Bioinformatics analysis of Transcriptome genes in osteoarthritis rats treated with pilose antler

Zhi-Dong Lin

Guangzhou University of Chinese Medicine

Li-Lun Zhong

Guangzhou University of Chinese Medicine

He Lu

Guangzhou University of Chinese Medicine

Yao-Ming Lu

Guangdong Hospital of Traditional Chinese Medicine

Wei Niu (✉ crazytstones@139.com)

Guangdong Hospital of Traditional Chinese Medicine <https://orcid.org/0000-0001-7652-0481>

Research article

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Abstract

Background

Pilose antler (PA) is a common Chinese medicine used to treat patients with osteoarthritis (OA) while its treating mechanism is still unknown. The purpose of our study is to clarify this mechanism by studying the effect of pilose antler on transcriptome genes expression in rats with knee osteoarthritis.

Methods

Ten Sprague Dawley (SD) rats are randomly divided into two groups, the pilose antler group (PA group) and the osteoarthritis group (OA group). The classic Hulth method was performed on the right knee of all the rats to establish the osteoarthritis model. Six weeks after the surgery, the PA group was given pilose antler and the OA group was given saline. This intervention lasted 6 weeks. Two hours after the last administration, cartilage tissues of all the rats were taken from their right knees. The samples were collected and sent to BGI, the genomic organization, to carry on the RNA-Seq measurement. The comparison of differentially expressed genes (DEGs), enrichment analysis, genes classification, and miRNA-mRNA linked network were studied within Dr.Tom system.

Results

Several DEGs(3226 in comparison between OA and PA group) were identified ($|\log_2FC| \geq 1, Q \text{ value} < 0.001$). Compared with the OA group, 711 up-regulated genes and 2615 down-regulated genes were found in the PA group. According to the enrichment analysis, the up-regulated genes mainly enriched in muscle contraction and the down-regulated-genes are involved in inflammatory response. The miRNA-mRNA regulatory network was performed and the key genes were identified.

Conclusions

Our studies have found that PA may alleviate the progression of OA through strengthening the muscle and positively regulate the inflammatory response. We also found some key genes, like *Tnni2*, *Mylpf*, *Myl1*, *Actn3*, *Ifitm6*, and *Retnlg* . These genes may be the potential target of pilose antler in the treatment of OA.

Background

Osteoarthritis is one of the most common joint diseases among elder people. OA is caused by many factors, like age, female sex, obesity and joint trauma[1]. OA is manifested as degeneration of articular cartilage and pathological changes often involve articular cartilage, subchondral bone, synovium, and other tissues. The pathological changes of OA are considered as the changes of subchondral bone, synovium and other tissues, in which the articular cartilage is the most commonly involved[2, 3]. The current drug therapy of osteoarthritis is purely to control symptoms, but long-term administration of these

medications may be harmful to human health. Hence, many patients choose an alternative or complementary medicine—pilose antler to prevent and treat osteoarthritis.

Pilose antler(PA), also known as velvet antler, is a Chinese medicine which is the unossified horn cut from deer. PA is one of the most famous traditional Chinese medicine and has been considered to possess many advantages, including promotion the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells[4], improvement of inflammation and oxidative stress[5, 6], modulatory effect on the immune system[7], inhibition growth of cancer cell[8], the anti-fatigue effect[9, 10], and anti-osteoporosis effect[11]. Pilose antler has been widely used in the treatment of the patient with knee osteoarthritis and it was proved that pilose antler could delay the degeneration of articular cartilage[12]. Xiu et al. have proved that pilose antler can inhibit chondrocytes apoptosis, decrease the levels of interleukin-1 β and tumor necrosis factor- α to delay the degeneration of articular cartilage in New Zealand white rabbits[13]. The study of Chun-Xia Zhang et al are consistent with the work of Xiu et al and proved that PA could inhibit reduction of the glycosaminoglycan and type II collagen in cartilage matrix to delay the degeneration of articular cartilage[12]. Our previous study has proved that the pilose antler could repair cartilages by regulating the expressions of Smad2 and Smad3 protein[14].

Although many studies have confirmed that PA could slow the progression of OA, the mechanism is still not clear and the molecule mechanism on genetic level is seldom studied by researchers. The bioinformatics analysis, including gene classification and gene enrichment, may be a powerful way to explore these complicated regulatory networks and molecule mechanisms.

In the current study, we aim to clarify the effect of pilose antler on transcriptome genes expression in rats with knee osteoarthritis and identify the DEGs between PA group and OA group to find out the molecule mechanism underlying it. These genes may act as the potential target of Pilose antler when treating OA.

Materials And Methods

Animals and Materials

Ten SD female rats(8 weeks, 180–200 g)were purchased from Guangzhou University of Chinese medicine laboratory animal center. All the rats were cage-reared in an SPF-level animal room at laboratory animal center in Guangzhou University of Chinese medicine laboratory. Animals were kept in an environment with 12 h-12 h light: dark cycle, temperature 22–24°C,and relative humidity 45–55%. All animals were fed daily and had free access to water. The pilose antler and saline were purchased from Guangdong provincial hospital of Chinese medicine. The pilose antler was ground to powder with the specific grinder. The pilose antler was dissolved in the saline to make a concentration of 420 mg·kg⁻¹. This concentration has been proved to be with the best treatment effect of rats with OA in our previous study[14].

Experimental design

This experiment was approved by the institutional animal care committee of Guangzhou University of Chinese medicine. The animals were randomly divided into two groups, the osteoarthritis group (OA group) and the pilose antler group (PA group). The right knees of all the rats were performed the classic Hulth method[15] to establish the model of OA, which through anterior cruciate ligament transection and partial medial meniscectomy. Six weeks after the surgery, the PA group was given pilose antler through oral administration. The OA group was given the same dosage of saline. The administration was given every other day and lasted 6 weeks. Two hours after the last administration, cartilage tissue of all the rats were taken and sent to BGI, the genomic organization, to perform the RNA-Seq measurement.

Enrichment analysis and gene classification for DEGs

Enrichment analyses and gene classification for DEGs ($|\log_2FC| \geq 1$, $Q \text{ value} \leq 0.001$) were carried on within Dr.TOM system(<http://biosys.bgi.com/#/report/login>), a powerful web-based tool, which involve in many processes, like gene enrichment, gene classification, gene heatmap and gene linked network. The available terms for enrichment include Kyoto Encyclopedia of Genes and Genomes (KEGG): KEGG pathway, KEGG disease, KEGG module, KEGG reaction, Gene Ontology (GO): GO component, GO molecular function, GO biological process. We perform the GO biological process gene enrichment analyses and GO component process function classification for DEGs in the current study.

Integration of miRNA-mRNA network and module analysis

We further analysis DEGs with cut-off criterion (gene expression $\geq 3, \log_2(A/B) \geq 3$) and get the top 10 gene. The heatmap of these genes was made to show the expression of each gene. To further identify the key genes, we performed the miRNA-mRNA network within Dr.TOM system and predicted the hub genes in the up-regulated group and the down-regulated group.

Results

Analysis of DEGs

several DEGs(3226 in comparison between the OA and the PA group) were identified ($|\log_2FC| \geq 1, Q \text{ value} < 0.001$) and showed in Fig.1.

Compared with the OA group, 711 up-regulated genes and 2615 down-regulated genes were found in the PA group($|\log_2FC| \geq 1, Q \text{ value} < 0.001$). We further perform the enrichment analysis for these DEGs and found that the up-regulated genes mainly enrich in muscle contraction (Fig.2), while the down-regulated genes mainly enrich in inflammatory response (Fig. 3).

Furthermore, we also perform the gene classification. Both the up-regulated genes and down-regulated genes are mainly involved in cellular processes, biological regulation and regulation of biological processes (Fig .4 and Fig. 5).

The expression heatmap of top ten genes

The top ten genes were selected from the DEGs (gene expression $\geq 3 \log_2 (A/B)$). According to the expression heatmap of the up-regulated genes (fig.6) and the down-regulated genes (fig.7), we found that the *Tnni2*, *Mulpf*, *Myl1*, and *Myh4* genes express highly among up-regulated genes. Furthermore, compared with the OA group, we found that the *Ifitm6*, *LOC257642*, *LOC1000360087* genes express less among down-regulated genes. These genes may act as the key genes in treating rats with OA.

miRNA-mRNA regulatory network

We further performed the miRNA-mRNA regulatory network with these genes. The miRNA-mRNA linked network of up-regulated genes was shown in Fig.8 and the down-regulated genes' were shown in Fig.9. According to the network, we predicted the hub genes may be *Tnni2* 51 nodes, *Mypf* 40 nodes, *Myl1* 57 nodes, *Myh4* 95 nodes, *Actn3* 74 nodes in up-regulated network. The hub genes in down-regulated network may be *Ifitm6* 62 nodes, *Retnlg* 38 nodes, *LOC257642* 91 nodes, *LOC100360573* 30 nodes, *LOC100360087* 51 nodes.

Discussion

Quantitative analysis of gene expression is crucial for understanding the molecular mechanisms underlying genome regulation. RNA-seq is a powerful platform for comprehensive investigation of the transcriptome[16]. In our study, we identified several DEGs between the OA group and PA group and perform the gene classification, gene enrichment and miRNA-mRNA linked network. Kyoto Encyclopedia of Genes and Genomes (KEGG) [17] and Gene ontology (GO)[18] were introduced as two effective bioinformatics tools to analyze DEGs.

Muscle weakness has been identified as a potential risk factor for OA development due to increased joint loading[19, 20]. It's reported that Deficits in muscle function, including muscle strength, activation, and proprioception, are found in people with knee osteoarthritis[19]. Further studies also indicated that there are positive associations between increased muscle strength and walking self-efficacy[21], reduced pain[22] and improved symptoms [23]. In our current study, we found that the up-regulated genes mainly enrich in muscle contraction which indicated that up-regulated genes are mainly involved in muscle contraction and may have the potential effect to strengthen muscle. Pilose antler was found to have the ability to strengthen muscle and muscular endurance, which was further verified by many previous studies[24, 25]. The results suggest that pilose antler may improve the symptoms of OA through strengthening the muscle.

OA is a chronic degenerative joint disease characterized by cartilage degradation, subchondral bone sclerosis, osteophyte formation and inflammation[26-28]. The exist of inflammation not only stimulates the production of enzymes that degrade the cartilage matrix but also inhibits repair of cartilage tissue[29]. Therefore, inhibit inflammation may be an effective way to treat OA. In our study, we found that the down-regulated genes mainly enrich in inflammatory response, which suggests that these genes may positively regulate the inflammatory response. Besides, several studies have proved that PA could ameliorate inflammation[5, 30-32]. Yu et al. revealed that PA could antagonize LPS-mediated inflammation and

oxidative stress, which was possible through mediating MAPK/NF- κ B pathway[32]. These findings are consistent with the results in our study and further prove that PA may delay the progression of OA by inhibiting inflammation response.

Furthermore, we also perform the gene classification of DEGs. Both the up-regulated genes and down-regulated genes are mainly involved in cellular processes, biological regulation and regulation of biological processes. To clarify the key hub genes, we also perform the gene heatmap to show the exact expression of the top ten genes. We found that *Tnni2*, *Mylpf*, *Myl1*, and *Actn3* genes express more in up-regulated genes, while *Ifitm6*, *Retnlg*, *LOC257642*, *LOC100360087* genes express less among down-regulated genes. To prove our hypothesis, we further perform the miRNA-mRNA linked network. According to the miRNA-mRNA linked network of up-regulated genes are mainly associated with muscle contraction and development. These genes were identified in previous studies, including *Tnni2*, *Mylpf*, *Myl1*, and *Actn3*. *Tnni2* has been identified in vertebrates as the fast skeletal muscle gene[33] and has been proved that it would prevent muscle contraction through binding to action and tropomyosin in the absence of Ca^{2+} [34, 35]. Besides, Zhu et al. revealed that mutation of *Tnni2* would result in impairment of angiogenesis, delay in endochondral ossification, and decrease in chondrocyte differentiation and osteoblast proliferation, indicating a vital role of *Tnni2* in the muscle development[36]. It is demonstrated that *Mylpf* is critically important for fast and slow skeletal muscle development[37]. It's also reported that *Mylpf* and *Myl1* play vital roles in the development of skeletal muscle[38]. *Tnni2*, *Mylpf*, and *Myl1* mainly enrich in muscle system process[39]. *Actn3* were confirmed that it could regular muscle metabolism[40]. The absence of α -actinin-3, which encoded with *Actn3*, has been associated with loss of muscle power[40] and strength[41] reduction of bone and muscle mass[42, 43]. These studies are consistent with the gene enrichment results of our studies that the DEGs mainly enrich in muscle contraction.

In our study, we also found that *Ifitm6*, *Retnlg*, *LOC257642*, *LOC100360087* genes express less among down-regulated genes. It's reported that *Ifitm6* was highest in the bone marrow of normal mice and its expression may be involved in macrophage functions[44], which implies that *Ifitm6* may be involved in inflammation response. Besides, it has been proved that *Retnlg*, identified as a novel member of the resistin-like molecule, was found in inflammatory zone (RELM/FIZZ) family in mice and rats[45, 46]. Furthermore, *Retnlg* is also a secreted molecule with a restricted expression pattern that may play a role in promyelocytic differentiation[46]. *LOC257642* and *LOC100360087* are mainly involved in the structural constituent of the ribosome. These findings are consistent with the results of gene enrichment, indicating that PA may alleviate the progression of OA through positive regulate the inflammatory response.

The OA rat model induced by the classic Hulth method[15] is one of the most common animal models in vivo. Therefore, it's convincing to use this model to unveil the potential molecular mechanisms of the treatment of PA. Besides, RNA-seq is a powerful platform for comprehensive investigation of the transcriptome. RNA-seq analysis based on next-generation sequencing data has recently become the defacto standard for the analysis of gene expression at the level of the whole transcriptome[16].

Of note, there are some limitations in our study. Firstly, the sample size of our study is limit (five in each group) and it's hard to exclude potential random error and false positive. Besides, our results are mainly based on bioinformatics analysis and lacked subsequent experimental verification, such as RT-qPCR, western blot, and immunohistochemistry. Owing to limited available materials in the current study, it was hard for us to verify our findings with these experiments. Anyway, our study may provide some potential useful orientation for future experimental studies.

Conclusions

Our studies have found that PA may alleviate the progression of OA by strengthening the muscle and positively regulating the inflammatory response. We also found some key genes, like *Tnni2*, *Mylpf*, *Myl1*, *Actn3*, *Ifitm6*, and *Retnlg*. These genes may act as the potential target of PA in the treatment of OA. Our findings may provide potential useful evidence for future researches in OA.

Abbreviations

PA—pilose antler—OA—osteoarthritis—SD—Sprague Dawley—DEGs—differentially expressed genes—KEGG—Encyclopedia of Genes and Genomes—GO—Gene Ontology—

Declarations

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Availability of data and materials

All the data were download from the Dr.Tom system—<http://biosys.bgi.com/#/report/login>.

Authors' contributions

Zhi-Dong Lin, Li-Lun Zhong and He Lu and participated in the design of this study. Zhi-Dong Lin and Li-Lun Zhong contributed equally to this work. They both carried out the study and Yao-Ming Lu help to collect important background information. Zhi-Dong Lin drafted the manuscript. Wei Niu contributed to editing the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This experiment was approved by the institutional animal care committee of Guangzhou University of Chinese medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Number of differently expressed genes

Figure 2

Enrichment analysis of the up-regulated genes

Figure 3

Enrichment analysis of down-regulated genes

Level2

Figure 5

Gene classification of down-regulated genes

Figure 6

Heatmap of 10 up-regulated genes

Figure 7

Heatmap of 10 down-regulated genes

Figure 8

miRNA-mRNA linked network of up-regulated genes

Figure 9

miRNA-mRNA linked network of down-regulated genes