Liuwei Dihuang pill cures postmenopausal osteoporosis with kidney-Yin deficiency

Potential therapeutic targets identified based on gene expression profiling
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Abstract
This study aimed to investigate the potential therapeutic targets of Liuwei Dihuang pill (LDP) in the treatment of postmenopausal osteoporosis with kidney-Yin deficiency (PMO-KY).

Gene expression data were downloaded from the GEO database, including 4 PMO-KY samples and 3 healthy postmenopausal controls from GSE56116, as well as 3 PMO-KY samples before LDP treatment and 3 PMO-KY samples after three months of LDP treatment from GSE57273. Limma package was used to identify differentially expressed genes (DEGs). Afterwards, the potential target genes of LDP (namely key DEGs) were identified according to the comparison of DEGs in PMO-KY group and the DEGs in LDP treatment groups. Subsequently, iRegulon plugin in Cytoscape software was used to predict potential transcription factors (TFs) that regulated the key DEGs, and Comparative Toxicogenomics Database was utilized to identify known PMO-related genes among the key DEGs.

Totally, 202 and 2066 DEGs were identified between PMO-KY and controls, as well as after-treatment and before-treatment groups, respectively. Among them, 52 DEGs were up-regulated in PMO-KY but down-regulated after LDP treatment, and 8 TFs were predicted to these DEGs. Furthermore, 34 DEGs were down-regulated in PMO-KY but up-regulated after treatment, and 7 TFs were predicted to regulate these DEGs. Additionally, 43 of the 86 key DEGs were known PMO-related genes.

NCOA3, TCF4, DUSP6, PEL12, and STX7 were predicted to be regulated by HOXA13. In the PMO-KY treatment, NCOA3, TCF4, DUSP6, PEL12, and STX7 might be the potential therapeutic targets of LDP. However, further investigation is required to confirm these genes.

Abbreviations: AU = approximately unbiased, BMD = bone mineral density, BP = bootstrap probability, CTD = Comparative Toxicogenomics Database, DEGs = differentially expressed genes, Dusp6 = dual specificity phosphatase 6, hMSC = human mesenchymal stem cells, LDP = Liuwei Dihuang pill, PMO = postmenopausal osteoporosis, PMO-KY = PMO with kidney-Yin deficiency, TFs = transcription factors.

Keywords: differentially expressed gene, kidney-Yin deficiency, Liuwei Dihuang pill, postmenopausal osteoporosis

1. Introduction
Postmenopausal osteoporosis (PMO) is characterized by deterioration of bone microarchitecture and chronic decrease of bone mass, and it generally leads to osteoporotic fracture, a major cause of disability and mortality in elderly women. Approximately 40% of postmenopausal women suffer from PMO, and patients with PMO may steadily increase in future due to population aging. PMO is a chronic and progressive process that involves osteocytes, lining cells, osteoclasts, osteoblasts, T lymphocytes, and so on. From the perspective of traditional Chinese medicine, kidney deficiency is considered as one of the factors causing PMO. According to the “Dialectical reference standards of traditional Chinese medicine deficiency syndrome,” patients with at least 3 symptoms of “aching pain in spinal column,” “limp in shin or pain in heel,” “tinnitus or epiphoria,” “hair loss or gomphiasis,” “dribble of urine or urinary incontinence” and “sexual dysfunction” are diagnosed with kidney deficiency. If the patients with kidney deficiency simultaneously have three of the symptoms “dysphoria in chestpalms-soles,” “mouth and throat dry,” “red tongue or little coating” and “pulse breakdown,” as well as one of the symptoms “hectic fever at afternoon,” “coprostasis or red-yellow urine” and “night sweat,” they will be diagnosed with kidney-Yin deficiency.

Currently, worldwide approved therapeutics for PMO are vitamin D, bisphosphonates (eg, alendronate, zoledronate, ibandronate, and risedronate), selective estrogen-receptor modulators, parathyroid hormone, anti-RANKL, calcitonin, parathyroid hormone (PTH1-34), teriparatide (PTH1-34), denosumab, menatetrenone, alfalcaldiol, and strontium ranelate. However, drug intolerance, adverse effect, or price of
these drugs limit their application and effectivity.\cite{9} Liwei Dihuang pill (LDP), a traditional Chinese medicine formula with a history of thousands of years, is an effective but lower-cost therapeutic for PMO, especially the PMO with kidney-Yin deficiency (PMO-KY).\cite{10,11,12} After 12 weeks of treatment with LDP, serum levels of osteocalcin and alkaline phosphatase were decreased, and bone mineral density (BMD) of femurs and biomechanics of lumbar vertebrae were improved in a PMO rat model.\cite{10} With a 95% curative rate, LDP can increase BMD, inhibit bone absorption, and promote bone formation in clinic.\cite{9} As LDP is a complex drug that is composed of 6 Chinese crude herbs, studies have been conducted to investigate its therapeutic mechanisms. Xia et al.\cite{10} suggested that LDP might alleviate PMO via up-regulating β-catenin, Runx2, Lrp-5, and Oss that were involved in canonical Wnt/β-catenin signaling pathway in osteoblast. Based on microarray analysis, Xie et al identified that PRLR, INSR, ASB1, CLCF1, PROK2, GPR27, C3orf35, JUN, and JUNB were down-regulated in patients with PMO-KY compared with healthy postmenopausal women, while ASB1, CLCF1, JUN, and JUNB were significantly up-regulated after LDP treatment for 3 months.\cite{13,14} In addition, 25 genes were differentially expressed in monocytes in peripheral blood of PMO-KY patients after LDP treatment, including the genes associated with immune response (eg, PRL, PRLR, OSM, ISG15, and IL4R), cell growth (eg, F2 and SOCS3), JAK/STAT pathway (eg, PIAS1 and SOCS4), and SH3/SH2 adaptor (eg, SRC and STAM), as well as regulator genes that interact with STAT proteins (eg, JUN, JUNB, SMAD3, SMAD5, SMAD4, SP1, and YY1).\cite{13} However, these studies mainly focused on several genes and regulatory mechanism of gene expression like transcription factor (TF) has not been studied. Thus, the precise therapeutic mechanisms of LDP in treatment of PMO-KY have not been fully understood.

In the present study, comprehensive bioinformatics methods were utilized to re-analyze 2 microarray datasets uploaded to the Gene Expression Omnibus (GEO) by Xie et al.\cite{10} (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56116; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57273) As a consequence, potential target genes of LDP in the treatment of PMO-KY were identified, providing a novel direction for drug design in future.

2. Materials and methods

2.1. Microarray data

Gene expression data about PMO-KY were obtained from the GEO database, including datasets GSE56116 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56116) and GSE57273 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57273). The dataset GSE56116 contained 4 peripheral blood samples from PMO-KY patients, 3 samples from PMO patients with kidney Yang deficiency, 3 samples from PMO patients without kidney deficiency, and 3 healthy postmenopausal controls. Only the 4 PMO-KY samples (PMO-KY group) and 3 healthy postmenopausal controls (control group) were utilized for this analysis as our study focused on only PMO-KY. Their average age were 61.15 ± 4.381 years old, GSE57273 contained 6 peripheral blood samples from 3 PMO-KY patients before LDP treatment (n = 3, before-treatment group) and after 3 months of LDP treatment (n = 3, after-treatment group). Both GSE56116 and GSE57273 data were produced by the platform of Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Feature Number version). They were approved by ethics committee Fujian Academy of Traditional Chinese Medicine.

2.2. Data preprocessing

As GSE56116 and GSE57273 included only Agilent chips with single-channel, the Agilent one-color method in limma package (version 3.26.3, http://www.bioconductor.org/packages/3.0/bioc/html/limma.html)\cite{15} was utilized to preprocess microarray data, including background correction, normalization between arrays, condensation of microarray data, transformation of probes into gene symbol, and expression calculation.

2.3. Identification of DEGs

Unpaired t-test method in limma package\cite{15} was utilized to obtain the DEGs between PMO-KY and control groups, as well as after-treatment and before-treatment groups. Only DEGs with \(|\log_2\) fold change\(| \geq 0.5 \) and \(P < .05\) were considered as significant DEGs, and they were clustered using gplots package (version 2.4)\cite{16} based on their expression levels.

2.4. Prediction of potential target genes of LDP

Based on the DEGs between PMO-KY and control groups, as well as DEGs between after-treatment and before-treatment groups, the key DEGs with opposite change patterns were identified using online software Venn-Diagram-online (http://www.bioinformatics.lu/venn.php#userconsent#). Expression change patterns of these DEGs between PMO-KY and control groups were different from those between after-treatment and before-treatment groups, indicating that they might be the target genes of LDP in the treatment of PMO-KY. In order to verify the accuracy of these key DEGs, Pvclust package (version 1.2-1, https://cran.r-project.org/)\cite{17} was utilized to cluster these DEGs. This package provides approximately unbiased (AU) P value, as well as bootstrap probability (BP) value. AU P value is calculated using multi-scale bootstrap resampling, whereas BP value is calculated using normal bootstrap resampling. Results based on AU P-value are more accurate than those based on BP value. In this study, Pvclust-clusters with AU P value > 95% were considered as significant clusters.

2.5. Functional analysis of key DEGs based on literature mining

For the key DEGs identified above, literature mining was performed using “Gene Cluster With Literature Profiles” module in the online software GenClaP 2.0 (http://iic.nmu.edu.cn/)\cite{18} to investigate their bio-functions. The corresponding criteria were \( Hir \geq 3 \) and \( P \) value < .05. “Gene Cluster With Literature Profiles” module can use a fuzzy cluster algorithm to generate statistically over-represented keywords and thus to annotate the input genes, and Medline abstracts are utilized to link genes and keywords.

2.6. Construction of TF-DEG regulatory network

iRegulon uses a genome-wide ranking-and-recovery approach to enrich TFs and their optimal targets based on TRANSFAC, ENCODE, HOMER, JASPAR, and SwissRegulon databases, and it is generally utilized to construct transcriptional regulatory network. Therefore, iRegulon plugin\cite{19} in Cytoscape (version
2.8; http://cytoscape.org[230] was applied to predict the potential TFs targeting the key DEGs identified above. The criteria for this analysis were false discovery rate on motif similarity ≤ 0.001, identity between orthologous genes ≥ 0.05, and normalized enrichment score (NES) > 4.

2.7. Known PMO-related genes

The Comparative Toxicogenomics Database (CTD; http://ctdbase.org/)[21] records various disease-related genes based on precise document processing and drug-target prediction. To examine whether the identified key genes were known PMO-related genes, CTD database was used to search genes related to “postmenopausal osteoporosis” at November 18, 2015.

3. Results

3.1. Identified DEGs

After data preprocessing, an expression matrix containing 12132 genes was generated from both GSE56116 and GSE57273. A total of 202 DEGs were identified between PMO-KY and control groups, including 101 up- and 101 down-regulated DEGs. Furthermore, 2066 DEGs were identified between After-treatment and Before-treatment groups, including 1157 up- and 909 down-regulated DEGs. After clustering analysis, the 202 DEGs clearly classified the PMO-KY and control samples (Fig. 1A), whereas the 2066 DEGs clearly distinguished the After-treatment and Before-treatment samples (Fig. 1B).

Figure 1. DEGs and key DEGs. A, Heatmaps of the 202 DEGs between PMO-KY and control groups. B, Heat maps of the 2066 DEGs between after-treatment and before-treatment groups. C, Clustering of the 86 key DEGs in PMO-KY and control samples. D, Clustering of the 86 key DEGs in after-treatment and before-treatment samples. Red letters on the edge stand for AU P-value (%), and green letters on the edge represent BP value (%). AU P-value > 95% are highlighted by red rectangles. AU P-value = approximately unbiased P-value, BP value = bootstrap probability value, DEGs = differentially expressed genes, LDP = Liuwei Dihuang pill, PMO-KY = postmenopausal osteoporosis with kidney-Yin deficiency.
3.2. Potential target genes of LDP

After comparing the 202 DEGs with the 2066 DEGs, a total of 86 genes were identified as key DEGs. Among these genes, 52 DEGs were up-regulated in PMO-KY but down-regulated after LDP treatment, and 34 DEGs were down-regulated in PMO-KY but up-regulated after LDP treatment. These DEGs might be the target genes of LDP. After clustering analysis, PMO-KY and control samples (Fig. 1C), as well as after-treatment and before-treatment samples (Fig. 1D), were clearly distinguished based on the 86 key DEGs, indicating the specificity of these DEGs. The total key DEGs are shown in Supplementary Table 1, http://links.lww.com/MD/C360.

3.3. Potential functions of the key DEGs

Based on literature mining, a total of 22 functions were enriched by the 52 key DEGs that were up-regulated in PMO-KY but down-regulated after LDP treatment (Fig. 2A and Table 1). These functions were mainly related to immune response and cell death. Furthermore, 14 functions were enriched by the 34 key DEGs that were down-regulated in PMO-KY but up-regulated after LDP treatment (Fig. 2B and Table 1). These functions were mainly associated with cell growth, apoptosis, and immune response.

3.4. TF-DEG regulatory networks

Based on TRANSFAC, ENCODE, HOMER, JASPAR, and SwissRegulon databases, 8 TFs were predicted to regulate 38 of the 52 key DEGs (Fig. 3A), and 7 TFs were predicted to target 27 of the 34 key DEGs (Fig. 3B). Expression levels of the TFs identified here were not significantly changed in PMO-KY or during the LDP treatment. In TF-DEG regulatory network of the 52 key DEGs, 7 DEGs (eg, NCOA3, TCF4, DUSP6, PELI2, and STX7) were predicted to be regulated by at least 5 TFs (Fig. 3A). Among them, all of NCOA3, TCF4, DUSP6, PELI2, and STX7 were predicted to be regulated by HOXA13 (target number = 11). Additionally, in TF-DEG regulatory network of the 34 key DEGs, 2 DEGs were targeted by at least 5 TFs (Fig. 3B).

3.5. Known PMO-related genes

Based on CTD database, 43 of the 86 key DEGs had been previously found to be associated with PMO (Table 2). Among the 43 known PMO-related genes, 37 genes were present in TF-DEG regulatory networks, such as NCOA3, TCF4, and DUSP6. In addition, PELI2 and STX7 were not known PMO-related genes, indicating that they were novel genes identified in this study.

4. Discussion

As a worldwide health problem associated with population ageing, PMO seriously impairs the quality of life and causes disability and mortality in postmenopausal women.[2] LDP, a traditional Chinese medicine, can effectively cure PMO and PMO-KY.[9-12] However, its therapeutic mechanisms underlying the treatment of PMO-KY have not been fully understood. Based on bioinformatics approaches, we re-analyzed the microarray data about PMO-KY and LDP treatment. As a result, 86
potential target genes of LDP in the treatment of PMO-KY were identified. Specially, NCOA3, TCF4, and DUSP6 were known PMO-related genes, whereas PEL12 and STX7 were newly-identified genes that might be related to PMO-KY. Additionally, NCOA3, TCF4, DUSP6, PEL12, and STX7 were predicted to be targeted by HOXA13.

NCOA3, namely, SRC3 or AIB1, encodes nuclear receptor co-activator 3 that is important in bone and mineral metabolism. NCOA3 interacts with osteoporosis-related nuclear hormone receptors (eg, vitamin D and thyroid hormone receptors), and it is associated with lumbar spine BMD. Furthermore, NCOA3 is located within chromosomal genomic regions for femoral neck cross-sectional geometric variables that are risk factors of osteoporosis. Furthermore, NCOA3 has also been reported to be located within quantitative trait locus of BMD in the mouse genome. These studies suggested that NCOA3 was associated with low BMD. In this study, NCOA3 was upregulated in PMO-KY samples, and became downregulated after LDP treatment, indicating that NCOA3 might be a potential target of LDP.

TCF4, also referred to as Tcf7L2, encodes transcription factor 4 (Tcf4), which is a nuclear effector of the Wnt signaling, which integrates with Notch signaling and BMP signaling pathways in osteoblast differentiation. In the present study, TCF4 was upregulated in PMO-KY samples, and became downregulated after LDP treatment. A similar previous study has found that TCF4 is up-regulated in human mesenchymal stem cells (hMSC) from osteoporotic donors in responding to 10% uniaxial cyclic tensile strain that is able to promote osteogenesis. Although there is no any other evidence that TCF4 is a...
responsive gene of LDP so far, we speculate that TCF4 may be a potential target of LDP in the treatment of PMO-KY based on the above results.

_DUSP6_ encodes dual specificity phosphatase 6 (Dusp6), the expression level of which in adipocytes that share the same cell lineage with osteoblasts is regulated by a local expression quantitative trait locus and correlates with BMD in mice,\(^\text{[29]}\) indicating the association of _DUSP6_ with osteoporosis. A recent study has reported that the expression of one homolog of _DUSP6_, _DUSP12_, in the neuroendocrine immunomodulation network is changed by Liuwei Dihuang decoction.\(^\text{[30]}\) Therefore, we speculate that _DUSP6_ may be also a responsive gene of LDP.

In this study, _PELI2_ and _STX7_ were newly-identified genes that might be related to PMO-KY. _PELI2_ encodes pellino-2 protein, a member of Pellino protein family, proteins in which can catalyze the poly-ubiquitylation of interleukin-1 receptor-associated kinases in the Toll-like receptor signaling pathway, which plays key roles in cytokines production and innate immune system.\(^\text{[31]}\) The important role of immunity in PMO has been previously demonstrated by multiple studies.\(^\text{[32–34]}\) Furthermore, SNP rs398652 next to _PELI2_ is significantly related to leukocyte telomere length, and its variant alleles are associated with longer telomere length.\(^\text{[35]}\) Telomere shortening causes cell apoptosis in osteoblasts and hMSCs and thus contributes to bone aging.\(^\text{[36]}\) Defects in Wrn, a telomere maintenance molecule, inhibit osteoblast differentiation and thus promote osteoporosis.\(^\text{[36]}\) Collectively, _PELI2_ may be closely related to PMO. _STX7_ encodes syntaxin 7, a member of soluble-N-ethylmaleimide-
sensitive-factor accessory-protein receptors that participate in membrane fusion and cytokine release in almost all aspects of innate and adaptive immune responses.\textsuperscript{137,138} As mentioned above, immunity plays key roles in PMO, thus, \textit{STX7} might be associated with PMO. Currently, there is no evidence to support the direct linkage between \textit{PELI2}/\textit{STX7} and LDP, whereas, both \textit{PELI2} and \textit{STX7} were significantly up-regulated in PMO-KY compared with healthy controls, and downregulated after LDP treatment. Therefore, \textit{PELI2} and \textit{STX7} might be potential targets of LDP.

Strikingly, all of \textit{NCOA3}, \textit{TCF4}, \textit{DUSP6}, \textit{PELI2}, and \textit{STX7} were regulated by the TF \textit{HOXA13}, a member of homeobox proteins. SNPs in homeobox A cluster are robustly associated with cortical volumetric BMD at the femoral neck trabecular and lumbar spine.\textsuperscript{139} \textit{HOXA13} is located on 11q14-25, a well-replicated osteoporosis susceptibility loci, and it is a likely candidate osteoporosis susceptibility gene based on bioinformatics tools.\textsuperscript{40} Therefore, \textit{HOXA13} might play a role in PMO-KY pathogenesis and LDP treatment via regulating the expressions of \textit{NCOA3}, \textit{TCF4}, \textit{DUSP6}, \textit{PELI2} and \textit{STX7}.

Despite the aforementioned results, there were several limitations in this study. The predicted results should be confirmed by laboratory data. Furthermore, the included samples for analysis should be more. In our further studies, more PMO-KY samples will be collected to validate the expression levels of \textit{NCOA3}, \textit{TCF4}, \textit{DUSP6}, \textit{PELI2}, \textit{STX7}, and \textit{HOXA13}. In conclusion, the above discussed genes (eg, \textit{NCOA3}, \textit{TCF4}, \textit{DUSP6}, \textit{PELI2}, and \textit{STX7}) might be the therapeutic targets of LDP in the treatment of PMO-KY, and their expressions might be regulated by \textit{HOXA13}. The potential identified target genes

Table 2
The 43 known PMO-related genes among the 86 key DEGs.

| Known gene    | Known gene    | Known gene    | Known gene    |
|---------------|---------------|---------------|---------------|
| NCOA3         | NCOA3         | NCOA3         | NCOA3         |
| DUSP6         | DUSP6         | DUSP6         | DUSP6         |
| TCF4          | TCF4          | TCF4          | TCF4          |
| TACC1         | TACC1         | TACC1         | TACC1         |
| SPTLC2        | SPTLC2        | SPTLC2        | SPTLC2        |
| HIPK2         | HIPK2         | HIPK2         | HIPK2         |
| NBF1F10       | NBF1F10       | NBF1F10       | NBF1F10       |
| RAB11F1P1     | RAB11F1P1     | RAB11F1P1     | RAB11F1P1     |
| API1S2        | API1S2        | API1S2        | API1S2        |
| SAMHD1        | SAMHD1        | SAMHD1        | SAMHD1        |
| XAF1          | XAF1          | XAF1          | XAF1          |
| SYK           | SYK           | SYK           | SYK           |
| CRHR2         | CRHR2         | CRHR2         | CRHR2         |
| CD93          | CD93          | CD93          | CD93          |
| GLPR1         | GLPR1         | GLPR1         | GLPR1         |
| HIST1H3C      | HIST1H3C      | HIST1H3C      | HIST1H3C      |
| HIST1H3A      | HIST1H3A      | HIST1H3A      | HIST1H3A      |
| PLEKH2B       | PLEKH2B       | PLEKH2B       | PLEKH2B       |
| CHIC2         | CHIC2         | CHIC2         | CHIC2         |
| ABCB10        | ABCB10        | ABCB10        | ABCB10        |
| NIPSNAP3B     | NIPSNAP3B     | NIPSNAP3B     | NIPSNAP3B     |
| MTNP          | MTNP          | MTNP          | MTNP          |
| RAB11F1P2     | RAB11F1P2     | RAB11F1P2     | RAB11F1P2     |
| MBOAT1        | MBOAT1        | MBOAT1        | MBOAT1        |
| SRP72         | SRP72         | SRP72         | SRP72         |
| IGS6F         | IGS6F         | IGS6F         | IGS6F         |
| OAK4P         | OAK4P         | OAK4P         | OAK4P         |
| ARS2          | ARS2          | ARS2          | ARS2          |
| UBASH3A       | UBASH3A       | UBASH3A       | UBASH3A       |
| TREF1F1       | TREF1F1       | TREF1F1       | TREF1F1       |
| ERCC5         | ERCC5         | ERCC5         | ERCC5         |
| IL21R         | IL21R         | IL21R         | IL21R         |
| MAP3K10       | MAP3K10       | MAP3K10       | MAP3K10       |
| SOX13         | SOX13         | SOX13         | SOX13         |
| DMPK          | DMPK          | DMPK          | DMPK          |
| AK1           | AK1           | AK1           | AK1           |
| PEBP1         | PEBP1         | PEBP1         | PEBP1         |
| SLC37A4       | SLC37A4       | SLC37A4       | SLC37A4       |
| PRSS23        | PRSS23        | PRSS23        | PRSS23        |
| EBP           | EBP           | EBP           | EBP           |
| DUSP2         | DUSP2         | DUSP2         | DUSP2         |
| SPTAN1        | SPTAN1        | SPTAN1        | SPTAN1        |
| CACNA2D2      | CACNA2D2      | CACNA2D2      | CACNA2D2      |

Letters in bold represent the key DEGs in transcription factor regulatory network. DEGs = differentially expressed gene(s), FC = fold change, PMO = postmenopausal osteoporosis, PMO-KY = postmenopausal osteoporosis with kidney-Yin deficiency.
might provide a novel direction for future drug design. However, beyond the scope of this study, further investigation should be performed to fully evaluate the roles of these genes in PMO-KY and LDP treatment.

Author contributions

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