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Expression profile analysis of new candidate genes for the therapy of primary osteoporosis

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Abstract. – OBJECTIVE: Primary osteoporosis is a progressive bone disease that is characterized by a decrease in bone mass and density which can lead to an increased risk of fracture. Most of present treatments are effective for osteoporosis, but have limitations and side-effects. With the aging of the world population is increasing, the incidence of osteoporosis is rising. Therefore, the purpose of this study was to identify new candidate genes used as the therapeutic targets of primary osteoporosis.

MATERIALS AND METHODS: In this study, microarray data GSE35958 were downloaded from Gene Expression Omnibus, then the differentially expressed genes (DEGs) were identified by limma package. Gene Ontology (GO) and KEGG pathway enrichment analyses were performed for both up- and down-regulated DEGs using DAVID. In addition, the transcription factor analysis was conducted for DEGs. The protein-protein interaction (PPI) network was constructed by STRING and Cytoscape. Finally, CFinder was used to analyze the PPI sub-network.

RESULTS: Totally, 327 up-regulated DEGs and 396 down-regulated DEGs were identified. The DEGs such as EGFR and AKT1 were mainly enriched in the pathway of focal adhesion. EGFR was also involved in cell adhesion based on GO enrichment analysis. Functional analysis of DEGs indicated that 26 transcription factors were screened. Moreover, EGFR, AKT1 and transcription factor CTNNB1 were the key nodes with high degrees according to PPI network and sub-network.

CONCLUSIONS: The literature suggested that AKT1, EGFR and CTNNB1 were closely related to osteoblastic differentiation and osteoclastogenesis. Some crucial DEGs such as EGFR, AKT1 and CTNNB1 might be connected with primary osteoporosis and could be used as therapeutic targets of osteoporosis.

Key Words:
Primary osteoporosis, Genes, Enrichment analysis, Protein-protein interaction network, Sub-network.

Introduction

Primary osteoporosis is a common bone disease that is characterized by a systemic impairment of bone mass, density and microarchitecture[1]. Osteoporosis has no symptoms, but it will lead to an increased propensity of fragility fractures, and fractures most commonly occur in the spine, hip, or wrist[2]. So osteoporosis is often diagnosed after the first clinical fracture has occurred[3]. The most important risk factors for osteoporosis are advanced age and gender besides a serious of other contributors, such as heredity, race and lifestyle[4-6]. The measurement of bone mineral density is an effective method to diagnose osteoporosis and to predict the risk of fracture[7]. Present antiresorptive and anabolic treatments are effective for osteoporosis, but most have limitations and side-effects[8]. Therefore, novel therapeutic targets are still needed for future osteoporosis treatments.

The basic pathogenetic mechanism of osteoporosis is an imbalance between bone resorption and bone formation[2]. Both excessive bone resorption and inadequate bone formation result in osteoporosis, which are closely related to bone remodeling. Bone remodeling is the major activity of bone cells in the adult skeleton[6] and comprises a coupled action of bone-resorbing cells (osteoclasts) and bone-forming cells (osteoblasts)[4]. Several key molecules coordinate activities of osteoblasts and osteoclasts during bone remodeling[2]. Hence, the cellular and molecular processes are new aspects used to study the pathophysiology of osteoporosis. As the essential regulator of osteoclast differentiation and activity, receptor activator of NF-κB ligand (RANKL) has been regarded as a standard therapy for osteoporosis[9,10]. However, the studies about the impact of osteoblasts on the pathophysiology of os-

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teoporosis have been relatively neglected. Osteoblasts derive from mesenchymal stem cells (MSC) which is the source for bone regeneration[11]. The rate of bone formation is determined by the speed and effectiveness of MSC differentiating into mature osteoblasts[2]. It is currently unknown if the molecular processes in MSC contribute to the inadequate bone formation of primary osteoporosis.

In this study, the microarray data GSE35958 were downloaded from Gene Expression Omnibus (GEO) database to detect new candidate genes for the therapy of primary osteoporosis in elderly people. Gene Ontology (GO) and KEGG pathway enrichment analysis were conducted for the screened differentially expressed genes (DEGs). Then analysis of transcription factor was performed for DEGs. In addition, protein-protein interaction (PPI) network and sub-network were constructed to identify the key genes related to osteoporosis.

Materials and Methods

Affymetrix Microarray Data

The gene expression profile data GSE35958 were extracted from the study of Benisch et al[12], which were deposited in Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). In this study, human mesenchymal stem cells (MSC) of non-osteoporotic donors (79-89 years old) were obtained from bone marrow of femoral heads after total hip arthroplasty. The MSC of patients (79-94 years old) suffering from osteoporosis were isolated from femoral heads after low-energy fracture of the femoral neck. The microarray data included 4 samples of MSC from non-osteoporotic donors (normal group) and 5 samples of MSC from osteoporotic patients (osteoporosis group). The GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array was used as microarray platform.

Data Preprocessing and DEGs Screening

The raw data were preprocessed utilizing limma package in Bioconductor[13] and affy annotation files from Brain Array Lab[14]. The pretreatment comprised background correction, quantile normalization and probe summarization of the microarray data, and the gene expression matrix was obtained eventually.

The normalized data were calculated by limma package. The genes with the $|\log_2 FC| > 1$ and $p < 0.05$ were considered as DEGs in osteoporosis group compared with normal group.

Enrichment Analysis and Functional Annotation of DEGs

DAVID (The Database for Annotation, Visualization and Integrated Discovery)[15] is a comprehensive set of functional annotation tools used for enrichment analysis. So GO[16] and KEGG pathway[17] enrichment analyses of DEGs were performed using DAVID based on hypergeometric distribution algorithm. The p-value < 0.05 was chosen as the cut-off criterion for both GO and KEGG enrichment analysis.

The transcription factor analysis was performed using the TRANSFAC database[18] to determine whether the DEGs were transcription factors.

Construction of PPI Network and PPI Sub-network Analysis

The online tool STRING version 9.1[19] was used to search interaction relationships of the screened genes, and the confidence score > 0.9 was considered as the cut-off criterion. Then the Cytoscape[20] was applied to construct the PPI network based on the protein interactions. Researches showed that most complex biology networks are scale-free and contain a small number of highly connected nodes (hubs) and a large number of poorly connected nodes (non-hubs)[21,22]. The hubs in PPI were obtained by the connectivity degree analysis.

CFinder[23] is a fast program locating and visualizing overlapping and can be used to predict functions of a single protein. The PPI sub-networks of DEGs were identified using clique percolation method (CPM) of CFinder. The clique size $k = 4$ was chosen as the cut-off criterion.

Results

DEGs analysis

The limma package was used for the identification of DEGs between osteoporosis group and normal group after preprocessing of microarray data. A total of 723 DEGs were obtained related to osteoporosis including 327 up-regulated DEGs and 396 down-regulated DEGs.

Table I. The top five Gene Ontology terms of differentially expressed genes (DEGs).

	Category	Term	Description	*p*-value	Genes
Up	BP	GO:0007155	cell adhesion	1.43E-13	*EGFR, IBSP, PCDHGA12…*
	BP	GO:0022610	biological adhesion	1.49E-13	*EGFR, IBSP, PCDHGA9…*
	BP	GO:0007156	homophilic cell adhesion	1.59E-12	*PKD1□PCDHGB7, PCDHGA8…*
	BP	GO:0016337	cell-cell adhesion	3.34E-12	*PKD1, EGFR, PCDHGA12…*
	MF	GO:0005509	calcium ion binding	3.61E-08	*FKBP8, TRPV2, LTBP4…*
Down	MF	GO:0046914	transition metal ion binding	3.57E-05	*PXDN, ILKAP, CMC1…*
	BP	GO:0016071	mRNA metabolic process	3.78E-05	*UPF2, TRA2A, PRPF39…*
	MF	GO:0008270	zinc ion binding	4.01E-05	*RSF1, RP9, ZNF518A…*
	CC	GO:0031981	nuclear lumen	6.36E-05	*ING3, SDAD1, LMNB1…*
	CC	GO:0070013	intracellular organelle lumen	1.15E-04	*CDC14B, INTS2, ANKRD1…*

Note: Up represents up-regulated DEGs; Down represents down-regulated DEGs.

Enrichment and Functional Analysis of DEGs

The top three enriched GO terms of up-regulated DEGs were mainly related to cell adhesion, included cell adhesion involving *EGFR*, *IBSP* and *PCDHGA12*, biological adhesion related to *EGFR*, *IBSP* and *PCDHGA9*, homophilic cell adhesion including *PKD1*, *PCDHGB7* and *PCDHGA8* (Table I). The results of KEGG pathway enrichment analysis indicated that up-regulated DEGs were mainly enriched in pathways of focal adhesion (e.g. *AKT1*, *EGFR* and *VEGFA*), pancreatic cancer (e.g. *EGFR*, *VEGFB* and *AKT1*) and regulation of actin cytoskeleton (e.g. *EGFR*, *MAP2K2* and *INS-IGF2*), as shown in Table II. Moreover, the top three GO terms of down-regulated DEGs included transition metal ion binding involving *PXDN*, *ILKAP* and *CMC1*, mRNA metabolic process related to *UPF2*, *TRA2A* and *PRPF39*, zinc ion binding including *RSF1*, *RP9* and *ZNF518A* (Table I). However, down-regulated DEGs such as *LSM5*, *THOC* and *TRA2A* were only enriched in one pathway: spliceosome (Table II).

Transcription factor analysis of DEGs showed that 9 transcription factors were significantly down-regulated in MSC of osteoporotic patients, such as *PLAGL1*, *CTNNB1* and *EGR1*. Meanwhile, 17 transcription factors were found significantly up-regulated, such as *ERF*, *STAT4* and *SMARCB1* (Table III).

PPI Network and PPI Sub-Network Analysis

To systemically analyze the functions of DEGs in MSC of osteoporotic patients, the PPI network of DEGs was constructed using Cytoscape in accordance with protein interactions. From Figure 1, we discovered that the key nodes with highest degrees were *TP53*, *INS*, *AKT1*, *CTNNB1*, *EGFR*, *VEGFA* and *TGFB1*. The degrees of these proteins were all larger than 30.

The obtained core sub-network included 101 genes, as shown in Figure 2. The top five proteins with the high degrees were *AKT1* (degree = 46), *EGFR* (degree = 36), *VEGFA* (degree = 35), *INS* (degree = 35) and *TP53* (degree = 33).

Table II. The top five KEGG pathways of differentially expressed genes (DEGs).

	Category	Term	Description	*p*-value	Genes
Up	KEGG	hsa04510	Focal adhesion	3.29E-08	*AKT1, EGFR, VEGFA…*
	KEGG	hsa05212	Pancreatic cancer	6.19E-05	*EGFR, VEGFB, AKT1…*
	KEGG	hsa04810	Regulation of actin cytoskeleton	2.13E-04	*EGFR, MAP2K2, INS-IGF2…*
	KEGG	hsa05220	Chronic myeloid leukemia	5.42E-04	*AKT1, CDKN1A, MAP2K2…*
	KEGG	hsa05219	Bladder cancer	0.00116	*EGFR, VEGFB, TP53…*
Down	KEGG	hsa03040	Spliceosome	0.021787	*LSM5, THOC, TRA2A…*

Note: Up represents up-regulated DEGs; Down represents down-regulated DEGs.

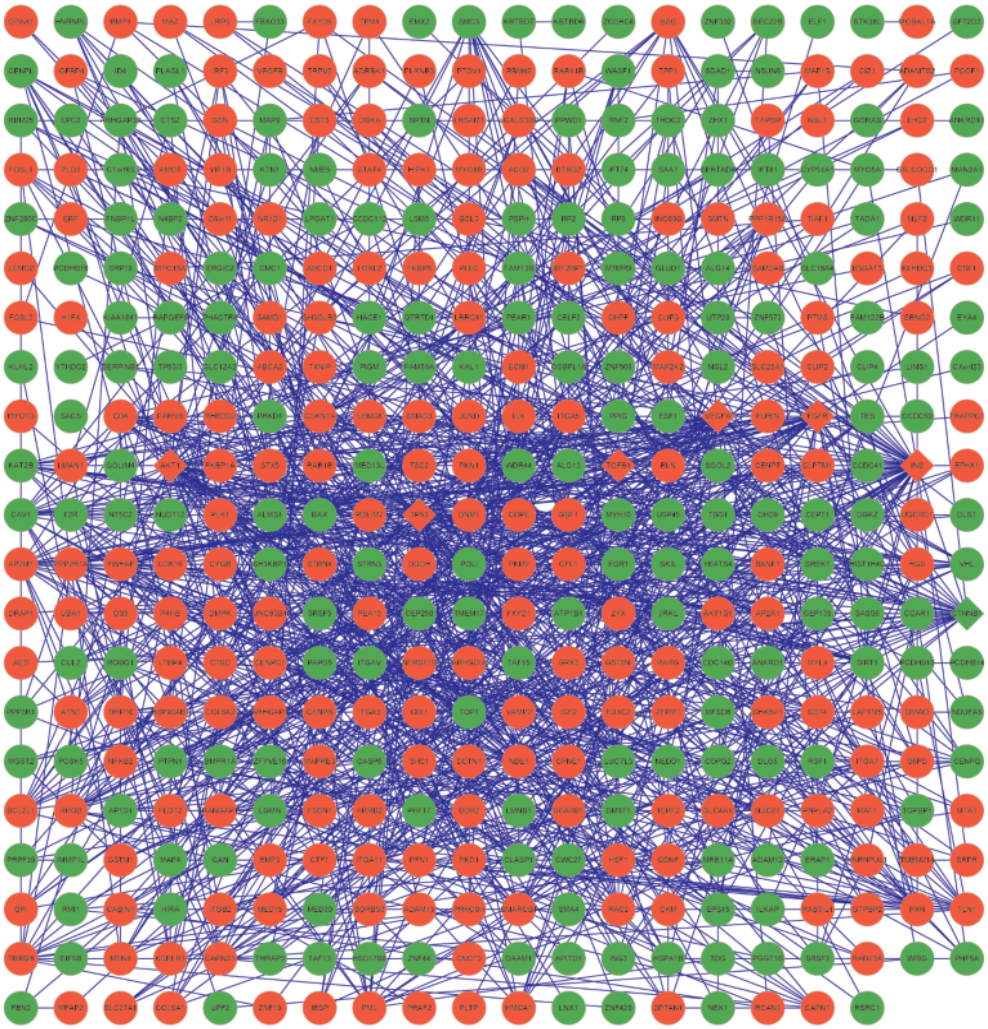

Figure 1. Protein-protein interaction network of differentially expressed genes (DEGs). Red nodes represent up-regulated DEGs; green nodes represent down-regulated DEGs; rhombus nodes represent key nodes.

Discussion

In this study, bioinformatics methods were used to identify potential biomarkers for clinical therapy of primary osteoporosis. The results showed that 723 DEGs were obtained in osteoporosis group compared with normal group. The up-regulated DEGs such as *AKT1* and *EGFR* were mainly enriched in the pathway of focal adhesion. Moreover, the results of GO enrichment analysis showed that the up-regulated DEGs such as *EGFR* and *PCDHGA12* were involved in cell adhesion. Transcription factors such as *CTNNB1*, *EGR1* and *STAT4* were identified by transcription factor analysis of DEGs. In addition, genes with high degrees were obtained according to the PPI network and sub-network, such as *AKT1*, *EGFR* and *CTNNB1*.

The pathway enrichment analysis showed that *AKT1*, *EGFR* were enriched in the pathway of focal adhesion which could regulate the osteogenic differentiation of human mesenchymal stem cells[24]. Furthermore, it has been reported that focal adhesion kinase signaling may be altered in osteoporotic osteoblasts compared with normal osteoblasts[25]. In addition, *AKT1* and *EGFR* were the key nodes with high degrees based on the PPI network and sub-network in our study. AKT, also termed protein kinase B (PKB), is a serine/threonine kinase has emerged as one of the most critical and versatile protein kinases at the core of human physiology and disease[26,27].

Table III. The transcription factors analysis of differentially expressed genes (DEGs).

	TF counts	TF genes
Down	9	*EGR1, EMX2, PLAGL1, CTNNB1, ELF1, ZNF44, ZHX1, SKIL, FOXC1*
Up	17	*ERF, STAT4, SMARCB1, HSF1, NR1D1, BCL3, SMAD3, PML, JUND, NFKB2, FOSL2, FOXC2, IRF3, RARG, HMGA1, ELK3, MAZ*

Note: Up represents up-regulated DEGs; Down represents down-regulated DEGs; TF represents transcription factor.

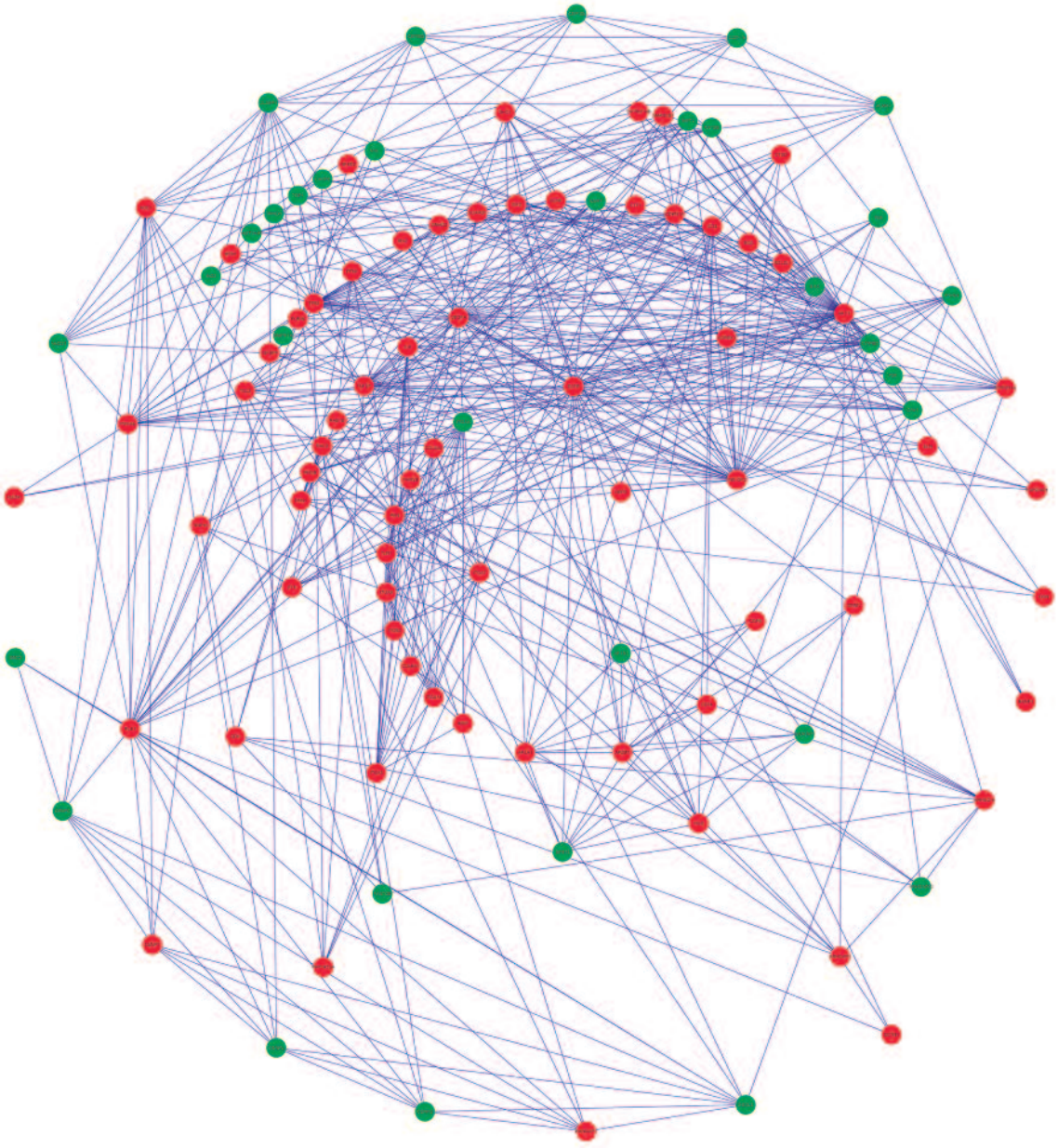

Figure 2. Protein-protein interaction (PPI) sub-network of differentially expressed genes (DEGs). Red nodes represent up-regulated DEGs; green nodes represent down-regulated DEGs.

It is also known as a key protein in the signaling of potent bone anabolic factors[28,29]. There have three mammalian isoforms (AKT1-3) and AKT1 is the major AKT isoform in bone cells[30]. Mukherjee et al[31] discovered that Akt1 was both a negative regulator of osteoblast differentiation and a robust positive mediator of osteoblast-coupled osteoclastogenesis in mice by experimental verification. Another study in mice indicated that Akt1 was the crucial regulator of osteoblasts and osteoclasts, and Akt1 deficiency could cause decreased bone mass and formation[30]. All the evidences demonstrated that *AKT1* might have an essential role in osteoporosis. However, the studies on the function of *AKT1* in human beings were relatively little. Therefore, further studies were needed to verify the potential role of *AKT1* in human osteoporosis.

In the present work, *EGFR* was also enriched in cell adhesion by the GO enrichment analysis. Previous studies reveal that the cell adhesion of MSC and osteoblasts are closely associated with the bone regeneration[32,33]. EGFR (epidermal growth factor receptor) is one member of transmembrane growth factor receptor proteins[34]. EGFR and its ligands form a complex EGFR networks which paly critical roles in key progresses of biology, such as growth, differentiation, survival, tissue homeostasis and repair[35]. In addition, the EGFR system has been reported to regulate the release of angiogenic factors in MSC[36]. Reports also showed[37] that EGFR network was closely associated with bone biology and pathology, for example it affected osteoblastic bone formation and bone mass in different ways. Recently, Zhu et al[38] found that EGFR system suppressed osteoblast differentiation and have crucial functions in skeletal homeostasis. Consequently, we speculated that EGFR system might be crucial in the treatment of osteoporosis.

CTNNB1 was both the transcription factor in DEGs and key nodes in PPI network. β-catenin, encoded by the *CTNNB1*, is the key downstream component of the canonical Wnt pathway. The activation of the canonical Wnt/β-catenin pathway regulates osteoblastic differentiation in human mesenchymal stem cells[39,40]. Moreover, β-catenin exerts the dosage-dependent regulation of osteoclastogenesis[41]. Thus, β-catenin signaling has been the potential therapeutic target for novel treatment of osteoporosis[2,42]. The essential role of β-catenin in osteoporosis also validated the reliability of our analysis.

Conclusions

We analyzed the gene expression profiles of the elderly people with and without osteoporosis to identify key disease-related genes. Some important DEGs such as *AKT1*, *EGFR* and *CTNNB1* were closely associated with osteoblast differentiation and bone formation. So they might be used as potential therapeutic targets of osteoporosis. However, these genes were only analyzed by bioinformatics methods, and validation studies were still needed.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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