Abstract. – OBJECTIVE: Despite improvements in research on osteoporosis in the elderly, the specific mechanism remains unknown. In order to develop better treatment regimens with better efficacy and fewer adverse reactions (ARs), it is vital to unravel the pathogenesis of osteoporosis in the elderly. The GEO chip was used to screen differential genes in senile osteoporosis and analyze their interaction mechanisms in order to obtain potential therapeutic pathways and targets.

MATERIALS AND METHODS: GSE35956 was downloaded from GEO database and used as the research object for KEGG pathway enrichment analysis, GO enrichment analysis and protein-protein interaction (PPI) network analysis, respectively, to explore the related mechanisms of the occurrence and development of osteoporosis in the elderly.

RESULTS: There were 156 differentially expressed genes in the elderly (72 years old) and middle-aged (42 years old) diagnosed with osteoporosis, of which 6 were up-regulated and 150 were down-regulated. An analysis of gene enrichment using GO (gene body) revealed that differentially expressed genes (DEG) were mainly distributed in extracellular matrix (ECM) and other cell structures. Its functions include ossification, parathyroid hormone metabolism, multicellular biological signaling pathway, vitamin catabolism, interleukin-5 metabolism, transmembrane transporter activity, receptor signaling pathway, calcium metabolism and other molecular functions. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), an online resource, signaling pathways associated with age-related osteoporosis (OP) are significantly enriched. The DEG enrichment pathways include Wnt, ECM-receptor interaction, cGMP-PKG, GAG degradation, and calcium signaling. A protein and protein interaction (PPI) network was constructed for 14 key genes, including CD44, GRIA1, KNG1 and IL7R.

CONCLUSIONS: The findings of this study indicate that CD44, GRIA1, KNG1, IL7R, and other differential genes affect the Wnt signaling pathway in the elderly, which can provide new targets for the follow-up basic research and treatment of osteoporosis in the elderly.

Key Words: Senile osteoporosis, GEO database, Genetics, Bioinformatics, Osteoporosis occurrence and development.

Introduction

Osteoporosis (OP) is a group of age-related health problems, the number of OP in the whole world is over 200 million, and the incidence of OP continues to increase with the growth of the number of elderly people1,2. There are about 30 percent of women postmenopausal in Europe and the United States who suffer from this condition. Age 50 years and older will be more likely to suffer from osteoporosis and low bone mass by 2050, with an increase of 32%3. Furthermore, as the total number of fractures has increased, the cost of treatment for OP has also increased. OP is a major issue for our ageing population and the OP associated with fractures of vulnerability is a significant health and economic burden4,5. Globally, hip fractures are expected to quadruple to 6.3 million cases by 2050, as life expectancy increases exponentially6. Among osteoporosis’ physical consequences are spinal compression fractures, hip fractures, distal forearm fractures, and proximal humerus fractures7. By one count, one third of women and 20% of men over 50 years of age have had osteoporotic fractures8. OP is distinguished from primary OP and secondary OP. The former has both type 1 (postmenopausal OP) and type 2 (senile OP)9. These two types are caused by estrogen deficiency and aging (at 70 years old)10. Malnutrition and chronic diseases are the most common sec-
ondary causes of osteoporosis, followed by metabolic diseases, endocrine dysfunction, and drug side effects. Metastasis or hematologic malignancies are also possible secondary causes.

Osteoporosis and osteoporotic fractures are very common, and bisphosphonates are commonly used to treat them. OP formation and its development have been studied more extensively in recent years, as well as the molecular mechanisms by which it forms and progresses. However, although the depth of research on its mechanism of action has been improved, it still needs to continue to explore its specific mechanism of action.

As a result, the pathogenesis of OP must be understood in order to find new treatments that are more effective and have fewer adverse reactions.

This project carried out bioinformatics analysis of the GEP (gene expression profile) of middle-aged bone marrow samples and middle-aged OP in selected GEO chips to more deeply analyze the underlying mechanism of OP in the elderly at the molecular level, so as to provide a potential new target for the treatment of OP.

**Materials and Methods**

**Selection and Acquisition of Gene Expression Profile Data**

GEP microarray of aged OP bone marrow tissue screened from the database GEO with the number “GSE35956” on the GPL570 platform [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc, Santa Clara, CA, USA). Five elderly patients with osteoporosis and five normal bone marrow tissue samples were used in the study. For future studies, gene expression information was extracted from normal control bone marrow tissue and elderly OP.

**Extraction and Analysis of Differential Genes**

For each GEP value, R software (version 3.40.2, University of Auckland, New Zealand) and limma package were used for normalization analysis to eliminate errors caused by non-experimental factors. The differential gene (DEG) screening conditions were set as follows: the difference fold (i.e., “log Fold Change”) was more than 4 times, and the p-value was lower than 0.01, so as to obtain DEG in normal bone marrow tissues and aged OP bone marrow tissues which matches the conditions. The visual screening of DEGs and the production of volcano and heat maps were completed by the relevant functions of the R software.

**Functional Enrichment and Annotation Analysis of Differential Genes**

The Cluster Profiler package in R software was used to analyze the GO function of potential mRNA and enrich the KEGG pathway. With the help of R language software function, the condition was set as p-value below 0.01 to obtain the GO result of DEG function annotation. The components of the results include molecular function (MF), cellular component (CC), biological pathway (BP). KEGG signal pathway analysis was carried out on the key DEGs obtained from the above screening, and then the analysis results were downloaded and exported. Then the GPLOT2 package contained in R software was used to complete the screening of the top 20 pathways according to the condition (p<0.01).

**Construct Protein-Protein Interaction Diagram among Differential Genes**

The interactions between DEGs were analyzed using the website STRING (available at: https://cn.string-db.org/), and a PPI network was generated online. The logarithms of the relationships between the different proteins in this network were counted, and the top 14 proteins were extracted based on the number of relationship logarithms to effectively screen the key DEGs related to ageing OP.

**Statistical Analysis**

All data in this paper were derived from GEO database and were statistically analyzed by R software (version 3.40.2) based on the limma package, ClusterProfiler package and the website STRING. Compared with normal bone marrow tissues, genes with up-regulated expression and down-regulated expression in senile osteoporosis bone marrow tissues were screened, and the difference was statistically significant (p < 0.01). And screened out the difference pathway, the difference was statistically significant (p < 0.05).

**Results**

**Screening of Significant Differentially Expressed Genes Between Osteoporotic Bone Marrow Tissues and Normal Bone Marrow Tissues in Middle-Aged and Elderly Patients**

With the help of limma and impute package contained in R software, the screening conditions were set as follows: log fold change above 4 and p-value below 0.01, 156 differential genes were
Screening and bioinformatics analysis of senile osteoporosis genes based on GEO database

obtained. Compared with normal bone marrow tissue, 6 genes were up-regulated and 150 genes were down-regulated in elderly osteoporotic bone marrow tissue (Table I). The selected differential genes were mapped to volcanoes and a heat map was made (Figure 1A-C).

Functional Enrichment and Annotation Analysis of Differential Genes

KEGG signaling pathway was studied by online analysis tool DADiD6.8 for key differential genes identified above, and obtained 20 KEGG signaling items. The top 18 most significant items with \( p < 0.05 \) were selected by R language and displayed in bubble diagram (Figure 2A), including Wnt signaling pathway, cGMP−PKG signaling pathway, calcium signaling pathway, Glycosaminoglycan degradation pathway, ECM− receptor interaction pathway, etc.

The first 26 entries of BP, CC and MF were presented in a bar chart with the help of R language for GO function annotation analysis (see Figure 2B). MF, BP and CC are related to ossification, parathyroid hormone metabolism, multilcellular biological signaling pathway, vitamin catabolism, interleukin-5 metabolism, transmembrane transporter activity, receptor signaling pathway, calcium metabolism and other molecular functions on BP. Catabolism of extracellular matrix occurs on CC. This pathway is related to transmembrane transporter activity, ion metabolism, retinoic acid metabolism, and receptor signaling pathways on MF.

Analysis of Protein Interaction Networks

The PPI network was constructed with the help of the website STRING, and the differential genes were imported into the online analysis website; the interaction score was set to be lower than 0.4, and the isolated nodes were eliminated to obtain the protein interaction network diagram, which was composed of 140 nodes and 127 protein interaction pairs (Figure 3A). Based on the logarithm of action of each protein in solution, the first 14 key genes were identified, including IL7R, CD44, KNG1 and GRIA1 (Figure 3B).

Discussion

Based on its etiology, OP can be classified into primary and secondary categories. The former mainly involves postmenopausal OP, senile OP and adolescent OP. The cause of the latter is mainly the long-term overapplication of drugs, such as the long-term application of glucocorticoids (GC)\(^2\). A high percentage of the elderly population suffers from osteoporosis, and the number of osteoporosis patients increases with age\(^3\). OP causes more than 8.9 million fractures worldwide every year, with Europe having the highest rate (34.8%). Osteoporotic fracture is the most com-

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<th>Senile osteoporosis vs. normal bone marrow tissue</th>
<th>Gene</th>
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<td>Upregulated (6 genes)</td>
<td>TEX26, KRT80, PKIB, CARD6, CNTN3, EMBOR2F2, STRC, CACNG8, BPIFB6, CKM, TTC16, MAGEB3, PPEF1, CD74, SF2B, MAB21L2, FOXE1, IGFB21, RAX, SMIM22, TGM4, TME5L9, LGY2, CFP, RNF208, NAPSI1, CT38, CTRB2, NPPC, FAM17E1, PERM1, BORCS8-MEF2B, MAPK4, FABP4, HMX1, PAQ9R, ASIC4, SOAT2, RNF148, CREG2, COL10A1, CTSW, SLC16A10, LCN1, CYP26A1, CLEC14A, FGF23, SMIM6, DOCK3, PRA1, TMEM238, LRR258, CACNG7, TCF24, PCDH8, IBSP, C9ORF131, ULBP3, BTBD16, NEUROG2, RDR1, SPATA19, ATCAY, FBXO17, LHX6, EGR2, DRYK1B, SPIB, FXD4, C11orf53, CT47a11, CDR1, CSN1S1, ODF3L1, FMR1B8, SRRM5, SAX01, ERVFRD-1, ITIH3, NPTX2, RIPFLY2, BRSK1, HRC, MOVI1L1, DYDC10, RENBP, AUBL4, WPB2NL1, EPF1A6, SCP3, CDK18, SOX8, KLB1, SHOX, CSF7, NPBW6R2, SCN3B, ZFPI4, CRLF2, PIPT, TTPA, PCDHGC5, MYBPH, ZNF208, SLC9C2, RARRES2, PHOSPH01, IFNL1, ZG6, CPN2, CAMK2A, SORCS3, IQCIF6, LRR259, CTXN1, CALM16, BBOX1, GPR84, ZDHHC23, FAT2, NME8, IZUMO1, PDCL2, DMBX1, CCK, PEN3, ZBTB32, PRAC2, XIRP2, PRAE, CA14, CLDNL8, SOST, NOXI1, SI100A5, LRR253, CPY1, CERS4, NLRP4, HPSE2, FZD10, INSL3, NECTIN4, LYLI, ELANE, PDE6H, MAGEC3, FN3K, CAPSL, SYOPL, HTN3, SLC17A8, TSSK1B, ASB11, CCDC3</td>
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Table I. The differential genes of bone marrow with osteoporosis and normal bone marrow in the geriatric patients were screened by using the limma package in R language.
mon clinical complication of OP\(^4\). The incidence of OVFVs (osteoporotic vertebral compression fractures) is higher in the elderly. By 2025, more than 3 million osteoporotic fractures will occur in the United States, and vertebral compression fractures will account for a quarter of these osteoporotic fractures\(^5\). Osteoporotic fractures are currently treated primarily with surgery and anti-osteoporosis drugs, according to Chinese and American clinicians\(^6\), but the related adverse reactions still cannot be ignored\(^7,8\). There are some risks associated with drugs, including gastrointestinal adverse reactions, vertebral height loss, kyphosis, secondary fractures, secondary vertebral fractures, nerve compression, and revision surgery\(^9,10\). In recent years, the number of studies on senile OP has been increasing, but the understanding of the molecular mechanism of this disease is still very limited. Analysis at the molecular level plays a positive role in understanding the pathogenesis of the disease, and may actively adjust the overall treatment of the disease and improve the prognosis by identifying new biomarkers\(^11\). Therefore, there is an urgent need to better understand the pathogenesis of osteoporosis in the elderly to identify novel biomarkers and develop new therapeutic strategies for the prevention and treatment of osteoporosis in the elderly.

This study screened 156 DEGs by analyzing GEP chips in 5 normal bone marrow tissue samples and 5 elderly OP bone marrow tissue samples. Differential gene GO functional annotation analysis showed that it was related to ossification, parathyroid hormone metabolism, multicellular biological signaling pathway, vitamin catabolism, interleukin-5 metabolism, transmembrane

Figure 1. Processing results of chip data. A, After normalization, the median of each expression spectrum chip is at the same level, eliminating the error of other interference experiments. B, The volcano diagram shows the differentially expressed genes in the chip. Compared with the normal bone marrow tissue, the red dots represent the genes with high expression of senile osteoporosis, and the blue dots represent the genes with low expression of senile osteoporosis. C, Clustering heat map shows the genes with the most significant differences. Red represents high expression signal, blue represents low expression signal, G1 represents the elderly osteoporosis group, G2 represents the normal group.
transporter activity, receptor signaling pathway, calcium metabolism, and other molecular functions in BP. A catabolic process associated with extracellular matrix is involved in CC. In MF, it is related to transmembrane transporter activity, ion metabolism, retinoic acid regulation, and receptor signaling pathway. DEG is mainly involved in ossification, metabolism, growth, and other regulatory functions, and such functions are key physiological functions of normal bone marrow tissue. Therefore, in the elderly OP, such functions appear abnormal to a certain extent, which leads to the occurrence of related diseases. In addition, the degradation of metabolic function and bone structure in the elderly easily leads to a series of co-existing diseases22,23. The results of GO analysis can be used to interpret the biological processes associated with osteoporosis in the elderly, such as the prevalence of impaired metabolism of fat-soluble vitamins24, parathyroid hormone and bone in elderly patients with osteoporosis25, which is consistent with the results of GO analysis and enables assessment of the relationship and criticality of fat-soluble vitamins (Vit) and parathyroid hormone (PTH) in relation to changes in bone metabolism.

KEGG pathway is mainly enriched in the following signaling pathways: ECM–receptor interaction pathway, Wnt signaling pathway, GAGs degradation pathway, calcium signaling pathway, cGMP–PKG signaling pathway, etc. Recent studies19,20,25 have demonstrated that canonical Wnt/β-catenin signaling plays an important role in the progression of osteoporosis. Because of the Wnt/β and beta, Beta-catenin signaling plays an important role in the development and tissue homeostasis. Mutations in the Wnt component of the signaling pathway are at risk of triggering severe disease, including cancer, related genetic diseases, diabetes mellitus (DM) and OP22. Isoporsalen can improve oxidative stress-induced injury through Wnt/β-catenin signaling pathway, suggesting that psoralen may be a new treatment for osteoporosis26. Alterations in components of the Wnt pathway can lead to skeletal abnormalities such as OP pseudogliomas associated with mutations in the Wnt co-receptor LRP5 (low density lipoprotein receptor-related protein 5)27. Low levels of VitD and calcium in patients may increase the incidence of OP28.

The first 14 key DEGs were screened based on protein interaction. Among these genes, CD44, as an SC (stem cell) marker, has been confirmed by GAO, etc29, that BMSCAM/P6 and BMSCBALB/C have the typical MSCs (mesenchymal stromal cell) phenotype (CD44+, 73+, 90+), CD45 was not expressed. BMSCSAM/P6 can be used as a suitable model for the study of age-dependent

**Figure 2.** Functional analysis of differential genes. **A**, Enrichment analysis results of KEGG pathway of different genes. **B**, GO enrichment analysis results of differential genes.
osteoporosis in vitro due to the progenitor characteristics of metabolic disorders. The remaining genes are not present today in studies related to OP in old age. These key genes may play a key regulatory role in the formation and progression of OP in the elderly and have the potential to be a new target for the treatment of OP in the elderly.

In this study, with the help of GEO expression profile data, we completed the screening of DEG in the aged OP and normal bone marrow tissues and explored the possible molecular mechanisms through enrichment analysis. At the same time, we identified key differential genes through the PPI network. At present, the molecular mechanism analysis of OP in the elderly is extremely insufficient globally30,31, and the key DEG obtained in this study is rarely mentioned. It is believed that the findings of this study can provide some help for the subsequent analysis of the biological process of the elderly OP, provide reference subjects for the exploration of new molecular markers, and provide new potential targets for the treatment of elderly OP.

Conclusions

The findings of this study indicate that CD44, GRIA1, KNG1, IL7R, and other differential genes affect the Wnt signaling pathway in the elderly, which can provide new targets for the follow-up basic research and treatment of osteoporosis in the elderly.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Ethics Approval
Not applicable.

Authors’ Contributions
Authors Leilei Wu and Jiaxuan Zhou have made the same contribution to this paper, which includes concept proposing, data management, formal analysis, survey development, research method development or design, management and coordination of research project planning and execution, and resource provision. Both of them supervised and led the whole research activity planning and execution. The other authors played a coordinating role in the above aspects.

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References


