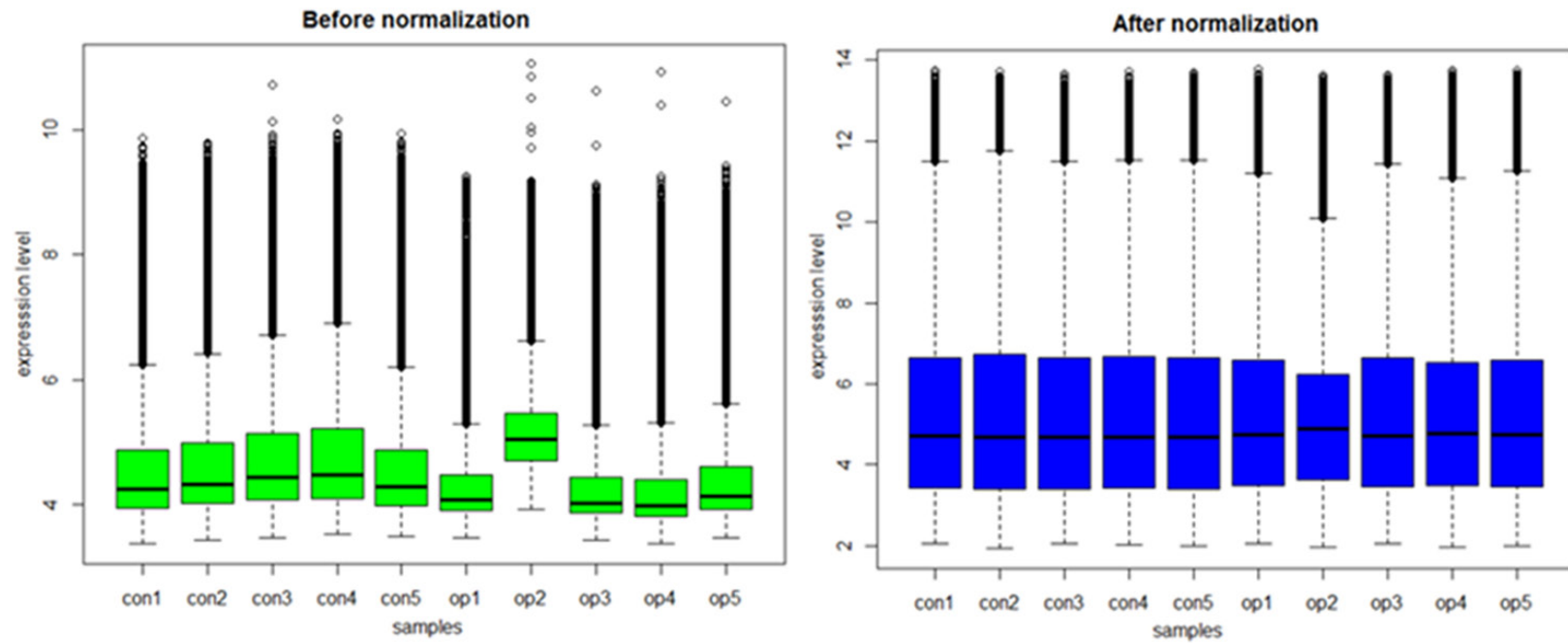
Figure 1. Box plot of gene expression in different samples before and after normalization. A. Gene expression in different samples before normalization. B. Gene expression in different samples after normalization. The vertical black line represent median of different samples.

Figure 2. Dendrogram of 10 different samples. The similarity was calculated by the Euclidean distance between different samples then applied for clustering analysis. Op2 and c2 were found out of the major cluster and defined as "ouliers".
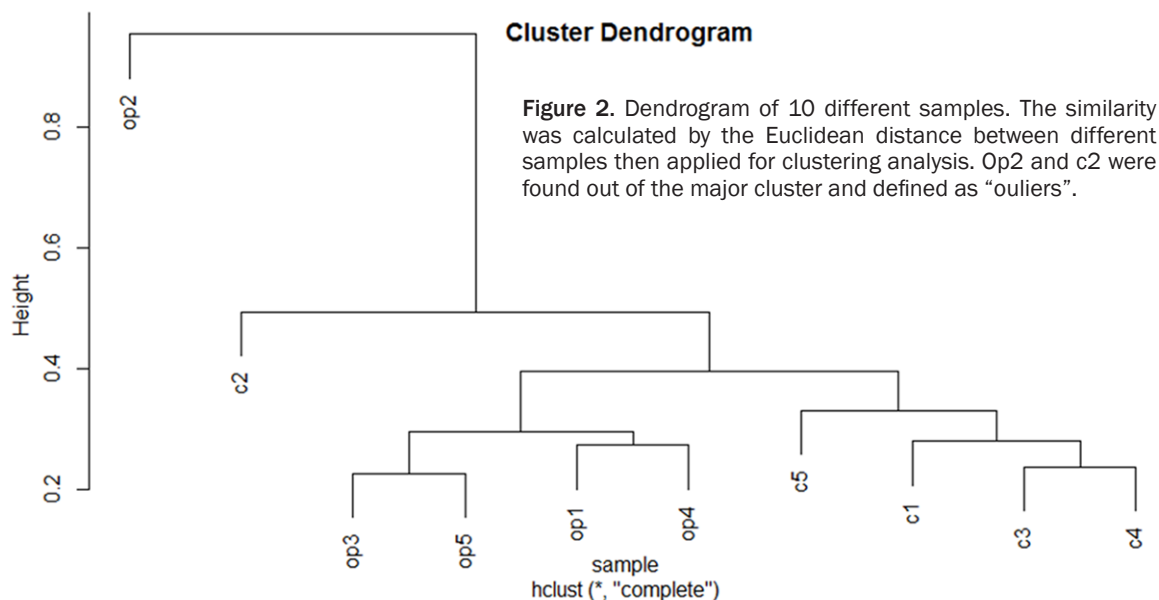
Table 1. Up and down-regulated genes

KEGG pathway	FDR	change
hsa04510 Focal adhesion	0.000869722	Up regulation
hsa04650 Natural killer cell mediated cytotoxicity	0.005093753	Up regulation
hsa04512 ECM-receptor interaction	0.006307736	Up regulation
hsa04974 Protein digestion and absorption	0.008414704	Up regulation
hsa04916 Melanogenesis	0.023696054	Up regulation
hsa04012 ErbB signaling pathway	0.029457201	Up regulation
hsa04912 GnRH signaling pathway	0.035288719	Up regulation
hsa03050 Proteasome	7.92E-10	Down regulation
hsa03040 Spliceosome	1.10E-05	Down regulation
hsa03060 Protein export	0.019054969	Down regulation

their environment. Potential pathways is recognized as a new target for the treatment of osteoporosis [9]. Important regulation of bone mass accrual by signal pathway is closely associated with the occurrence of osteoporosis. By understanding cell signaling of osteoporosis, we can treat the disease more effectively. In the past few years, many researches have been conducted on the extensive biological pathway/funtion related to protein export and DNA repairing, in order to reveal the molecular mechanism of osteoporosis [10, 11]. However, the information was not sufficient enough to clearly reveal the mechanism.

In the present study, we analyzed the differentially expressed genes (DEGs) involved in the progression of osteoporosis upon the expression profiling of mesenchymal stem cells of five osteoporosis patients and five normal samples. Protein-protein interaction network was constructed by investigating the critical DEGs in the progression. What's more, essential biological process, signal pathway and transcription factors associated with osteoporosis disease were figured out by using GO enrichment and analysis and GSEA method. Herein, by these bioinformatics methods, we can advance the understanding about the molecular mechanism of osteoporosis disease and therefore promote the therapy development.

Materials and methods

Data source and preprocessing

The gene expressing profiles of primary osteoporosis GSE35956 [12] was downloaded from
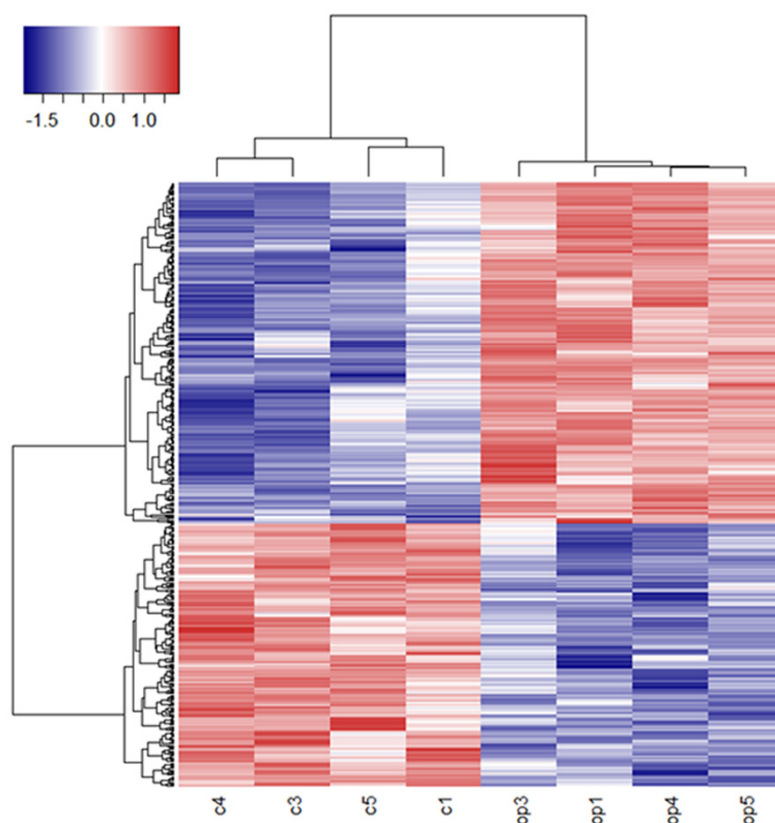
Figure 3. Heatmap of differentially expressed genes. The blue color represents down-regulated gene and the red represents up-regulated gene in osteoporosis samples. Legend on the top left indicate log fold change of genes.

<0.5 was used as the threshold to determine the significance of gene expression difference. FDR (False Discovery Rate) of q-value was adjusted to 0.05. Heatmaps were made to ensure that the screened genes have significant differences.

Construction of PPI (protein-protein interaction) network

STRING (Search Tool for the Retrieval of Interacting Genes) (http://string-db.org/) database [15] was used to build the PPI network to predict the interactions of these differentially expressed genes. The interactions were searched and visualized by Cytoscape [16] and then reactome pathway-based analysis [17] was performed to obtain the protein functions in the network by Cluepedia [17].

GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) database. A total of ten gene chips from mesenchymal stem cells were used for analysis, including five gene chips from osteoporosis patients and five genes chips from non-osteoporosis samples. The sequencing was based on the Affymetrix GeneChips Human Genome U133_Plus_2.0 platform, which is the first and most comprehensive whole human ge-nome expression array.

Data preprocessing

All the data was analyzed by AFFY package [13] and normalized in a cassette figure. hgu 133 plus2.db was used to convert the raw data into prove-level data. Finally, abnormal samples were filtered out according to the result of dendrogram.

Differentially expressed genes screening

Limma package of R language [14] was used to identify the DEGs between osteoporosis samples and normal samples. Fold change >2 or

GO enrichment and KEGG pathway analysis of DEGs

The result from DEGs analysis and GSEA (Gene Set Enrichment Analysis) were integrated with previous studies [18]. Our research used all the information contained in the transcriptome, and the whole GO or pathways were analyzed in a unit, which made an advantage over other researches. At the same time with Gene Ontology enrichment analysis, we identified the over-represented KEGG categories in pathways using GSEA to find affected biological process, signal pathway and possible signal factors involved in the regulation of osteoporosis. FDR=0.0001 was used as the threshold.

Results

Data source and preprocessing

The expression value of each probe was obtained after normalization. As is shown in boxplot (**Figure 1**), the median of different samples was almost on the same line after normalization, which shows an excellent degree of
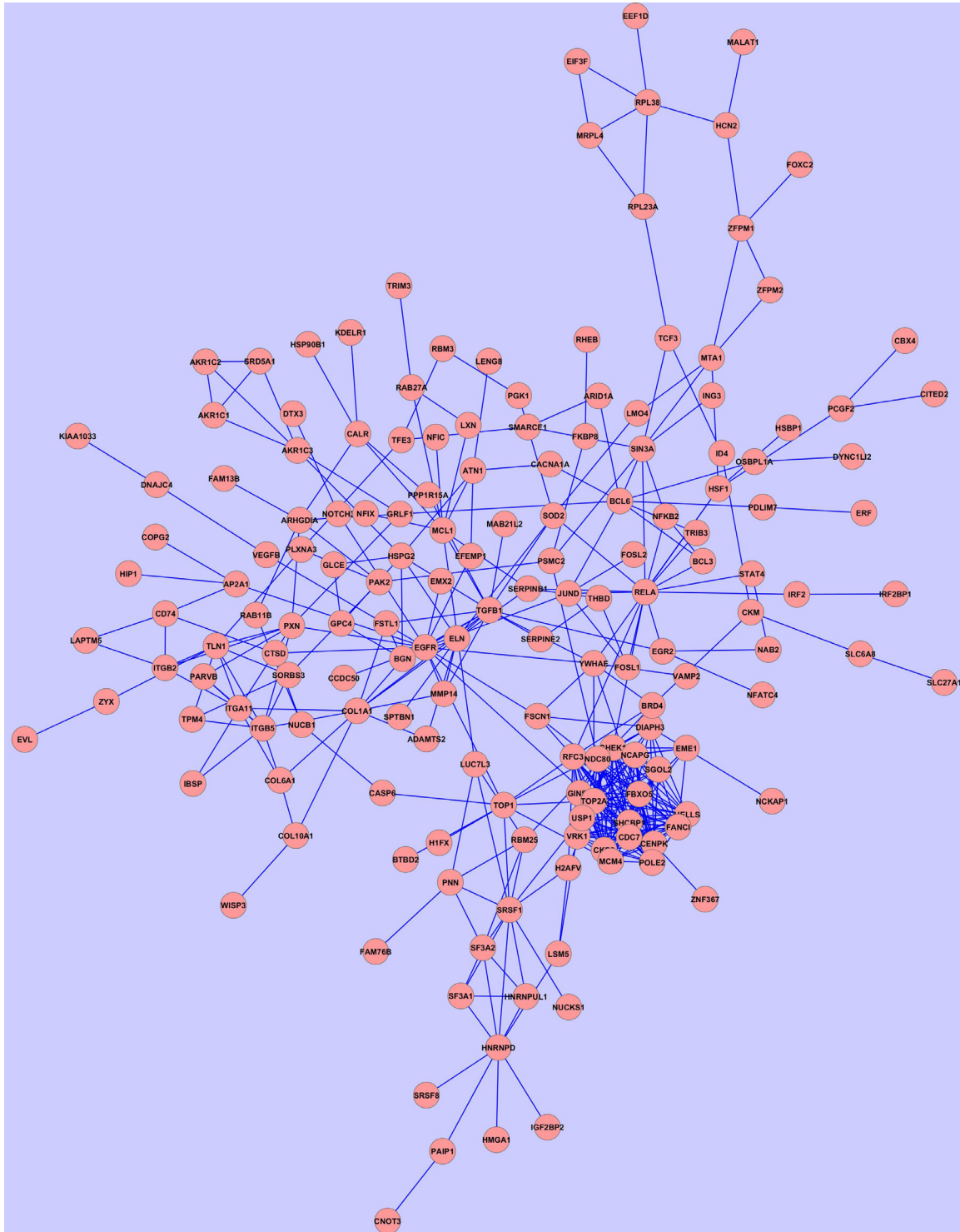
Figure 4. Protein-protein interactions between differentially expressed genes. Nodes represent differentiated genes and the blue edge represent summarized relationship according to algorism in String.

standardization. The cluster dendrogram showed that one osteoporosis sample and its corresponding control group is outlier (samples out of the major cluster) (**Figure 2**), which were discarded in later analysis.

DEGs screening

One osteoporosis sample and its corresponding control specimen were filtered out after clustering analysis. Total 406 DEGs were ex-
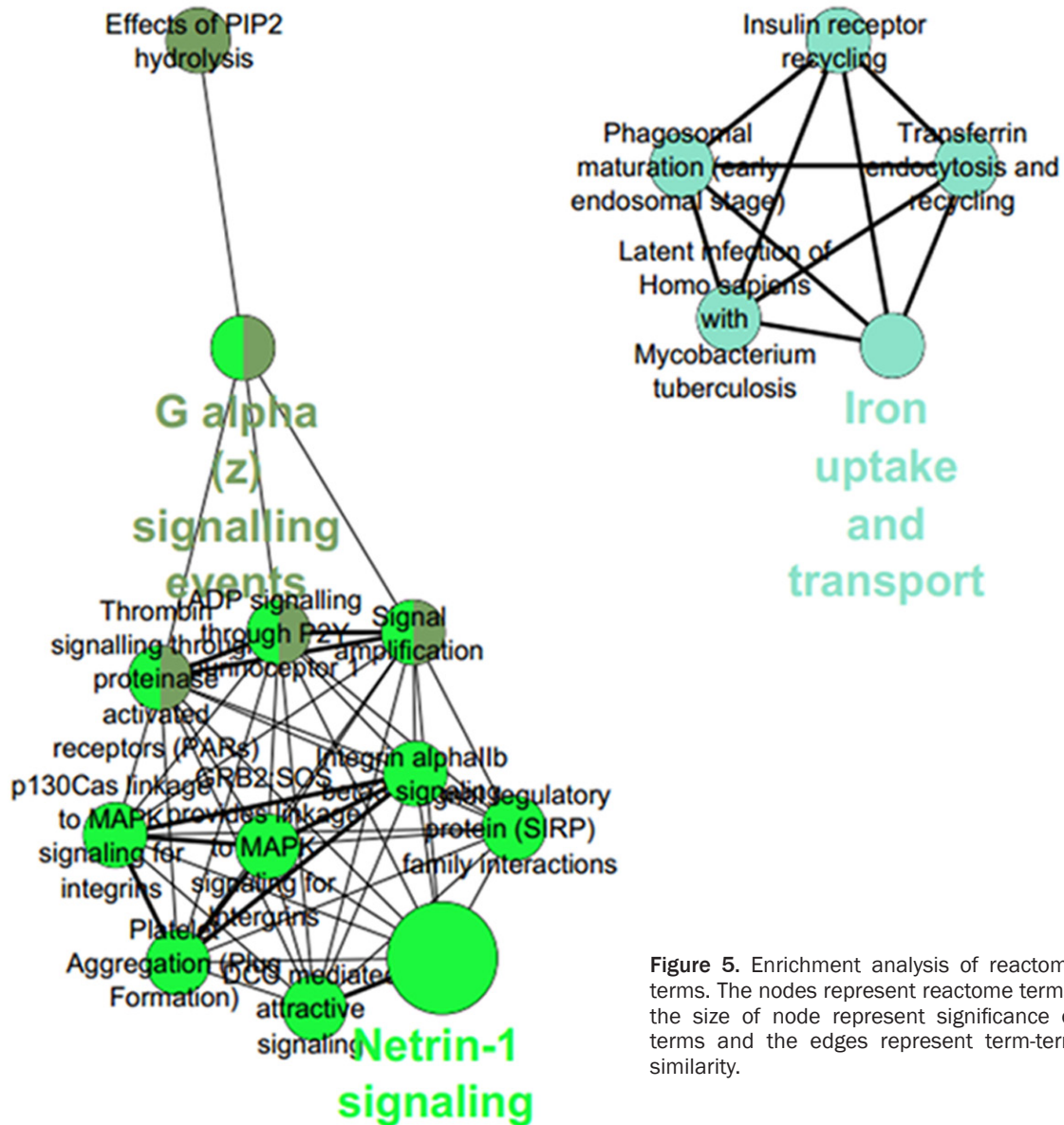
Figure 5. Enrichment analysis of reactome terms. The nodes represent reactome terms, the size of node represent significance of terms and the edges represent term-term similarity.

tracted out, including 168 down-regulated genes and 238 up-regulated genes in mesenchymal stem cells, accounting for 41.4% and 58.6% respectively (**Table 1**). As can be seen in the heatmap, all the DEGs can be divided into two groups, the control group and osteoporosis samples (**Figure 3**). Besides, the up- and down-regulated genes is significantly distributed (**Figure 4**), indicating the chara-cter of differentiated expression.

Protein-protein interaction analysis

In the PPI network costructed with these DEGs by STRING, 166 genes were found to be involved in main network enrichment module

(**Figure 5**). Reactome enrichment analysis was performed on the specific module extracted out and two main functions, nitrin-1signallin and iron uptake were found out (**Figure 6**).

GO enrichment analysis

All the GO terms were displayed in dendrogram form by DAG method. According to the result of GO enrichment analysis, in biological process, 10 GO terms were significantly up-regulated, including immune and skeletal system development and circulatory system process; while 27 GO terms as mitotic cell cycle, RNA processing, nuclear division, DNA metabolic process, DNA

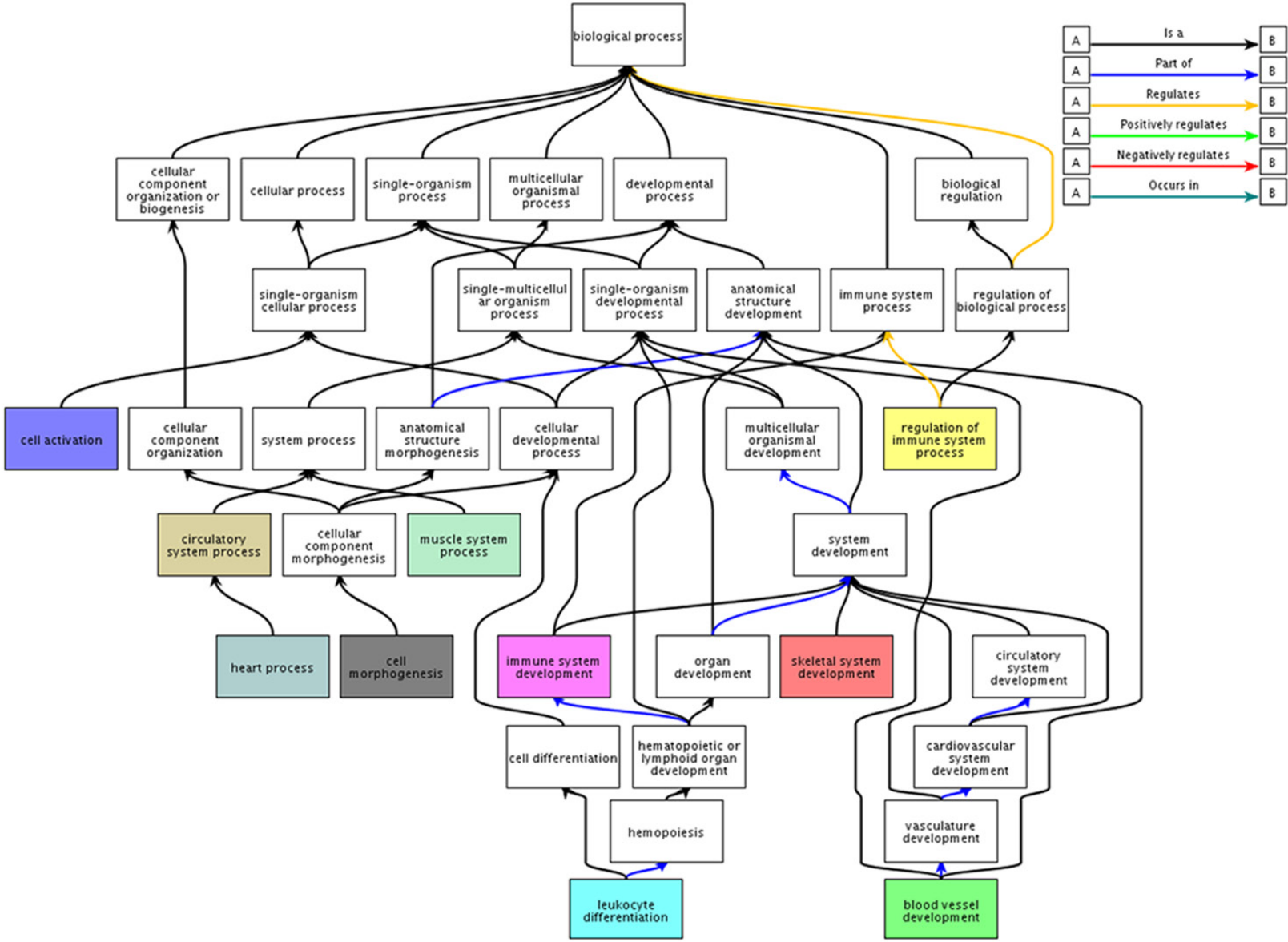# Potential signaling pathway analysis of primary osteoporosis

Figure 6. Directed acyclic graph of significant up-regulated GO terms. The upper terms are ancestor terms and the lower terms are offspring terms. The colored boxes are the significant enriched terms. Edges represent different relationships according to the keys on up right.

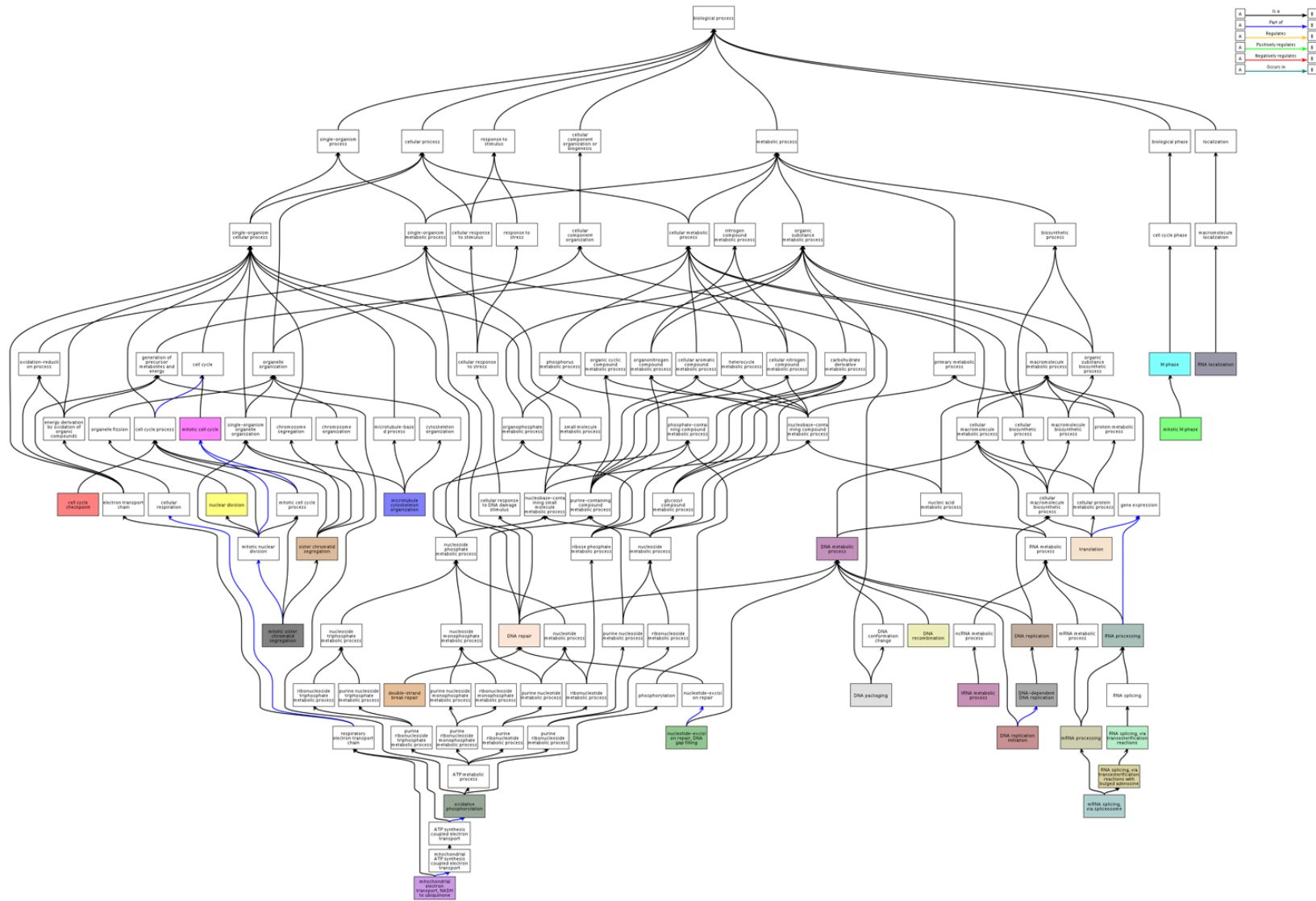# Potential signaling pathway analysis of primary osteoporosis

Figure 7. Directed acyclic graph of significant down-regulated GO terms. The upper terms are ancestor terms and the lower terms are offspring terms. The colored boxes are the significant enriched terms. Edges represent different relationships according to the keys on up right.

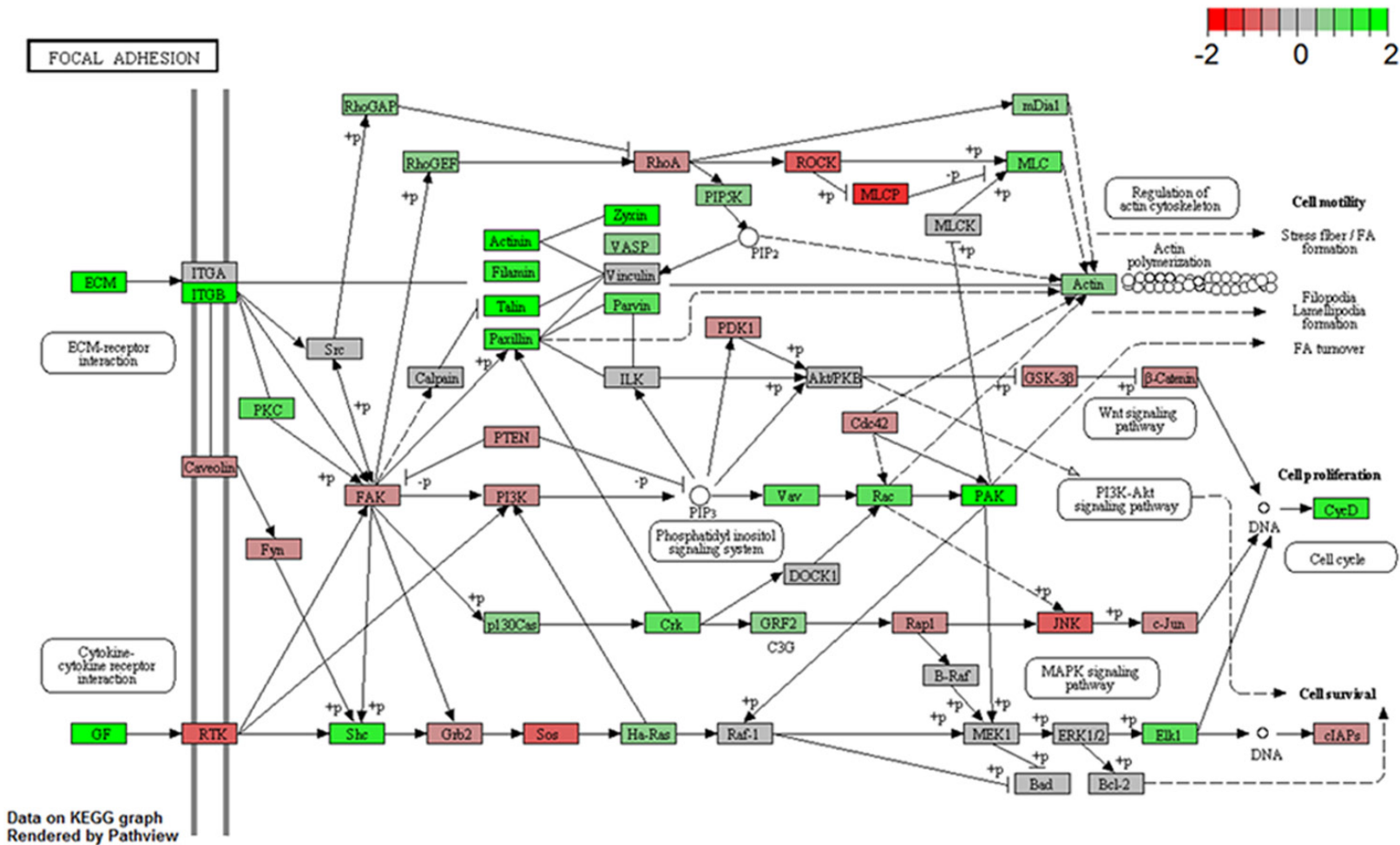# Potential signaling pathway analysis of primary osteoporosis

Figure 8. The gene expression in Focal Adhension pathways. The genes are showed by colored in log-fold change according to the keys on up right.

Table 2. Transcription factors (TFs) predicted

	Mean of fold change	P value	FDR	Set size
V$AP4_Q6	2.998566	1.28E-09	7.35E-07	216
V$TFIII_Q6	2.863622	6.33E-09	1.09E-06	198
V$MYOGENIN_Q6	2.837612	8.42E-09	1.09E-06	251
V$CACCCBINDINGFACTOR_Q6	2.834745	8.44E-09	1.09E-06	266
V$AP2ALPHA_01	2.825753	9.48E-09	1.09E-06	237
V$HEB_Q6	2.754005	2.12E-08	2.02E-06	252
V$COUP_DR1_Q6	2.658859	6.05E-08	4.96E-06	239

repairing were significantly down-regulated (**Figure 7**). The up-regulated genes were associated with immune regulation while the down-regulated genes were mainly involved in three sets: the function of DNA repairing, such as double-strand break repair; processing of DNA and RNA, including DNA replication, RNA processing, etc; cell cycle, such as mitotic M Phase, etc.

KEGG enrichment analysis

Diverse down-regulated pathways were screened out, including proteasome (hsa03050), spliceosome (hsa03040) and protein export (hsa03040); while in the up-regulated pathways, focal adhension (hsa04510), natural killer cell mediated cytotoxicity (hsa04650) and ECM-receptor interaction (hsa04512) were highly enriched (**Figure 1**). For example, most genes enriched in focal adhesion pathway were significantly up-regulated while those down-regulated genes were not in much degree, thus resulting in the up-regulation of the whole pathway (**Figure 8**).

Transcription factor analysis

According to the database of transcription factor in MsigDB, (http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=TFT) the method of GEDA was performed on these transcription factors. It could be found that several transcription factors, such as AP_4, TFIII, MYOGENIN, AP2ALPHA, HEB and COUP_DR1, might be involved in the regulation of osteoporosis process (**Table 2**).

Discussion

Osteoporosis, the most common metabolic bone disease, poses a great threat to the aging society. Although several markers have been reported to participate in osteoporosis, such as IL-1 (interleukin-1), IL-6 and TNF-α (tumor necrosis factor-alpha) [19-21], the evaluation or treatment with osteoporosis patients is still very poor.

Signal pathways, involved in gene regulations, participate in a series of intracellular pathologic process. Besides, the extensive and complicated interactions among these pathways in different diseases have been recognized as important intervention targets and predictive tools. Through screening expression profiles of five mesenchymal stem cells from osteoporosis patients and five normal samples, 168 up-regulated genes and 238 down-regulated genes were identified. By analyzing interactions among the DEGs by GSEA method, signal pathways as netrin-1 signaling,iron uptake, cell cycle, focal adhesion, natural killer cell mediated, ECM-receptor interaction were significantly enriched by KEGG enrichment analysis.

The pathogenic mechanism of osteoporosis is recognized as the imbalance of osteoblast and osteoclast in number. Netrin-4 was reported to prevent the bone loss in vitro and vivo by inhibiting osteoblast differentiation and decreasing the number of osteoclast [22]. The result of its up-regulation in osteoporosis samples in our study confirmed its close association with the occurrence of osteoporosis, thus we predicted that the metal suppresses osteoblast formation of bone and may also stimulate osteoclast absorption of bone [23].

Osteoporosis is recognized as a complication of iron taking conditions and through the results of PPI network and KEGG pathway, we found that the function of iron-taking process is affected, which is consistent with the result. Given the symptom of abnormal bone metabolism of the disease, we predicted that the metal suppresses osteoblast formation of bone and may also stimulate osteoclast absorption of bone [23]. Furthermore, there has been report that bone mineral density decreases according to age, which can possibly explain the high incidence of osteoporosis in people aged 50 and above.

Another two pathways, focal adhesion and ECM-receptor interactions were significantly up-regulated in the enrichment analysis. The genes related to focal adhesion and ECM-receptor pathway were observed to be up-regulated, resulting in the up-regulation of the whole pathway. Focal adhesions is reported to play a critical role in cell survival, migration and in sensing physical force [24]. Osteogenic differentiation is more prevalent in human mesenchymal stem cells (hMSC) with greater number of focal adhesion [25]. Bone-marrow-derived hMSC, which are able to differentiate into several committed phenotypes, osteogenic included [26-28], have significant clinical potential in cellular therapies and tissue regeneration. On the other hand, cell contact with extracellular matrix (ECM) proteins also plays a critical role in regulating hMSC osteogenesis [29-31]. Since ECMP (extracellular matrix receptor) is highly enriched in our enrichment analysis and the interaction between ECMP and osteoporosis hasn't been researched thoroughly, we try to analyze its role in osteoporosis through the effect of ECM in regulating osteogenesis of mesenchymal stem cells.

It's reported that ECMR expression may direct cell localization to specific tissue domains. Whether ECMP leads to age-related osteoporosis has not been evaluated. Given the associations between ECM and the cell-surface-associated component, we indicate that variation in ECMP will possibly influence bone tissue differentiation. Since the occurrence of osteoporosis is a result of imbalance of osteoblast and osteoclast in number, the finding of corresponding pathway association with bone tissue differentiation can be meaningful. Thus, the identification of ECM-receptor as a potential network biomarker for osteoporosis will be of great interest. And these findings will be beneficial in disclosing the molecular mechanisms of the osteoporosis disease and thus advancing the therapy development.

In conclusion, we identified netrin-1, iron taking, focal adhension and ECMP interaction signal pathways associated with pathogenesis of osteoporosis disease. More importantly, we analyzed the availability and rationally of ECMP interaction as a novel and meaningful diagnostic and prognostic marker to clinical outcome of osteoporosis patients. However, further researches on the specific mechanism were still in need.

Disclosure of conflict of interest

None.

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References

[1] Kanis JA, Melton LJ, Christiansen C, Johnston CC and Khaltaev N. The diagnosis of osteoporosis. J Bone Miner Res 1994; 9: 1137-1141.

[2] De Laet CE, van Hout BA, Burger H, Hofman A and Pols HA. Bone density and risk of hip fracture in men and women: cross sectional analysis. BMJ 1997; 315: 221-225.

[3] Eisman JA. Genetics of osteoporosis. Endocr Rev 1999; 20: 788-804.

[4] Lewiecki EM and Silverman SL. Redefining osteoporosis treatment: who to treat and how long to treat. Arq Bras Endocrinol Metabol 2006; 50: 694-704.

[5] Kim HW, Kim YH, Han K, Nam GE, Kim GS, Han BD, Lee A, Ahn JY and Ko BJ. Atrophic Gastritis: A Related Factor for Osteoporosis in Elderly Women. PLoS One 2014; 9: e101852.

[6] Cotter MM, Loomis DA, Simpson SW, Latimer B and Hernandez CJ. Human evolution and osteoporosis-related spinal fractures. PLoS One 2011; 6: e26658.

[7] Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 2005; 115: 3318-3325.

[8] Jules J, Ashley JW and Feng X. Selective targeting of RANK signaling pathways as new therapeutic strategies for osteoporosis. Expert Opin Ther Targets 2010; 14: 923-934.

[9] Rawadi G and Roman-Roman S. Wnt signalling pathway: a new target for the treatment of osteoporosis. Expert Opin Ther Targets 2005; 9: 1063-77.

[10] Nicolaije C, Diderich KE, Botter S, Priemel M, Waarsing JH, Day JS, Brandt RM, Schilling AF, Weinans H and Van der Eerden BC. Age-related skeletal dynamics and decrease in bone strength in DNA repair deficient male trichothiodystrophy mice. PLoS One 2012; 7: e35246.

[11] Hsu YH, Niu T, Terwedow HA, Xu X, Feng Y, Li Z, Brain JD, Rosen CJ, Laird N and Xu X. Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men. Hum Genet 2006; 118: 568-577.

[12] Benisch P, Schilling T, Klein-Hitpass L, Frey SP, Seefried L, Raaijmakers N, Krug M, Regens-

burger M, Zeck S and Schinke T. The transcriptional profile of mesenchymal stem cell populations in primary osteoporosis is distinct and shows overexpression of osteogenic inhibitors. PLoS One 2012; 7: e45142.

[13] Gautier L, Cope L, Bolstad BM and Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004; 20: 307-315.

[14] Smyth GK. Limma: linear models for microarray data. Bioinformatics and computational biology solutions using R and Bioconductor. Springer; 2005. pp. 397-420.

[15] Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A and Simonovic M. STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 2009; 37: D412-D416.

[16] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003; 13: 2498-2504.

[17] Bindea G, Galon J and Mlecnik B. CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. Bioinformatics 2013; 29: 661-663.

[18] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR and Lander ES. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.

[19] Cohen-Solal M, Graulet A, Denne M, Gueris J, Baylink D and De Vernejoul M. Peripheral monocyte culture supernatants of menopausal women can induce bone resorption: involvement of cytokines. J Clin Endocrinol Metab 1993; 77: 1648-1653.

[20] Cohen-Solal M, Boitte F, Bernard-Poenaru O, Denne M, Graulet A, Brazier M and De Vernejoul M. Increased bone resorbing activity of peripheral monocyte culture supernatants in elderly women. J Clin Endocrinol Metab 1998; 83: 1687-1690.

[21] Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Mine Res 1996; 11: 1043-1051.

[22] Enoki Y, Sato T, Tanaka S, Iwata T, Usui M, Takeda S, Kokabu S, Matsumoto M, Okubo M and Nakashima K. Netrin-4 derived from murine vascular endothelial cells inhibits osteoclast differentiation in vitro and prevents bone loss in vivo. FEBS Lett 2014; 588: 2262-9.

[23] Weinberg E. Role of iron in osteoporosis. Pediatr Endocrinol Rev 2008; 6: 81-85.

[24] Zintzaras E, Doxani C, Koufakis T, Kastanis A, Rodopoulou P and Karachalios T. Synopsis and meta-analysis of genetic association studies in osteoporosis for the focal adhesion family genes: the CUMAGAS-OSTEOporosis information system. BMC Med 2011; 9: 9.

[25] Wu X, Guo S, Shen G, Ma X, Tang C, Xie K, Liu J, Guo W, Yan Y and Luo E. Screening of osteoprotegerin-related feature genes in osteoporosis and functional analysis with DNA microarray. Eur J Med Res 2013; 18: 15.

[26] Jaiswal N, Haynesworth SE, Caplan AI and Bruder SP. Osteogenic differentiation of purified, culture - expanded human mesenchymal stem cells in vitro. J Cell Biochem 1997; 64: 295-312.

[27] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.

[28] Salasznyk RM, Williams WA, Boskey A, Batorsky A and Plopper GE. Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. J Biomed Biotechnol 2004; 2004: 24-34.

[29] Mizuno M and Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J Biochem 2001; 129: 133-138.

[30] Salasznyk RM, Klees RF, Hughlock MK and Plopper GE. ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. Cell Commun Adhes 2004; 11: 137-153.

[31] Klees RF, Salasznyk RM, Kingsley K, Williams WA, Boskey A and Plopper GE. Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. Mol Biol Cell 2005; 16: 881-890.