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Detection and analysis of knee osteoarthritis in mice with gene expression profile

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Abstract: When gene expression profile is used for gene detection, the probe on the chip can emit fluorescence with different wavelengths. Under the action of confocal laser scanner, a clear gene change image can be obtained, by which the gene changes of the sample to be tested can be observed directly. First, the knee osteoarthritis (KOA) models of mice are established by the method of collateral ligament and meniscus resection (MLI-OA). Then, Bushen Huoxue formula is given by gavage, and ribonucleic acid (RNA) is routinely extracted and purified. Finally, the gene expression changes of KOA tissues of mice are detected by Agilent SurePrint G3 Mouse GE V2.0 gene expression profile. The results show that Bushen Huoxue formula has significant regulation effect on gene expression of KOA tissue. Among the genes with significant up-regulation effect of Bushen Huoxue formula, there are 56 genes of traditional Chinese medicine (TCM) groups up-regulated more than twice compared with model groups. Among the genes with significant down-regulation effect, there are 119 genes of TCM groups down-regulated more than twice compared with model groups. The experimental results indicate that Bushen Huoxue formula may promote the metabolism of arthritic factors and delay cartilage degeneration to treat KOA by regulating genes that are currently unknown in the pathological process of KOA.

Key words: gene expression profile; Bushen Huoxue formula; knee osteoarthritis (KOA); gene chip; mice

0 Introduction

Osteoarthritis (OA) is the most common joint disease in clinical practice. It is characterized by degeneration of articular cartilage with joint pain, stiffness, and deformity. It has become a huge social problem causing heavily economic burden[1-2]. Its incidence is extremely high[3]. Improper treatment often leads to irreversible joint deformities[4], which seriously affects the patient's ability to work and quality of life. The number of patients undergoing total knee/hip arthroplasty (TKA/THA) due to OA has steadily increased[5-6]. So far, there is still a lack of effective drugs. Traditional Chinese medicine (TCM) has accumulated rich clinical experience in treating OA. TCM can often achieve good effects in relieving inflammation and improving clinical symptoms such as edema and pain, but its mechanism of action has not been elucidated[7]. In order to further reveal the intervention effect of Bushen Huoxue formula on knee OA in mice, we use Agilent sureprint G3 mouse Ge v2.0 gene expression profile to detect the knee OA joint tissue of mice. First, total ribonucleic acid (RNA) is reverse-transcribed into double-stranded complementary desoxyribonucleic acid (cDNA), and then cRNA labeled with Cyanine-3-CTP (Cy3) is synthesized[8-10]. The labeled cRNA is hybridized with the chip, and the original image is obtained by scanning with Agilent Scanner G2505C (agilent technologies) after elution. Finally, the data are read. By the up-regulated, down-regulated and unchanged gene images, the gene changes can be observed intuitively in the knee samples of mice[11]. Thus, we can get the general distribution of the gene regulation targets of Bushen Huoxue formula for OA, which is of great significance for the further study of it[12].

1 Materials and methods

1.1 Experimental animals

Twelve clean grade male C57BL/6 mice, 10 weeks old, with body mass (22 ± 3) g, are provided by

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Shanghai Si Laike experimental animals. Place the mice in a clean laboratory at a temperature of 5 ℃—15 ℃, feed them with standard feed and let them drink water normally. After two weeks of adaptive feeding in a clean animal room, the mice are randomly divided into two groups: model group (MLI-OA) and Bushen Huoxue formula treatment group (BSHXF), with six mice in each group.

1.2 Composition and administration of Bushen Huoxue formula

Here is the composition of Bushen Huoxue formula: Achyranthes bidentata, 12 g; Drynaria Rhizoma, 12 g; Psoralea, 10 g; leech, 5 g; Caulis Spatholobus, 30 g; Astragalus membranaceus, 24 g; Chuanxiong, 12 g; Angelica, 24 g; Radix Rehmanniae, 24 g and Glycyrrhizae, 9 g. The conventional Chinese medicine dosage of mice (20 g) and human (60 kg) is converted according to "Experimental Animal Pharmacology" (conversion factor: 9.01), therefore the concentration of the decoction of Bushen Huoxue formula (166 g) taken by the mice is 1.75 g/ml. Take five doses of the above-mentioned traditional Chinese medicine, add five times of water (4 150 ml), soak for 30 min, boil and continue to decoct for 30 min and then filter with sterile gauze to get decoction 1. The remaining residue is added with five times of water (4 150 ml) and then repeat the above steps to obtain decoction 2. The decoctions 1 and 2 are mixed, heated and concentrated to obtain 477 ml of Bushen Huoxue formula decoction. Then it is stored in refrigerator at −80 ℃ for standby.

1.3 Modeling method and grouping

Twelve clean-grade male C57BL/6 mice are randomly divided into MLI-OA group and BSHXF group, with six mice in each group. The model is established according to the OA model of meniscal collateral ligament injury (MLI-OA)[8-9]. From the second day after the end of modeling, BSHXF group is given the concentrated decoction of the traditional Chinese medicine by gavage with the dose of 0.2 ml per mouse, while the MLI-OA group is given the same dose of 0.9% sodium chloride solution once a day for 12 weeks. The mice are killed 12 weeks after operation, and the knee joint tissues are harvested.

1.4 RNA extraction and purification

The total RNA of synovial tissue is extracted by the Trizol method, and the total RNA of the sample is quantified by NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity is tested by Agilent Bioanalyzer 2100 (Agilent Technologies). After passing the RNA quality inspection, the labeling of the sample, the hybridization of the chip and the elution refer to the standard process of the chip.

2 Detection and analysis with gene chip

The microarray is detected by Agilent sureprint G3 mouse Ge v2.0 (8×60 K, design ID: 074809) gene expression profile and its kit from Shanghai Ouyi Biomedical Technology Co., Ltd. It is summarized as follows: first, total RNA is reverse-transcribed into double-stranded cDNA, and then cRNA labeled with Cyanine-3-CTP (Cy3) is synthesized. The labeled cRNA is hybridized with the chip, and the original image is obtained by scanning with Agilent Scanner G2505C (Agilent Technologies) after elution. Finally, the data are read. (The above chip detection and data reading are completed by Shanghai Ouyi Biomedical Technology Co., Ltd.)

2.1 RNA quality detection of specimens

The RNA quality detection results of knee joint specimens of mice are shown in Table 1.

Table 1 RNA quality detection results of knee joint specimens of mice

Code	Sample name	Concentration (μg/μl)	A260/280	A260/230	Volume (μl)	Total mass (μg)	2100		Result	Fluorescent label	CV(%)	Result
							28S/18S	RIN				
1	C-g	1.777 2	2.10	2.18	25	44.43	2.1	9.6	A	cy3	4.23	pass
2	F-g	1.411 5	2.13	1.88	25	35.29	2.1	9.3	A	cy3	3.84	pass
3	EG-g	1.823 6	2.10	2.15	25	45.59	1.6	9.1	A	cy3	2.94	pass
4	A-i	1.549 8	2.11	1.81	25	38.75	1.8	9.0	A	cy3	2.45	pass
5	H-i	1.638 3	2.11	1.87	25	40.96	1.7	9.5	A	cy3	2.74	pass
6	AC-i	1.557 5	2.12	1.92	25	38.94	1.8	9.6	A	cy3	2.98	pass

Over 56 000 genes are detected and no obvious RNA degradation occurs. According to the statistic results, there are 175 differentially expressed genes (DEGs) whose variations are more than two times,

as shown in Table 2, which indicates that the effect of Bushen Huoxue formula on gene expression profile of knee joint in MLI-OA mice is significantly different.

It is noteworthy that the reference index for RNA quality detection is that if 2 100 RIN≥7.0, it is qualified and if 28S/18S≥0.7, it is qualified. CV(%) represents the variation coefficient of repetitive probe, and the smaller the CV (%) value, the higher the experimental accuracy will be. C-g, F-G and EG-g are the samples of the BSHXF treatment group while A-i, H-i and AC-i are the samples from the MLI-OA model group .

Table 2 Statistics results of gene variatons from MLI-OA model group and Bushen Huoxue formula treatment group

Experiment group	MLI-OA group	Up-regulated genes	Down-regulated genes	Total genes
BSHXF	Placebo	56	119	175

2.2 Data standardization and evaluation

To further analyze the gene variation, the detected data are normalized and the results are shown in Figs. 1 and 2.

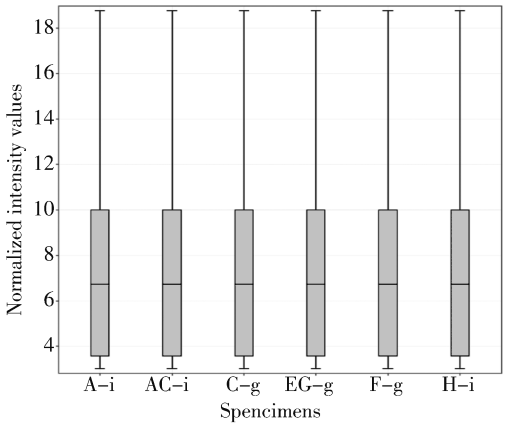

Fig. 1 Intensity values of spencimens

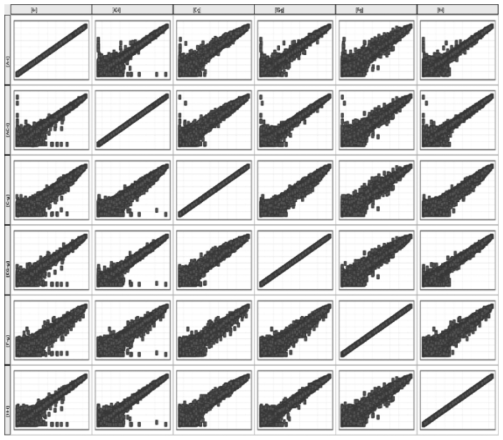

Fig. 2 Scattering chart of overall distribution trends of two groups of data

Fig. 1 shows the normalized intensity values of the specimens and their baselines are at roughly the same level, therefore the data have high consistency. Fig. 2 shows the overall distribution trends of the two groups of data. It can be seen that the distributions of the two groups of data are concentrated.

2.3 Differential gene screening

Before screening DEGs, the probe detection is performed, and the difference significance P value obtained by T test and the difference multiple Fold change value of standardized signal value are used to screen. The standard is that Fold change≥2.0 and P value≤0.05, and the results are shown in Fig. 3. The gray dots (bottom) are the genes with P value greater than 0.05, light gray dots (top) are the genes with Fold change absolute value less than 2 and P value less than or equal to 0.05. the dark gray dots (right)are the genes with Fold change greater than or equal to 2 and P value less than or equal to 0.05. They are significant up-regulated differential genes. The black dots (left) are the genes with Fold change less than or equal to −2 and P value less than or equal to 0.05. They are significantly down-regulated differential genes.

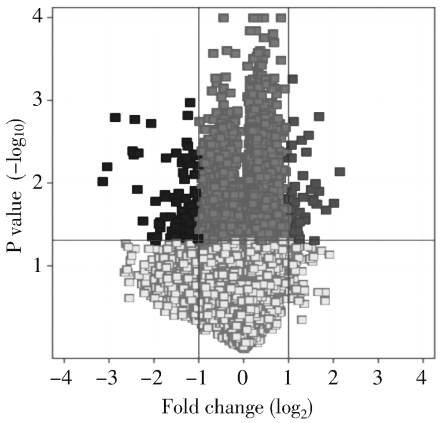

Fig. 3 Differential screening of volcano plot

The enrichment analysis of gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathways are performed[13-16], as shown in Table 3. The GO can be divided into biological process (BP), cellular component (CC) and molecular function (MF).

It can be seen from Table 3 that the up-regulated genes mainly include Chrm3; Foxa2; Pax6; Sorbs2; Clic4; Il20ra; Trpm2; Neurog3, etc. and the down-regulated genes include Cnga3; Tomm40; Trpc4; Hcn4; Fxyd6; Nrsn2, etc.

Table 3 GO analysis of DEGs

Gene classification	Go category	Go ID	Number of genes	Gene name	P values	Gene description
Up-regulated genes	BP	6810	6	Nup160; Slc2a3; Agap1; Trpm2; Clic4; Abca13	0.035 635	Transport
	BP	7219	3	Timp4; Sorbs2; Foxa2	0.000 538	Notch signaling pathway
	BP	55085	3	Trpm2; Abca13; Slc2a3	0.018 853	Transmembrane transport
	CC	5886	10	Psd4; Npy1r; Il20ra; Sorbs2; Slc2a3 Sytl3; Ipcef1; Trpm2; Chrm3; Clic4	0.024 782	Cytoplasmic membrane
	CC	5829	6	Npy1r; Agap1; Trpm2; Ipcef1; Fcor; Clic4	0.016 049	Cytosolic
	CC	5578	3	Spock2; Timp4; Impg2	0.010 146	Protein extracellular matrix
	MF	1077	3	Pax6; Neurog3; Foxa2	0.006 284	Transcription activator activity, the specific binding of proximal region sequence of RNA polymerase II core promoter
	MF	3690	3	Pax6; Neurog3; Foxa2	0.000 786	Double-stranded DNA binding
	MF	19904	3	Ipcef1; Sorbs2; Foxa2	0.008 051	Specific binding of protein region
Down-regulated genes	BP	6811	5	Cnga3; Tomm40; Trpc4; Hcn4; Fxyd6	0.011 692	Ion transport
	BP	5085	4	Cnga3; Tomm40; Trpc4; Hcn4	0.015 805	Transmembrane transport
	BP	35023	2	Rasgrf1; Mcf2l	0.011 228	Regulation of Rho protein signal transduction, etc.
	CC	43025	4	Cnga3; Nrsn2; Hcn4; Rasgrf1	0.028 558	Body of neuron
	CC	16323	3	Rasgrf1; Trpc4; Hcn4	0.008 184	Basal plasma membranes
	CC	34704	1	Trpc4	0.040 939	Calcium channel complex
	MF	5216	4	Cnga3; Trpc4; Hcn4; Fxyd6	0.000 746	Ion channel activity
	MF	5222	2	Cnga3; Hcn4	0.000 368	Intracellular cAMP activated cation channel activity
	MF	5089	2	Rasgrf1; Mcf2l	0.011 342	Nucleotide exchange factor activity

Fig. 4 shows the gene expression variation of the specimens from low color scale to high color scale. The horizontal coordinates represent the specimens and the vertical coordinates represent the probes.

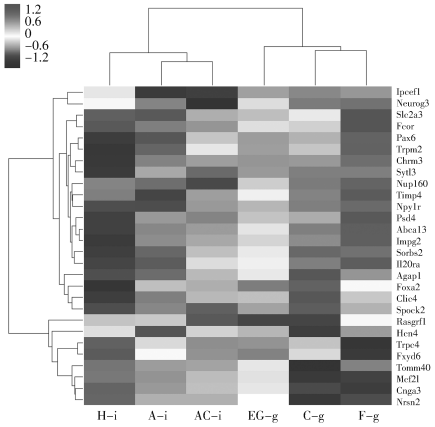

Fig. 4 Enrichment analysis of KEGG pathway of gene expression

3 Conclusions

According to GO analysis, the up-regulated genes include Chrm3, Foxa2, Clic4, Impegg2, Neurog2, etc. Further analysis reveals that most of the genes focus on smooth muscle signal regulation. For example, Foxa2 gene is a typical transcription factor and is characterized by a pterygoid DNA binding domain, which can simulate histone binding to nucleosome targets enteing the nuclear chromatin, thus achieving positive regulation of smooth muscle signaling pathway as well as anatomical structure formation and morphogenesis, etc. In conclusion, the up-regulated and down-regulated genes are mainly concentrated in smooth muscle, transmembrane and intracellular regions. They are closely related to transmembrane and intracellular signaling pathways such as cAMP, Notch and Jak-STAT. Gene regulation of smooth muscle contraction signaling pathway is especially common. Because Bushen Huoxue formula may be regulate the contraction of lymphatic smooth muscle, promote lymphatic circulation, accelerate the metabolism of knee osteoarthritis arthritis factors and decompose enzymes to alleviate OA by regulating related genes. Therefore, the smooth muscle signaling pathway is very likely to be one of the targets of Bushen Huoxue formula to improve OA.

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基因表达谱芯片用于小鼠膝关节炎组织的检测与分析

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摘　要： 通过副韧带及半月板切除方法(MLI-OA)建立小鼠 KOA 模型，补肾活血方灌胃给药、常规抽提和纯化 RNA，采用 Agilent SurePrint G3 Mouse GE V2.0 基因表达谱芯片检测小鼠膝 OA 关节组织的基因表达变化。检测结果显示补肾活血方对 KOA 组织基因表达具有明显调控作用：补肾活血方有明显上调作用的基因中，中药组比模型组上调 2 倍以上的有 56 个；明显下调作用的基因中，中药组比模型组下调 2 倍以上的有 119 个。研究结果表明补肾活血方可能通过调控目前在 KOA 病变过程中作用尚不明确的基因，促进关节炎性因子代谢，延缓软骨退变，治疗骨关节炎的作用。

关键词： 基因表达谱检测；补肾活血方；膝关节炎；基因芯片；小鼠

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