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Xuemei Jin
Department of Pharmacy, Affiliated Hospital of Yanbian University, Yanji 133000, Jilin, P. R. China

Chunyan Liu
Tongliao People's Hospital, Tongliao 028000, Inner Mongolia, P.R. China

Huanhuan Du
Public Health College of Inner Mongolia Minzu University, Tongliao 028000, Inner Mongolia, P. R. China

Xiaojun Chen
Tongliao People's Hospital, Tongliao 028000, Inner Mongolia, P.R. China

Bin Yu
Affiliated Hospital of Inner Mongolia Minzu University, Tongliao 028000, Inner Mongolia, P. R. China

Corresponding Author:
Bin Yu
Affiliated Hospital of Inner Mongolia Minzu University, Tongliao 028000, Inner Mongolia, P. R. China

Transcriptome sequencing reveals the effect of Mongolian remedy ruxian-I on rat breast hyperplasia via regulation of HPO axis and immunity pathways

Xuemei Jin, Chunyan Liu, Huanhuan Du, Xiaojun Chen and Bin Yu

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Abstract
The traditional Mongolian remedy Ruxian-I has exhibited significant therapeutic efficacy on breast hyperplasia. To elucidate the potential pathogenesis of breast hyperplasia and the therapeutic mechanism of Ruxuan-I, we employed transcriptome technology to analyze alterations in gene expression profiles, functional categories, and molecular pathways associated with both untreated breast hyperplasia and its treatment with Ruxuan-I. Our findings suggest that the upregulation of ER-α gene expression levels regulated by HPO and pathways related to inflammation and immunity may play pivotal roles in the development of breast hyperplasia. Furthermore, our drug testing results indicate that Ruxian-I may exert its therapeutic effects through immune-related pathways. This study provides valuable insights into the potential causes of breast hyperplasia as well as the therapeutic mechanism of Ruxian-I. Further investigation into these gene alterations and active ingredients within Ruxian-I will be instrumental in advancing our understanding of both the pathogenesis and treatment strategies for breast hyperplasia.

Keywords: Transcriptome sequencing, mongolian remedy, ruxian-I, breast, hyperplasia

1. Introduction
Hyperplasia is considered a physiological response involving an increase in the number of cells and is a common preneoplastic reaction. Unlike hypertrophy, which involves an increase in cell size, hyperplasia results in an increased number of cells. Various factors can induce breast hyperplasia, including chronic inflammatory response, hormones, lactation behavior, etc [1]. It is now accepted by traditional Chinese medicine that the hypothalamic-pituitary-ovarian (HPO) axis is a key pathway to regulate the physiological and pathological changes of the breast, and its dysfunction is closely related to the occurrence and development of breast hyperplasia [2-3]. In addition to normal physiological proliferation, there are instances of usual ductal hyperplasia and atypical ductal hyperplasia (ADH) [4]. Atypical ductal hyperplasia differs from usual ductal hyperplasia as it represents an abnormal pattern of growth and is associated with an increased risk of developing breast cancer. The development of breast tumors generally progresses sequentially from normal tissue to ductal hyperplasia (DH), then to atypical ductal hyperplasia (ADH), followed by ductal carcinoma in situ (DCIS), and finally invasive ductal carcinoma (IDC) [5]. The risk of breast cancer among women with hyperplasia varies depending on the presence or absence of atypia. Women with atypia-hyperplasia have a 2.6-fold increased risk for breast cancer (OR = 2.6; 95% CI 1.6–4.) [6]. Traditional Mongolian medicine plays an important role within the current medical system of Mongolia [7, 8], and has been officially recognized as part of the heritage of traditional Mongolian medicine [9]. In regions with a significant Mongolian population, hospitals and clinics apply both Western and traditional medical treatment methods [7]. The coverage of traditional Mongolian healthcare services stands at 23.6% [7]. A clinical study involving 400 cases of breast hyperplasia with the treatment of Ruxian-I, a traditional Mongolian remedy, achieved an impressive cure rate of 98% with minimal side effects [8]. WANG Zhong-Chao *et al*. reported Ruxian-I can effectively reduce estradiol and estrogen receptor α (ER-α) mRNA expression and the sensitivity of breast tissue to estradiol in rat model of mammary gland hyperplasia [10]. Previous research has identified 17 proteins affected by Ruxian-I in breast hyperplasia rats [8], while the gene expression profiles remains unreported. Transcriptomic analysis offers insights into genes involved in specific biological processes to be identified.
This study aims to investigate the changes in gene expression related to breast hyperplasia

using transcriptome technology and to elucidate the therapeutic mechanism of Ruxian-I. Considering that breast hyperplasia is considered to be an early potential precursor of breast cancer, identifying the transcriptome and genes related to breast hyperplasia may help reveal treatment strategies for breast hyperplasia and breast cancer and reveal how Ruxian-I affects HPO axis.

2. Materials and Methods

2.1 Drugs and reagents

Ruxian-I was derived from the Mongolian medicine manufacturing department of the affiliated hospital of Inner Mongolia Minzu University. The components and production process of Ruxian-I were as described by Wang *et al* [8].

2.2 Rat model of breast hyperplasia and medications

40 virgin female Wistar rats weighing 180–220 g, supplied by Experimental Animal Center of Jilin University, were allowed to acclimate in our facility for 2 weeks. The rats were housed in a room at temperature of 21 ± 1˚C, relative humidity of 50 ± 5% and a 12h light/12h dark cycle. Animals had free access to rodent food and tap water. The rats were randomly allocated into four groups, each consisting of 10 individuals. The control group received standard feeding for 56 days and samples were collected on day 28 (Control Group, Con_1_T). Rats in the disease model group were administered estradiol benzoate (0.5 mg/kg) for a continuous period of 24 days, followed by progesterone injection (4 mg/kg, ig) for 4 days. Samples were obtained from these rats on day 28 (Disease Model Group, Mod_1_T), after which they received standard feeding for an additional 28 days before sample collection on day 56 (Treatment Group, Tre_3_T). Rats in the drug model group were fed Ruxian-I and sampled on day 28 (Medicine Group, Med_1_T), followed by standard feeding for another 28 days. Rats in the prevention treatment group received Ruxian-I and were injected with estradiol benzoate (0.5 mg/kg) for 24 days, followed by progesterone injection (4 mg/kg, ig) for 4 days. Samples were collected from these rats on day 28 ((Preventive Treatment Group, Tre_1_T), after which they received standard feeding for an additional period of 28 days. 3 rats were randomly selected from each group for sampling with right breast tissue being harvested. Nipple diameter measurements were taken on days 1, 14, 28, 42, and 56. After the rats were sacrificed, the right breast tissues were immediately snap-frozen in liquid nitrogen and stored at -80˚C. The Inner Mongolia Minzu University Animal Care and Use Committee approved all animal procedures.

2.3 RNA isolation

Total RNA was isolated using TRIZOL reagent (Invitrogen) based on the manufacturer's protocol, and mRNA was purified by oligotex (Qiagen). The total RNA samples were treated with DNase to degrade the possible genomic DNA contamination. The ratios of A260/A280 and A260/A230 (1.8-2.0) were used to ascertain the RNA purity. The RNA quality was assessed by 1.2% agarose gel electrophoresis using the RNA 6000 Pico LabChip Kit on the Agilent 2100 BioAnalyzer. Meanwhile, the ratio of 28S-to-18S rRNA (>2) and the RNA integrity number (RIN) (RIN>7) were calculated to evaluate RNA integrity using the Agilent 2100 BioAnalyzer.

2.4 cDNA library preparation and transcriptome sequencing

The mRNA in RNA samples was enriched using magnetic beads conjugated with oligo (dT). Following purification, the mRNA was divided into short fragments at 94℃ in the 5×fragmentation buffer (Illumina, USA). Synthesis of first-strand cDNA was generally performed by random hexamer-primers. The first strand cDNA was used for the second-strand cDNA synthesis in the reaction system with DNA Ligase, DNA polymerase I, RNAse H, and dNTPs. The double-stranded cDNA was extracted using magnetic beads. End reparation and 3'-end single nucleotide A (adenine) addition were performed. Finally, the fragments were ligated to sequencing adaptors. The fragments were enriched by PCR amplification. During the QC step, the sample libraries were evaluated qualitatively and quantitatively using Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System. The library templates were ready for paired-end sequencing via Illumina HiSeqTM 2000.

2.5 Quality control of sequencing reads and functional annotation

We defined the raw sequencing reads which contained the sequence of adapter, high content of unknown bases and low quality reads. The raw reads were subjected to quality control (QC) to determine if a resequencing step was required. Filtering steps are as follows: 1) Remove reads with adaptors. 2) Remove reads in which unknown bases are more than 10%. 3) Remove low-quality reads. The reads with over 50% low-quality bases will be defined as low-quality reads. Bases with sequencing quality≤5 were defined as low-quality bases. After filtering, clean reads were mapped to the reference genome using Bowtie2 software [10]. The gene expression levels were calculated with RSEM [11].

2.6 Screening of differentially expressed genes (DEGs)

We screened genes that are differentially expressed among samples using the method as described at Audic S, *et al* [12]. The threshold values of "FDR≤0.001 and |log2Ratio| ≥1" were used to judge the significance of gene expression difference. The GO functional enrichment analysis and KEGG pathway enrichment analysis were performed on these DEGs to identify significantly enriched GO terms and DEGs-related pathways.

3. Results

3.1 Comparison of nipple diameters among four groups of rats

The diameter of the nipple may reflect hyperplasia and the impact of treatment. Before the experiment, the nipples in each group were similar in size, white, small, soft, close to the skin, and nearly invisible. There were no significant changes in diameter or appearance before and after the experiment for rats between the Control Group and the Medicine Group. However, rats in the Disease Model Group and the Preventive Treatment Group showed redness, swelling, and increased size of their nipples. The nipple diameters of rats in the Disease Model Group were significantly larger than those of the Control Group (P<0.01), reaching the maximum value (1.83±0.16 mm) on day 28, and decreased after RX-I treatment. The nipple diameters of the Preventive Treatment Group were significantly smaller than those of the Disease Model Group on days 14, 28, and 42 (P<0.01)(Table 1).

3.2 Sequencing, assembly, and detection of differentially expressed genes

A total of five samples were RNA-seq sequenced in this study. There were more than 13 million pure reads (Supplementary Table 1), with an average of 0.2% low-quality data. To objectively assess and quantify differences in gene expression, all samples were quantified using RSEM10 (version 1.2.30). The numbers of expressed genes with detectable transcripts were similar across the five groups of samples, ranging between 12984 and 13465, with an average of 13254 expressed genes detected across the five samples. Bioinformatics analysis identified differentially expressed genes (DEGs) (Fig. 1): 2327 DEGs between the Control Group and the Disease Model Group (Supplementary Table 2, Supplementary Fig. 1), 1936 DEGs between the Disease Model Group and the Treatment Group (Supplementary Table 3, Supplementary Fig. 2), 2254 DEGs between the Control Group and the Preventive Treatment Group (Supplementary Table 4, Supplementary Fig. 3), 883 DEGs between the Control Group and the Treatment Group (Supplementary Table 5, Supplementary Fig. 4), and 814 DEGs between the Control Group and the Medicine Group (Supplementary Table 6, Supplementary Fig. 5).

3.3 Comparison of DEGs between normal control and disease model

To objectively identify candidate target genes of hyperplasia, we evaluated and quantified the differences in gene expression among the Control Group, Disease Model Group, and Treatment Group. There were 479 up-regulated genes and 1848 down-regulated genes in the Disease Model Group compared to the Control Group. We performed an enrichment analysis of Gene Ontology (GO) terms in the set of 2327 DEGs to characterize hyperplasia in rats.

Categories about biological process in the set of DEGs were enriched in some kinds of cellular responses, including "developmental process" (P=9.95×10^{-22}), "response to chemical" (P=1.91×10^{-12}), "cell differentiation" (P=5.79×10^{-11}), "response to endogenous stimulus" (P=1.92×10^{-10}), "circulatory system development" (P=2.57×10^{-07}), "cell motility" (P=8.29×10^{-07}), "chemical homeostasis" (P=1.67×10^{-06}), "response to lipid" (P=3.50×10^{-6}), "energy derivation by oxidation of organic compounds" (P=3.60×10^{-6}), "response to hormone" (P=3.65e×10^{-6}), "biological adhesion" (P=6.11×10^{-6}), "response to organic cyclic compound" (P=7.07×10^{-6}), "response to steroid hormone" (P=1.01×10^{-5}), "oxidation-reduction process" (P=1.32×10^{-5}), "response to wounding" (P=0.00013), "organ morphogenesis" (P=0.00042), "vasculature development" (P=0.00223), "response to stress" (P=0.00455), "chemotaxis" (P=0.01089), "blood vessel development" (P=0.011), "response to estrogen" (P=0.02258) (Supplementary Figure 6, Supplementary Table 7).

Molecular function categories in the set of DEGs were enriched in functions of binding and transporter, including "protein binding" (P=2.83×10^{-23}), "cytoskeletal protein binding" (P=1.17×10^{-11}), "transporter activity" (P=5.34×10^{-06}), "substrate-specific transporter activity" (P=1.53×10^{-05}), "transmembrane transporter activity" (P=0.00014), "ion binding" P=0.00203), "ion transmembrane transporter activity" (P=0.00213), "glycosaminoglycan binding" (P=0.00474), "cargo receptor activity" (P=0.00497), "identical protein binding" (P=0.01102), "cation binding" (P=0.01669) (Supplementary Figure 6, Supplementary Table 8).

Cellar component categories in the set of DEGs were enriched in mitochondrion, including "mitochondrial inner membrane" (P=1.15×10^{-12}), "mitochondrial membrane part" (P=1.43×10^{-10}), "mitochondrial envelope" (P=2.80×10^{-10}), "mitochondrial membrane" (P=4.63×10^{-10}), "mitochondrial respiratory chain" (P=6.21×10^{-08}), "respiratory chain" (P=6.21×10^{-08}), "mitochondrion" (P=1.06×10^{-07}), "mitochondrial part" (P=1.25×10^{-07}), "proton-transporting two-sector ATPase complex" (P=0.01822) (Supplementary Figure 6, Supplementary Table 9).

We identified 40 enriched pathways of DEUs based on a Q-value threshold of 0.05. 40 overrepresented pathways of DEUs could be assigned to metabolisms and signaling pathways including "calcium signaling pathway" (Q = 1.13×10^{-6}), "metabolic pathways" (Q = 3.27×10^{-6}), "ECM-receptor interactions" (Q = 1.65×10^{-4}), "glycolysis / gluconeogenesis" (Q = 3.50×10^{-3}), "citrate cycle" (Q = 5.33×10^{-3}), "MAPK signaling pathway" (Q = 6.10×10^{-3}), "pathways in cancer" (Q = 1.68×10^{-2}), "glutathione metabolism" (Q = 1.82×10^{-2}), "transcriptional misregulation in cancer" (Q = 2.76×10^{-2}), "Galactose metabolism" (Q = 3.04×10^{-2}), "TGF-beta signaling pathway" (Q = 3.73×10^{-2}), "p53 signaling pathway"(Q = 3.91×10^{-2}) (Supplementary Table 10; Supplementary Figure 7).

3.4 Comparison of DEGs between disease model and treatment group

We performed an enrichment analysis of Gene Ontology (GO) terms in the set of DEGs between the Disease Model Group and Treatment Group. Categories about biological process in the set of DEGs were enriched in some kinds of development and cellar responding, including "anatomical structure development"(P=1.07×10^{-31}), "system development" (P=9.09×10^{-28}), "multicellular organismal development" (P=3.52×10^{-24}), "tissue development" (P=1.01×10^{-22}), "organ development" (P=3.59×10^{-22}), "response to chemical" (P=9.24×10^{-19}), "anatomical structure formation involved in morphogenesis" (P=6.30×10^{-15}), "response to organic substance" (P=1.27×10^{-09}), "response to endogenous stimulus" (P=9.51×10^{-08}), "response to stress" (P=5.33×10^{-05}), "response to lipid" (P=7.80×10^{-05}), "response to growth factor" (P=0.00015), "response to external stimulus" (P=0.00022), "response to hormone" (P=0.00022), "epithelium development" (P=0.00058), "response to oxygen-containing compound" (P=0.00101), "regeneration" (P=0.00104), "cellular response to organic substance" (P=0.00578) (Supplementary Table 11, Supplementary Figure 8).

Molecular function categories in the set of DEGs between the Disease Model Group and Treatment Group were enriched in functions of binding and kinase activity, including "protein binding" (P=8.26×10^{-19}), "cytoskeletal protein binding" (P=4.01×10^{-11}), "glycosaminoglycan binding" (P=3.16×10^{-06}), "transmembrane receptor protein tyrosine kinase activity" (P=3.63×10^{-06}), "receptor binding" (P=0.00225), "cation binding" (P=0.00283), "metal ion binding" (P=0.00942), "antioxidant activity" (P=0.01847), "growth factor binding" (P=0.0418) (Supplementary Table 12, Supplementary Figure 8).

For the cell component ontologies, the enriched categories in the DEGs between the Disease Model Group and Treatment Group were associated with "extracellular matrix" (P=3.18×10^{-26}), "contractile fiber" (P=5.62×10^{-24}), "myofibril" (P=4.26×10^{-22}), "sarcomere" (P=6.56×10^{-17}), "plasma membrane" (P=7.24×10^{-11}), "intermediate filament cytoskeleton" (P=5.25×10^{-08}), "actin cytoskeleton" (P=0.00193), "collagen trimer" (P=0.0322), etc

(Supplementary Table 13, Supplementary Figure 8).
A total of 38 enriched pathways of DEGs between the Disease Model Group and Treatment Group were identified, including "ECM-receptor interaction" (Q =1.23×10^{-07}), "focal adhesion" (Q=6.04×10^{-07}), "MAPK signaling pathway" (Q=5.18×10^{-04}), "TGF-beta signaling pathway" (Q = 5.58×10^{-04}), "glycolysis / gluconeogenesis" (Q=1.11×10^{-03}), "fructose and mannose metabolism" (Q=1.64×10^{-03}), "transcriptional misregulation in cancer" (Q=3.30×10^{-03}), "calcium signaling pathway" (Q=3.30×10^{-03}), "glutathione metabolism" (Q=3.53×10^{-03}), "arachidonic acid metabolism" (Q=3.87×10^{-03}), "cytokine-cytokine receptor interaction" (Q=5.60×10^{-03}), "hedgehog signaling pathway" (Q= 5.63×10^{-03}), "pathways in cancer" (Q =5.63×10^{-03}), "chemokine signaling pathway" (Q=1.19×10^{-02}), "Wnt signaling pathway" (Q=2.85×10^{-02}), "p53 signaling pathway" (Q=3.91×10^{-02}) (Supplementary Table 14; Supplementary Figure 9).

3.5 Comparison of DEGs between normal control and treatment group

For identifying the gene influenced by Ruxian-I, we compared the DEGs between the Control Group and the Treatment Group. Categories about biological process in the set of DEGs were enriched in some kinds of cell differentiation and cellar responding, including "epidermal cell differentiation" (P=5.67×10^{-06}), "response to chemical" (P=8.12×10^{-06}), "keratinocyte differentiation" (P=1.50×10^{-05}), "epithelial cell differentiation" (P=1.60×10^{-05}), "response to lipid" (P=.61×10^{-05}), "response to organic substance" (P=0.0001), "cell differentiation" (P=0.00016), "cellular response to chemical stimulus" (P=0.00161), "response to organic cyclic compound" (P=0.00191), "cellular chemical homeostasis" (P=0.00203), "response to reactive oxygen species" (P=0.00498), "response to oxidative stress" (P=0.00691), "response to external stimulus" (P=0.01002), "defense response" (P=0.01124), "response to endogenous stimulus" (P=0.01713), "response to stress" (P=0.02768), "response to hormone" (0.04843) (Supplementary Table 15; Supplementary Figure 10).
Molecular function categories in the set of DEGs between the Control Group and the Treatment Group were enriched in metal ion binding and transporter, such as "protein binding" (P=0.0002), "transporter activity" (P=0.00108), "transmembrane transporter activity" (P=0.00176), "metal ion binding" (P=0.00272), "cytoskeletal protein binding" (P=0.0039) (Supplementary Table 16; Supplementary Figure 10).
For the cell component ontologies, the enriched categories in the DEGs between the Control Group and the Treatment Group were associated with cytoskeleton and membrane, including "contractile fiber" (P=1.94×10^{-13}), "myofibril" (P=2.73×10^{-11}), "intermediate filament" (P=2.49×10^{-06}), "intermediate filament cytoskeleton" (P=4.64×10^{-06}), "plasma membrane" (P=1.69×10^{-05}), "intrinsic component of membrane" (P=0.04373) (Supplementary Table 17; Supplementary Figure 10).
We identified 16 enriched pathways of DEUs between the Control Group and the Treatment Group. Some representative pathways include "fructose and mannose metabolism" (Q= 2.33×10^{-03}), "arachidonic acid metabolism" (Q= 6.48×10-03), "cell adhesion molecules (CAMs)" (Q= 6.48×10^{-03}), "cell adhesion molecules (CAMs)" (Q= 6.48×10-^{03}), "mineral absorption" (Q= 1.16×10^{-02}), "linoleic acid metabolism" (Q= 1.99×10^{-02}), "Fc epsilon RI signaling pathway" (2.28×10^{-02}) and "Fc gamma R-mediated phagocytosis" (Q= 2.28×10^{-02}) (Supplementary Table 18; Supplementary Figure 11).

3.6 Comparison of DEGs between normal control and medical group

To investigate the effects of Ruxian-I on the normal rats, we compared the expression levels between the Control Group and the Medicine Group. Categories about molecular function in the set of DEGs were enriched in some kinds of development and cellar responding, including "cytoskeletal protein binding"(P=9.68×10^{-09}), "protein binding" (P=9.44×10^{-05}), "actinin binding" (P=0.01473), "tetrapyrrole binding" (P=0.01574), "glycosaminoglycan binding" (P=0.02099) (Supplementary Table 19; Supplementary Figure 12).
Categories about biological process in the set of DEGs were enriched in multiple developments and processes, including "immune system process" (P=4.22×10^{-8}), "developmental process" (P=1.38×10^{-6}), "anatomical structure development" (P=1.73×10^{-5}), "response to chemical" (P=1.99×10^{-5}), "immune effector process" (P=6.87×10^{-5}), myofibril assembly (P=0.00035), actomyosin structure organization (P=0.00035), regulation of response to external stimulus (P=0.00053), response to stress (P=0.00054), homeostatic process (P=0.0006), response to organic substance (P=0.00071), response to external stimulus (P=0.00729), regulation of immune system process (P=0.0097), response to reactive oxygen species (P=0.03423) (Supplementary Table 20; Supplementary Figure 12).
The enriched cell component categories in the DEGs between the Control Group and the Medicine Group were assigned to cytoskeleton, such as contractile fiber (P=1.16E-^{37}), myofibril (P=3.09×10^{-33}), sarcomere (P=6.83×10^{-31}), actin cytoskeleton (P=1.42×10^{-6}), cytoskeleton (P=0.00134) (Supplementary Table 21; Supplementary Figure 12).
A total of 12 enriched pathways of DEUs between the Control Group and the Treatment Group were identified. Some representative pathways include "staphylococcus aureus infection" (Q= 3.69×10^{-7}), "glycolysis / gluconeogenesis" (Q= 2.32×10^{-3}), "Arachidonic acid metabolism" (Q= 7.61×10^{-3}), "Pathogenic Escherichia coli infection" (Q= 2.28×10^{-2}), "Herpes simplex infection" (Q= 3.43×10^{-2}) (Supplementary Table 22; Supplementary Figure 13).

4. Discussion

Breast hyperplasia is a very common disease in adult women and a precursor of breast cancer. In our study, DEGs were enriched in cancer-associated pathways, "MAPK signaling pathway", "pathways in cancer", "transcriptional dysregulation in cancer", "p53 signaling pathway", "TGF-beta signaling pathway" between the Control Group and the Disease Model Group. This result supports the idea that breast hyperplasia is a risk factor for developing breast cancer [13-15].
Previous analyses suggested that estrogen and ER-α regulated by HPO axis may be important in the development of hyperplasia [10, 17]. Estrogen plays an important role in stimulating cell proliferation and suppressing cell apoptosis [17, 18]. Elevated ER-α, an estrogen mediator, causes hyperplasia. Overexpression of the ER-α gene in normal breast epithelium induces the development of hyperplasia [19]. In our study, DEGs were enriched in "response to estrogen" (P=0.02258) between the Control Group and the Disease Model Group, supporting the notion that elevated ER-α gene expression may be responsible for breast hyperplasia. Previous studies have shown that several chemokines are consistently down-regulated in breast hyperplasia. Several

immune-related pathways were enriched, including "Staphylococcus aureus infection", and "Pathogenic Escherichia coli infection", which supports the view that inflammation- and immune-related pathways may also be important in the development of breast hyperplasia.

The therapeutic mechanism of Ruxian-I is still unknown. 883 DEGs were identified between the Control Group and the Treatment Group. The biological processes occurring in rats in the Treatment Group included a series of drug stress responses, such as "response to chemical", "response to lipid", "response to organic substance", and "response to reactive oxygen species" (Supplementary Table 15), which correlate with Ruxian-I's complex compound composition. Previous studies have shown that Ruxian-I treats breast hyperplasia in rats by regulating the cell cycle, immune system metabolism, and signal transduction[6]. In our study, DEGs between the Control Group and the Treatment Group were enriched in the immune-related pathways, including "*Staphylococcus aureus* infection", "Pathogenic *Escherichia coli* infection", "Arachidonic acid metabolism", "Malaria", "Amoebiasis", "Fc epsilon RI signaling pathway", and "Fc gamma R-mediated phagocytosis". This indicates that Ruxian-I may play a therapeutic role through immune-related pathways.

5. Conclusion

In this study, we found that the possible etiology of breast hyperplasia is related to inflammation and immunity, and Ruxian-I may exert therapeutic effects through regulation of HPO axis and immune-related pathways. Further studies on the changes of these DEGs and the active ingredients of RX-I will be of great help in elucidating the pathogenesis and therapeutic mechanisms of breast hyperplasia.

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7. Author Contributions

XUEMEI JIN and CHUNYAN LIU did all the experiments; HUANHUAN DU and XIAOJUN CHEN analysed the data; BIN YU designed all the work, directed the experiments and and wrote the article.

8. Conflict of Interest

The authors declare that they have no conflict of interest.

9. References

1. Wang XY, Lei HB, Qi XP, *et al*. Simultaneous determination of five bioactive components of Xiaojin capsule in normal and mammary gland hyperplasia rat plasma using LC-MS/MS and its application to a pharmacokinetic study. Biomed Chromatogr. 2021;35(3):e5000.
2. Ning J, Li L, Di R, Dandan L. Effects of the massage manipulation along meridians combined with tamoxifen on the HPO axis in MGH rats. Jilin J Chin Med. 2022;42(8):945-949.
3. Xinrong G, Xiaojun M, Wei F, Xiaowei M, Hailong S. Effect of Tongtiao Manipulation Electroacupuncture on Regulating the Hypothalamus Pituitary Axis Hormone Secretion in Rats with Mammary Gland Hyperplasia. Shandong J Chin Med. 2018;37(7):602-606.
4. Biernacka A, Lerwill MF. Diagnostic evaluation of usual ductal hyperplasia and atypical ductal hyperplasia. Springer International Publishing AG, part of Springer Nature; 2018. p. 205-225.
5. Shackney SE, Silverman JF. Molecular evolutionary patterns in breast cancer. Adv Anat Pathol. 2003;10(5):278-290.
6. McDivitt RW, Stevensm JA, Lee NC, Wingo PA, Rubin GL, Gersell D. Histologic types of benign breast disease and the risk for breast cancer. Cancer. 1992;69(6):1408-1414.
7. Pitschmann A, Purevsuren S, Obmann A, Natsagdorj D, Gunbilig D, Narantuya S, *et al*. Traditional Mongolian Medicine: history and status quo. Phytochem Rev. 2013;12(4):943-959.
8. Wang Z-C, Batu D-L, Saixi Y-L, Zhang B, Ren L-Q. 2D-DIGE proteomic analysis of changes in estrogen/progesterone-induced rat breast hyperplasia upon treatment with the Mongolian remedy Ruxian-I. Molecules. 2011;16(4):3048-3065.
9. Kletter C, Glasl S, Thalhammer T, Narantuya S. Traditional Mongolian medicine-a potential for drug discovery. Sci Pharm. 2008;76(1):49.
10. Zhongchao W, Bin Z, Liqun R, Chengxi W, Du E, Guiyu C. Effect of Mongolian medicine Ruxian -I on estrogen and estrogen receptor mRNA expression in rats with mammary gland hyperplasia. Matern Child Health Care China. 2014;29:2797-2799.
11. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.
12. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12(1):323.
13. Audic S, Claverie J-M. The significance of digital gene expression profiles. Genome Res. 1997;7(10):986-995.
14. McLaren BK, Gobbi H, Schuyler PA, Olson SJ, Parl FF, Dupont WD, *et al*. Immunohistochemical expression of estrogen receptor in enlarged lobular units with columnar alteration in benign breast biopsies: a nested case-control study. Am J Surg Pathol. 2005;29(1):105-108.
15. Page D, Dupont W, Rogers L. Breast cancer risk of lobular-based hyperplasia after biopsy:" ductal" pattern lesions. Cancer Detect Prev. 1986;9(5-6):441-448.
16. Shaaban A, Sloane J, West C, Moore F, Jarvis C, Williams E, *et al*. Histopathologic types of benign breast lesions and the risk of breast cancer: case–control study. Am J Surg Pathol. 2002;26(4):421-430.
17. Lee S, Mohsin SK, Mao S, Hilsenbeck SG, Medina D, Allred DC. Hormones, receptors, and growth in hyperplastic enlarged lobular units: early potential precursors of breast cancer. Breast Cancer Res. 2005;8(1):R6.
18. Anderson E, Clarke RB. Steroid receptors and cell cycle in normal mammary epithelium. J Mammary Gland Biol Neoplasia. 2004;9(1):3-13.
19. Gompel A, Somaï S, Chaouat M, Kazem A, Kloosterboer HJ, Beusman I, *et al*. Hormonal regulation of apoptosis in breast cells and tissues. Steroids. 2000;65(10):593-598.
20. Frech MS, Halama ED, Tilli MT, Singh B, Gunther EJ, Chodosh LA, *et al*. Deregulated estrogen receptor α expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma in situ. Cancer Res. 2005;65(3):681-685.