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ORIGINAL ARTICLE

Immunomodulatory Mechanism of *Cordyceps militaris* Polypeptide through Regulating Gene Hist1h2bp, Ctsg, and Elane in Mice

Guangyu Xu, Guangxin Yuan, Liping An, Peige Du, Liya Xie, Hongyu Li, Yu Sheng, Xiao Han

Department at the College of Pharmacy, Beihua University, Jilin, 132013, China

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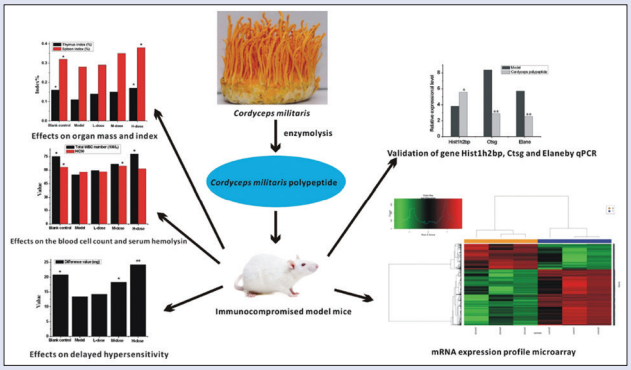
ABSTRACT

Background: *Cordyceps militaris* and *Cordyceps sinensis* belong to the same genus, but the different species, with immunity improvement, antibacterial, and antihypertensive effect, and the studies on the functions of *C. militaris* mainly primarily focus on those of its polysaccharides and polypeptides currently. The latest studies have found that some of the polypeptides with immunomodulatory effect can widely regulate immune functions at multiple levels, improve immunity, and enhance immune functions to ensure the healthy body, showing an important significance. **Materials and Methods:** *C. militaris* polypeptide prepared with the enzymolysis method was taken as the research object in this study. The differentially expressed genes and the related cell signal transduction pathway were screened by mRNA expression microarray. STEM software V1.3.6 (short time-series expression miner, http://www.cs.cmu.edu/jernst/stem/) was used for the clustering of the gene functions, and David and KEGG database were applied for the analysis of the related functions. **Results:** One thousand seven hundred and forty-eight differentially expressed genes were selected finally and three of them were validated by quantitative polymerase chain reaction. The results showed that gene Hist1h2bp, Ctsg, and Elane were involved in the regulation of *C. militaris* on the immune activity of mice. **Conclusion:** Gene Hist1h2bp, Ctsg, and Elane may be the potential targets of *C. militaris* polypeptide, which may provide an important theory basis for the further research and development of *C. militaris* polypeptide.

Key words: *Cordyceps militaris,* mRNA microarray, polypeptide, real-time quantitative polymerase chain reaction, systemic lupus erythematosus

SUMMARY

- Preparation of *Cordyceps militaris* polypeptide by enzymolysis method
- *Cordyceps militaris* polypeptide can improve the immunity of mice
- Gene Hist1h2bp, Ctsg, and Elane may be the potential targets of *C. militaris* polypeptide.



Abbreviations used: *C. militaris*: *Cordyceps militaris*; ConA: Concanavalin A; SRBC: Sheep red blood cells; PCR: Polymerase chain reaction; RT PCR: Real time quantitative; HC50: Half values of hemolysin; CMP: Cordyceps militaris polypeptide; SLE: Systemic lupus erythematosus; GO: Gene Ontology.

Correspondence:

Dr. Guangxin Yuan,
College of Pharmacy, Beihua University, No. 3999 East Binjiang Road, Fengman District, Jilin, 132013, China. E-mail: yuanguangxin2007@163.com
Prof. Peige Du, College of Pharmacy, Beihua University, No. 3999 East Binjiang Road, Fengman District, Jilin, 132013, China.
E-mail: dupeige2001@126.com

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INTRODUCTION

Cordyceps militaris, also known as *Cordyceps* or worm grass, is the complex composed of the fruiting body (the grass part) and the sclerotium (the dead body of insect). *C. militaris* and *Cordyceps sinensis* belong to the same genus, but the different species, with immunity improvement,[1-3] antibacterial,[4,5] and antihypertensive effect.[6] The studies on functions of *C. militaris* mainly primarily focus on those of polysaccharides and polypeptides from *C. militaris*. The latest studies have found that some polypeptides with immunomodulatory activity can widely regulate immune functions from multiple levels, which may be significate to improve immunity and enhance immune functions for the guarantee of human health.[7-11]

The mechanism of immune regulation is the key to maintain the homeostasis of the body and has an important physiological significance to the normal function of the body. Moreover, modern pharmacology studies have shown that *C. militaris* modulate immunity mainly by improving the phagocytes is of macrophages and stimulating the formation of antibodies in the body.[12,13] However, most of studies on the polypeptides of *C. militaris* focus on the immune regulation at an animal level,[2,14,15] not on the signal pathways and related key regulation genes, which may limit the application of them as new drugs or the popularization of them as health products.

With the approach of postgenome era and the application of gene microarray,[16] to screen differential genes and related pathways by

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data mining has become a reality. In this study, taking a *C. militaris* polypeptide prepared by enzymolysis method as the research object, the differential genes and related pathways were screened by mRNA expression microarray, and effects of the *C. militaris* polypeptide on the immune function and the related mechanisms in mice were investigated, in order to provide an important theory basis for the further research and development of *C. militaris* polypeptide.

MATERIALS AND METHODS

Experimental materials

Animal

Male ICR mice (SPF grade), weighing 16–18 g, were purchased from Changchun City Yisi Experimental Animal Technology Co., Ltd (license number: SCXK (Ji)-2011–0004).

Drugs and reagents

C. militaris was purchased in Shenyangnizi *C. militaris* plant base and identified by Pharmacist Chief Jin Yinglan at Institute for Drug Control of Jilin City based on the identification standards specified by Local standard of Chinese herbal medicine in Jilin Province. (2017) *C. militaris* polypeptide from the fruiting body of *C. militaris* was prepared by the enzymolysis with pepsin in our laboratory. The enzymolysis conditions included 37°C, 5 h for the hydrolysis, 2.5% volume of enzyme, 12% substrate concentration (80 mg/ml, measured after the weight loss on drying), and pepsin (activity: 3000–3500 U/mg; Beijing DingGuo Changsheng Biotechnology Co., Ltd.); Yunzhijun Capsule (ShenyangShuangdingPharmaceuticalCo.,Ltd.);petroleumether(60°C–90°C); concanavalin A (ConA); sheep red blood cells (SRBC).

Instruments

THZ-C thermostatic oscillator (Taicang City Experiment Equipment Factory); UV-2550 ultraviolet-visible spectrophotometer (Shimadzu International Trade Co., Ltd.); XE-2100 automatic blood cell analyzer (Japan Sysmex Corporation); infinite M200 microplate recorder (TECAN); 5430R low-temperature high-speed centrifuge (Eppendorf Company, USA); AL204 electronic balance (Mettler-Toledo Instruments Co., Ltd.).

Experimental methods

Animal grouping, drug administration, and model establishment

The experimental animals were fed under the conditions with a relative humidity of 40%–70%, at 20°C ± 1°C and in a sterile environment, with *ad libitum* access to water and food. After they adapted to the environment for 1 week, the mice were randomly divided into blank control group, model group, and low-, medium-, and high-dose *C. militaris* polypeptide (CMP) groups. Mice in low-, medium-, and high-dose *C. militaris* polypeptide were orally given 32, 160, and 800 mg/kg of CMP once daily continuously for 45 days, respectively, and those in the blank control group and model group were given equal volume of distilled water in the same way. In addition to those in the blank control group, mice in the other groups were injected with 40 mg/kg of cyclophosphamide intraperitoneally once daily, successively for 2 days for the establishment of mice immunodeficiency model. The mice's body weights were measured every week regularly for the observation on changes in their body weights, and their general states were also observed at the same time.

Observation of immunological activities

Measurement of organ index

After the last administration, the mice were sacrificed and their body weights were weighed, and then, their thymus and spleen were removed and weighed for the calculation of the ratio of organ/body.

Organ index/% = Organ mass/body mass × 100%

Measurement of blood cells

After the last administration, the blood samples were collected by removing the mice's eyeballs, in which the eyeball on one side was removed with a clean and sterile ophthalmic forceps, the blood was left to freely drop into a tube containing an anticoagulant, the blood dropped into the tube was gently mixed unceasingly during the blood collection process, and about 1 ml of the anticoagulated whole blood was collected in each mouse. Within 24 h, a whole blood cell analyzer was used to detect the total number of white blood cells in the blood samples.

Determination of serum hemolysin

After the last administration, the mice were given 0.2 ml of 2% (v/v) SRBC in intraperitoneal injection for the immunization. Five days later, the mice were sacrificed, and the blood samples were collected in centrifuge tubes by removing eyeballs, left to stand until the full precipitation of serum, and then centrifuged at 2000 r/min for 10 min to obtain the serum. 10 μl of the serum were diluted with 1 ml of 1:5 SA buffer, 100 μl of the diluted serum solution were drawn into a centrifugal tube, and then 50 μl of 10% (v/v) SRBC and 100 μl of the complement (diluted with SA buffer at a dilution ratio of 1:8) were added into the tube, respectively, which was kept in a constant temperature water bath at 37°C for 30 min, and then centrifuged at 1500 r/min for 10 min. 50 μl of the supernatant taken from each tube were placed in wells of another 96-well culture plate, and 150 μl of Drabkin's reagent were added to the wells; in addition, the wells for half value of hemolysin (HC50) were set, in which 12.5 μl of 10%(v/v) SRBC and then 200 μl of Drabkin's reagent were added, which were fully mixed for the measurement of the values of optical density (OD) at the wavelength of 540 nm by a microplate recorder.

HC50 = (OD of samples/OD of SRBC half hemolysis) × dilution ratio

Concanavalin A-induced spleen lymphocyte transformation (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide method)

Spleen cell suspension preparation: After the last administration, the mice were sacrificed, the spleen was removed under a sterile condition, and the spleen cell suspension was prepared and adjusted to the cell concentration of 3×10^6/ml. Lymphocyte proliferation reaction: The cell suspension was added into wells of 24-well culture plates, 1 ml in each well; the wells were divided into two parts, one part for the samples, in which 75 μl of ConA liquid were added to, and another part for the control, and the culture lasted continuously for72 h. According to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide method, the OD values were measured at the wavelength of 570 nm by a microplate recorder. Before the detection, the cell suspension was separated into the wells of 96-well culture plate, and three parallel wells were set for each sample. The difference in the OD between the well with ConA and that without ConA was calculated and used for the evaluation of the proliferation of lymphocytes.

Sheep red blood cells-induced delayed hypersensitivity (paw swelling method)

After the last administration, the mice were intraperitoneally injected with 0.2 ml of 2% (v/v) SRBC. Four days later, the right hind plant was subcutaneously injected with 20 μl of 20% (v/v) SRBC, and 24 h later, the mice were killed and their left and right hind paws were removed and weighed. The difference in the mass between the left and right hind paws was calculated and used for the evaluation of foot swelling degree.

Swelling degree of paw = right hind paw mass (mg)–left hind paw mass (mg)

RNA extraction and quality control

The total RNA of mice's spleen was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD, USA). Briefly, the spleen tissue was ground into powders under the frozen condition with liquid nitrogen, and before the liquid nitrogen was volatilized, the spleen powder was transferred into a 1.5 ml microcentrifuge tube-containing Trizol reagent (500 mg of the liver tissue per 0.5 ml Trizol reagent). The spleen powder in the microcentrifuge tube was drawn and reinjected vigorously several times with a 5 ml disposable syringe until the tissue homogenate was no longer sticky, in favor of the full breaking down of the cells and genomic DNA. The tissue homogenate was left to stand at room temperature for use. According to the ratio of 200 ul chloroform per 1 ml Trizol reagent, chloroform was added to the homogenate. The mixed solution was oscillated violently for 30 s for evenly mixing and then left to stand at room temperature for 5 min. The mixed sample solution was centrifuged at 12000 r/m for 15 min to obtain the supernatant, and the obtained supernatant was carefully transferred to another centrifuge tube, in which it was noticeable not to draw the intermediate phase. 500 ul isopropanol was added to the supernatant, which was left to stand at the room temperature for 10 min for its precipitation, and then centrifuged at 12000r/m for 15 min, and the supernatant was discarded to obtain the precipitation. A volume of 1 ml of 75% ethanol was added to the precipitation for washing the RNA by oscillating it with a vortex, which was left to stand at room temperature for 5 min and then centrifuged at 7500r/m for 5 min to discard the supernatant. Finally, 75% ethanol was added to the precipitation to wash the RNA again, the precipitation solution was centrifuged at 7500r/m for 5 min, the supernatant was removed, and the final precipitation was dried at room temperature, in which the RNA precipitation should not be too dried, otherwise it was not easy to dissolve.

The RNA precipitation was dissolved in an appropriate amount of TE buffer (1 mmol/L Tris, pH 7.6 and 10 mmol/L EDTA). The total mass of RNA was detected by agarose gel electrophoresis. The mRNA was purified with RNeasy mini kit (Qiagen, Valencia, CA, USA). By referring to the kit instructions, the quality of RNA samples was assessed, and the RNA integrity, inhibitors, and DNA contamination were detected. The mass of mRNA was detected by formaldehyde-denatured agarose gel electrophoresis, and the content of mRNA was determined by UV spectrophotometry.

Microarray test

Sample labeling and hybridization

The samples were labeled according to the instructions of Agilent Quick Amp Labeling kit and the hybridization experiment was conducted with Agilent SureHyb.

Data acquisition and standardization

After washing, the microarray was scanned by an Agilent DNA microarray scanner. Agilent feature extraction software (v 11.0.0.1) was used for the acquisition of microarray probe signal values, and Agilent Gene Spring standard GX v12.1 software was applied for the standardization of microarray. The points located outside of the 95% confidence interval were considered to be differentially expressed genes.

Clustering analysis

The clustering analysis of significantly differential genes screened was carried out with STEM 1.3.6 software (short time-series expression miner, http://www.cs.cmu.edu/jernst/stem/). Based on the norms of software operation, the gene list to be analyzed was made, Mouse (European Bioinformatics Institute [EBI]) was selected as the functional annotation gene pool, Mus musculus (ensemble/Biomart) was used for the gene mapping database, and the operation was carried out after setting the number of time expression modules to obtain the expression modules of significantly differential genes (also known as the clustering phase), in which $P < 0.001$ was taken as the screening standard for a significance.

Analysis on the significance in the function of differential genes

Based on NCBI gene ontology database, the GO annotation of these genes was conducted to obtain all GOs in which the genes were involved, Fisher's exact test and Chi-square test were used to calculate the significance level and misjudgment rate of each GO, and the *P* values were calibrated with the misjudgment rate to screen out the significance ($P < 0.05$) of differential genes.[17] The results were artificially analyzed with European Bioinformatics Institute (EBI) database.

Analysis of the function and biological pathway enrichment of differential genes screened by mRNA expression microarray using DAVID

The DAVID database (http://david.abcc.ncifcrf.gov) was opened, and 1748 genes were presented as the gene set for further analysis. At the same time, the corresponding gene identifier (the gene identifier corresponding to the gene name was OFFICIAL_GENE_SYMBOL) was selected; the whole genome of mouse was ticked as the background gene, and then "functional annotation tool" was selected as the analytical tool, through which the results of GO and biological pathway enrichment analysis of differentially expressed genes could be obtained.[18] In the appeared page, "Gene_Ontology" was clicked and then the "Chart" of "GOTERM_MF_FAT" was clicked to download the listed information analysis files, where the significance level $P < 0.05$ was calculated. Moreover, the results of multiple hypothesis tests were corrected and the False Discover Rate (FDR) was obtained. At last, the significant Gene Ontology (GO) of all differential gene enrichment was obtained.

Analysis of the KEGG pathway enrichment of differential genes screened by mRNA expression microarray using DAVID

The DAVID database (http://david.abcc.ncifcrf.gov) and KEGG database (http://www.kegg.jp/) were opened. First, in the website, https://david.ncifcrf.gov/, the Functional Annotation was clicked. In addition, the corresponding gene identifier (the gene identifier corresponding to the gene name was OFFICIAL_GENE_SYMBOL) was selected; the whole mouse genome mice was marked as background genes, and the "functional annotation tool" was selected as the analytic tool, through which the results of GO and biological pathway enrichment analysis of differentially expressed genes were obtained. At last, on that page clicked "Pathways," then clicked "Chart" in "KEGG_PATHWAY" to download the listed information file, where the significance level $P < 0.05$ was calculated from the hypergeometric distribution of the hypergeometric distribution. The results of multiple hypothesis tests were corrected. FDR and significance pathways of all the differences were obtained.

KEGG pathway analysis of lupus erythematosus

All pathways used in this study were downloaded from the KEGG databases.[19] Relevant KEGG pathways of systemic lupus erythematosus (SLE) were downloaded from http://www.kegg.jp/kegg-bin/show_pathway? map05322 in March 2016.

Validation by real-time quantitative polymerase chain reaction

The differentially expressed genes relevant to the regulation of *C. militaris* polypeptide on mice immune functions were validated by real-time quantitative polymerase chain reaction (PCR). The operation of real-time quantitative PCR was strictly in accordance with the manual

Table 1: Primer pairs for the real-time quantitative polymerase chain reaction

Gene name	Sequences of two way primers	Annealing temperature (°C)	Product length (bp)
Hist1h2bp	F: 5' AGTTCCTGACCTAACATGCCTGAG3'	60	103
	R: 5' GCTTCTTGCCATCCTTCTTTTG3'		
Ctsg	F: 5' CCCTGGTATGTAGCAATGTGG3'	60	136
	R: 5' TATCTTGGTGCAAAGCGTCTC3'		
Elane	F: 5' GCTTTGACCCATCACAACTGC3'	60	133
	R: 5' AGACATGGAGTTCTGTCACCCAC3'		

of Real-time PCR Master Mix (SYBR Green, TOYOBO Company), and the instrument used was Roche Light Cycler 1.5. The primer design is shown in Table 1.

Data processing

Statistical Product and Service Solutions (SPSS) 16 statistical software was used for the analysis of data. The single factor analysis of variance and q test of measurement data were conducted with ANOVA procedure, LSD was used for the pairwise comparison, the graded data were analyzed with Relative to an identified distribution unit (Ridit), and it was considered to be a significant difference in statistics when $P < 0.05$.[20]

RESULTS

Effects of *Cordyceps militaris* polypeptide on organ indexes in mice

Effects of the different doses of *C. militaris* polypeptide on mice spleen and thymus indexes are shown in Table 2. The data showed that low-, medium-, and high-dose *C. militaris* polypeptide shave different effects on the immune organs of mice, in which compared with those in the model group, the effects of high-dose *C. militaris* polypeptide were significantly different ($P < 0.05$), indicating that *C. militaris* polypeptide could promote the growth of immune organs in mice.

Effect of *Cordyceps militaris* polypeptide on the blood cell count in mice

Effects of the different doses of *C. militaris* polypeptide on the total number of white blood cells in mice are shown in Table 3. The results showed that the total number of white blood cells of mice in groups treated with the different dose of *C. militaris* polypeptide was higher than that in the model group, and that in high-dose C. militaris polypeptide group was signifcantly higher than that in the model group ($P < 0.05$), indicating that the C. militaris polypeptide could increase the number of white blood cells in mice.

Effect of *Cordyceps militaris* polypeptide on the serum hemolysin in mice

Effects of the different doses of *C. militaris* polypeptide on the serum hemolysin in mice are shown in Table 3. The data showed that half values of hemolysin (HC50) in the serum of mice in groups treated with the different doses of *C. militaris* polypeptide were affected in varying degrees, of which the effect of medium-dose *C. militaris* polypeptide was significantly different compared with that in the model group ($P < 0.05$), suggesting that *C. militaris* polypeptide could increase the content of serum hemolysin.

Effects of *Cordyceps militaris* polypeptide on cellular immune functions in mice

OD difference values of the spleen lymphocyte transformation in low-, medium-, and high-dose *C. militaris* polypeptide groups, the model group, and the blank control group were 0.035 ± 0.014, 0.039 ± 0.021, 0.042 ± 0.014, 0.027 ± 0.022, and 0.053 ± 0.034, respectively. The OD difference values in groups treated with the different doses of *C. militaris* polypeptide showed no significant difference compared with those in the model group ($P > 0.05$), indicating that *C. militaris* polypeptide has no obvious effect on the transformation of spleen lymphocytes in mice. Effects of *C. militaris* polypeptide on delayed hypersensitivity in mice are shown in Table 4. The results showed that the different doses of *C. militaris* polypeptide had effects in varying degrees on the foot swelling in mice, of which the effects of medium- and high-dose *C. militaris* polypeptide were significant compared with those in the model group ($P < 0.05$ and $P < 0.01$), indicating that *C. militaris* polypeptide could increase the degree of SRBC-induced paw swelling and improve the immunity of mice.

Table 2: Effects of *Cordyceps militaris* polypeptide on organ mass and index in mice

Group	Spleen mass (mg)	Spleen index (%)	Thymus mass (mg)	Thymus index (%)
Blank control	90.3±8.3	0.32±0.02*	42.6±8.4	0.16±0.02*
Model	75.1±6.3	0.28±0.03	33.7±7.7	0.11±0.02
CMP				
Low dose	85.5±3.6	0.29±0.02	41.3±4.6	0.14±0.04
Medium dose	95.3±13.1	0.35±0.06	43.1±8.4	0.15±0.02
High dose	112.0±17.0	0.38±0.03*	44.5±9.3	0.17±0.03*

*$P<0.05$ versus model group. CMP: *Cordyceps militaris* polypeptide

Table 3: Effects of *Cordyceps militaris* polypeptide on the blood cell count and serum hemolysin in mice

Group	Total WBC number (10^9/L)	HC50
Blank control	7.54±0.85*	63.4±3.8*
Model	5.50±0.42	57.9±3.9
CMP		
Low dose	5.96±0.60	58.2±4.2
Medium dose	6.68±0.65	64.7±4.1*
High dose	7.81±0.79*	61.6±3.5

*$P<0.05$ versus model group. HC50: Half value of hemolysin; WBC: White blood cell; CMP: *Cordyceps militaris* polypeptide

Table 4: Effects of *Cordyceps militaris* polypeptide on delayed hypersensitivity in mice

Group	Right paw mass (mg)	Left paw mass (mg)	Difference value (mg)
Blank control	166.0±10.3	145.2±9.4	20.8±4.4*
Model	150.7±7.5	137.3±6.1	13.4±1.6
CMP			
Low dose	167.6±13.6	153.5±12.8	14.2±6.4
Medium dose	182.3±11.8	164.0±5.5	18.3±6.3*
High dose	195.7±12.4	171.6±7.5	24.2±4.9**

*$P<0.05$; **$P<0.01$ versus model group. CMP: *Cordyceps militaris* polypeptide

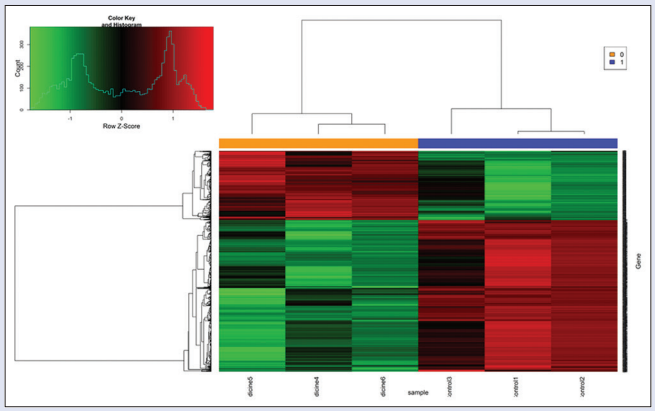

Figure 1: mRNA expression profile microarray. (The red parts mean upregulate, the green parts mean downregulate)

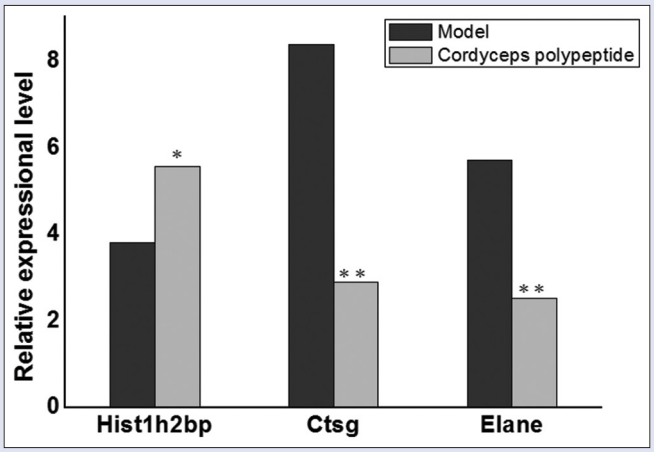

Figure 2: Validation of gene Hist1h2bp, Ctsg, and Elane by quantitative polymerase chain reaction. *$P < 0.05$, **$P < 0.01$: versus model group

Results of mRNA microarray

The results of mRNA expression profile microarray indicated that compared with the model group, there were 1748 differentially expressed genes to tall yin *C. militaris* polypeptide-treated groups, the expression of 533 (30.49%) genes of them was significantly upregulated, and that of 1215 (69.5%) genes of them was significantly downregulated [Figure 1].

As shown in Figure 1, the distinction of microarray between *C. militaris* polypeptide-treated groups and model group was obvious, and *C. militaris* polypeptide lowered the expression level of genes related to immune activities (the expression of 69.51% genes was significantly downregulated). The related document shave demonstrated that the majority of these genes show a negative regulation on immune activities.

Clustering analysis results

The clustering analysis of 1748 differentially expressed genes was conducted with STEM 1.3.6 software first. It was found that most of the differentially expressed genes were closely related to SLE cell signaling pathway.[21] Therefore, the SLE cell signaling pathway was mainly investigated in this study. There were 18 differentially expressed genes in SLE [Table 5], 2 of them showed a significantly higher expression and the other 16 genes showed a low expression. By referring to the literature and the related *P* value in lupus erythematosus systemic, we finally determined the Hist1h2bp, Ctsg, and Elane genes for real-time quantitative PCR validation.

Validation by real-time quantitative polymerase chain reaction

Hist1h2bp, Ctsg, and Elane genes were chosen for the validation by real-time quantitative PCR. As shown in Table 6, genes Ctsg and Elane presented an obvious effect in the spleen of mice with the hypoimmunity induced by cyclophosphamide ($P = 0.0065$ and $P = 0.0024$), a negative regulation on the immune activity (the ratio of *C. militaris* polypeptide-treated group to the model group was 0.35 and 0.44, respectively) [Figure 2]. Hist1h2bp gene showed an obvious effect in the spleen of cyclophosphamide-induced immunodeficient mice ($P = 0.03055$), an upregulation (the ratio of *C. militaris* polypeptide-treated group to the model group was 1.47).

DISCUSSION

Spleen and thymus are important immune organs in the body, and the mass can reflect the level of immune cells in the body to a certain extent. The spleen index and thymus index were used as the preliminary indexes to assess the immune function.[9] Our animal experiments found that *C. militaris* polypeptide could significantly improve the immunity of mice. In fact, many clinical studies[14,15,22-24] have showed that *C. militaris* polypeptide has a regulatory effect on the immune function of mice, but the key factors and regulatory pathways related to the effect have not been investigated fully.

In this experiment, 1748 differentially expressed genes in the spleen tissue were screened with mRNA expression profile microarray and by comparing the effects of *C. militaris* polypeptide with those in the blank control group. Then, most of the differentially expressed genes were found to be closely related to SLE cell signal transduction pathway by the clustering analysis of gene functions with 1.3.6 STEM software. SLE is an autoimmune disease, characterized by the production of a large amount of pathogenic autoantibodies and the involvement of multiple organs and systems, such as skin, kidney, blood system, and nervous system.[25,26] The abnormality of immune system is the important pathogenesis of SLE. The GO annotation and functional analysis of these genes were conducted using NCBI gene ontology database. It was found that 13 of the differentially expressed genes were closely related to nucleosomes, in which Hist1h2bp was not only related to the assembly of nucleosomes but also involved in the DNA replication, transcription regulation, and apoptosis.[27] The other 5 genes were found to be related to neutrophils and nervous system, in which Ctsg[28] and Elane[29] were related to the tissue damage and destruction of neutrophils. Further real-time quantitative RT-PCR was used to validate Hist1h2bp, Ctsg, and Elane. The experimental results demonstrated that these three genes were the key factors in the immunomodulation of *C. militaris* polypeptide in the spleen model of mice.

The main function of Elane gene is a regulatory effect on neutrophils.[30] Some documents also report that Elane is a pathogenic gene for severe congenital neutrocytopenia, and neutrocytopenia can lead to cellular immunosuppression and decreased plasma gamma globulin.[31] The results from PCR (qPCR) [Figure 2 and Table 5] found that Elane in the *C. militaris* polypeptide-treated group versus the model group presented a negative regulation (the ratio of *C. militaris* polypeptide-treated group/model group was 0.35 and the *P* value was 0.00665), suggesting that *C. militaris* polypeptide could inhibit the expression of Elane gene, thereby inhibiting the onset of some immune diseases and significantly improving the immune activity of mice, which was also in consistency with the results of animal experiments.

Table 5: Different expression gens of mRNA microarray in systemic lupus erythematosus

EntrezGeneID	Gene symbol	Regulation	*P*	FDR	Description
319188	Hist1h2bp	Down	0.001585237	0.044128375	Histone cluster 1, H2bp (Hist1h2bp), transcript variant 1
13035	Ctsg	Down	0.006938864	0.081948512	Cathepsin G (Ctsg)
50701	Elane	Down	6.46052E-05	0.01879113	Elastase, neutrophil expressed (Elane)
319170	Hist1h2an	Down	0.011638189	0.099653906	Histone cluster 1
319167	Hist1h2ag	Down	0.00979902	0.093051255	Histone cluster 1, H2ag (Hist1h2ag)
665433	H2ao	Down	0.020550549	0.126704826	Histone cluster 1, H2ao (Hist1h2ao)
232440	H2afj	Down	0.013410374	0.105657709	H2A histone family, member J (H2afj)
360198	Hist1h3a	Down	0.004078971	0.065366441	Histone cluster 1, H3a (Hist1h3a)
665596	Hist1h2bq	Down	0.007220097	0.082638879	Histone cluster 1, H2bq (Hist1h2bq)
319192	Hist2h2aa2	Down	0.007548118	0.083933856	Histone cluster 2, H2aa2 (Hist2h2aa2)
319168	Hist1h2ah	Down	0.01285363	0.104444644	Histone cluster 1, H2ah (Hist1h2ah)
319169	Hist1h2ak	Down	0.008963194	0.090379789	Histone cluster 1, H2ak (Hist1h2ak)
319186	Hist1h2bm	Down	0.003394851	0.061080195	Histone cluster 1, H2bm (Hist1h2bm)
319178	Hist1h2bb	Down	0.000180843	0.022944659	Histone cluster 1, H2bb (Hist1h2bb)
547160	Gm14484	Down	0.044054902	0.181155221	Predicted gene 14484 (Gm14484)
76383	1700012L04Rik	Down	0.012609702	0.103354174	RIKEN cDNA 1700012L04 gene (1700012L04Rik)
97122	Hist2h4	Up	0.022514968	0.132393527	Histone cluster 2, H4 (Hist2h4)
14811	Grin2a	Up	0.002529413	0.053639464	Glutamate receptor, ionotropic, NMDA2A (epsilon 1) (Grin2a)

FDR: False Discover Rate

Table 6: Results of real-time quantitative polymerase chain reaction

Data comparison scheme	Hist1h2bp	Ctsg	Elane
Model	3.81E-04	8.39E-04	5.73E-04
Cordyceps polypeptide	5.58E-04	2.90E-04	2.54E-04
Experiment/control	1.47	0.35	0.44
P	0.03055	0.00665	0.00244

Gene Ctsg encodes CtsG, a proteolytic enzyme,[32] and the proteolytic enzyme is the main inhibitor of neutrophils. The results from real-time quantitative PCR showed a negative regulation of gene Ctsg, indicating that the inhibition of the *C. militaris* polypeptide-treated group could further increase the expression of neutrophils, which should be conducive to improve the immunity of the body. Our experiments confirmed that *C. militaris* polypeptide could improve the index of immune organs, increase the number of white blood cells in the blood, and improve the immune functions in mice, which was in line with the experimental results of gene Ctsg by real-time quantitative PCR.

Histone is a constitutive protein of the chromosome, and histone and the nucleosome composed of DNA are basic structural units of the chromosome.[33,34] Histone with many functions plays an important role in the gene regulation, the proliferation and apoptosis of tumor cells, and the regulation of autoantigens.[35] Hist1h2bp is one of the histones, and gene Hist1h2bp may be related to the body's immune regulation.[36] In addition, we got to know through the KEGG website that gene Hist1h2bp may be involved in the regulation of the phosphatidylserine, and the functions of serine are to ease the pressure and improve the memory and so on. In 2015, neuroscientists at Medical School of University of Virginia found that there were two lymphatic tubes directly connected to the peripheral immune system in the endocranium of mice.[37] Therefore, we speculated that gene Hist1h2bp might be involved in the regulation of the lymphatic system.

It is believed that *C. militaris* polypeptide may regulate the immunity mainly through regulating gene Hist1h2bp, Ctsg, and Elane in mice, in which gene Ctsg and Elane are for the regulation of neutrophils, and gene Hist1h2bp is one of the histones involved in the multiple functions but without obvious specific association with immunity. However, the related studies have found that gene Hist1h2bp is involved in the regulation of phosphatidylserine, and phosphatidylserine may be related to the body's immunity. The regulation of *C. militaris* polypeptide on the immunocompetence of mice has been confirmed by many experiments. This study can further demonstrate at the gene level that the key genes for the immune activity regulation pathway through which *C. militaris* polypeptide can exert its effect should be Hist1h2bp, Ctsg, and Elane, which is expected to provide potential drug targets and theoretical basis for the medicinal application of *C. militaris* polypeptide.

CONCLUSION

Different doses of *C. militaris* polypeptide were intragastrically given to mice. The results showed that *C. militaris* polypeptide could improve the index of immune organs, increase the number of white blood cells in the blood, enhance the delayed type hypersensitivity, and improve the content of serum hemolysis in mice, indicating that *C. militaris* polypeptide can improve the immune function of mice. Furthermore, through the analysis with mRNA expression spectrum chip, the clustering of gene functions with STEM 1.3.6 software, the correlation function analysis based on DAVID database, the functional analysis of KEGG regulation network, and the validation of qPCR experiment, it may be verified that the mechanism of action of *C. militaris* polypeptide should be related to gene Hist1h2bp, Ctsg, and Elane, and the three genes may be the potential targets of *C. militaris*.

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Conflicts of interest

There are no conflicts of interest.

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