

The Benefit of the Optimized Formula of Yinxieling in Psoriasis Vulgaris via Regulation on Autophagy Based on microRNA Expression Profile and Network Pharmacology Analysis

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Background: Psoriasis is a widespread chronic, immune-mediated skin disease with frequent recurrences, and is extremely harmful to the physical and mental health of patients, causing enormous suffering and exerting considerable economic burdens on the health care system as a whole. In more than a decade of clinical use, the optimized formula of Yinxieling (PSORI-CM01) has consistently demonstrated its effectiveness for treating psoriasis. However, its underlying mechanism remains largely unexplored.

Methods: The network pharmacology analysis was conducted to predict the mechanism and protective effect of PSORI-CM01 in treating psoriasis. Subsequently, we collected blood samples from 21 patients with psoriasis as part of a randomized, double-blind, and double-dummy clinical trial for microRNA expression profiling. Finally, it was experimentally confirmed that PSORI-CM01 improved psoriasis by regulating miR-20a-3p and miR-3184-3p expression.

Results: As a result of the network pharmacology analysis, PSORI-CM01 improved psoriasis through the regulation of autophagy, cellular apoptosis, cellular proliferation, and anti-inflammatory processes. In the target-miRNA regulatory network, these key targets were mainly associated with the regulation of hsa-miR-20a-3p, hsa-miR-155-5p, hsa-miR-3184-3p, hsa-miR-328-3p and hsa-miR-124-3p. Based on the microRNA expression profiling results, the PSORI-CM01 treatment group exhibited five up-regulated genes and 16 down-regulated genes compared with the healthy control group. In particular, miR-20a-3p and miR-3184-3p were the primary differentially expressed microRNAs, and they were significantly enriched in the signaling pathways involving autophagy, apoptosis, proliferation, and anti-inflammation. Further experiments confirmed that PSORI-CM01 effectively regulates miR-20a-3p and miR-3184-3p, resulting in increased autophagy.

Conclusion: We demonstrated by combining network pharmacology and clinical studies of miRNA expression profiles in PBMCs that PSORI-CM01 effectively modulated miR-20a-3p and miR-3184-3p, leading to an increase in autophagy and a decrease in keratinocyte proliferation.

Keywords: PSORI-CM01, Psoriasis vulgaris, MicroRNA expression profile, network pharmacology, autophagy

Introduction

Psoriasis is a widespread chronic, immune-mediated skin condition characterized by symmetrical, erythematous, scaling papules and plaques.¹ It is known that there are five main types of psoriasis, based on their morphological characteristics: psoriasis vulgaris, guttate psoriasis, inverse psoriasis, pustular psoriasis, and erythrodermic psoriasis. Psoriasis vulgaris, being the most prevalent type, accounts for approximately 90% of cases.² The disease is characterized by frequent recurrences, significantly impacting the physical and psychological well-being of those affected, causing considerable suffering, and placing a substantial economic burden on the public health system.³ Despite the unclear pathogenesis of psoriasis, it is closely associated with factors such as immune system abnormalities, genetic factors, abnormal proliferation of skin cells, and environmental influences. In particular, the abnormal proliferation of skin cells plays a crucial role in the onset and progression of psoriasis.⁴

Autophagy is a lysosomal-mediated catabolic system that is commonly found in eukaryotic cells and plays a crucial role in cell growth and metabolism, proliferation and differentiation, and inflammatory regulation.⁵ Dysregulated autophagy has been associated with a variety of diseases due to its fundamental role in maintaining cellular homeostasis, including cancer, metabolic disorders, neurodegenerative conditions, cardiovascular and hepatic diseases, infections, and autoimmune disorders.⁶ Moreover, in recent years, an increasing number of studies have identified the role of autophagy in the pathogenesis of skin diseases.^{7,8} The immune cells in psoriatic lesions produce excessive amounts of pro-inflammatory factors, which contribute to chronic inflammation and KC proliferation. Autophagy-related protein ATG16L1 deficiency affects the upstream autophagy mechanism, leading to the production of cytokines, which further results in the accumulation of damaged proteins and organelles, triggering cell death, tissue damage, and inflammation.⁹ However, the specific mechanisms regulating autophagy require further investigation.

A growing body of evidence indicates that micro-RNAs (miRNAs) play a significant role in autophagy regulation.¹⁰ It plays a significant role in regulating a variety of aspects of disease progression, including proliferation and apoptosis of disease-related cells, the release of inflammatory factors, oxidative stress, and signaling pathways.¹¹ Under stressful conditions, certain miRNAs can modulate autophagy by altering the levels of critical autophagy-related proteins within the cells. MiR-20 is known to be significantly expressed in numerous cancer cells, including small cell lung cancer, prostate cancer, and gastric cancer. Notably, recent studies have demonstrated its involvement in platinum resistance in ovarian cancer¹² and its abnormal expression in Hashimoto's thyroiditis¹³ and β -thalassemia in elderly breast cancer patients.¹⁴ Exploring the mechanism of action and function of specific miRNAs related to psoriasis may provide new clues for the treatment of psoriasis.¹¹

At present, the main drugs for patients with psoriasis are corticosteroids, vitamin D analogues, calcineurin inhibitors, and keratolytic. In addition, biological therapy is typically reserved for severe cases due to its high cost and may not be suitable for long-term treatment. Additionally, dermatologists and patients are also looking for safer and more cost-effective treatment options for psoriasis.¹⁵ Psoriasis Optimization Formula (PSORI-CM01) is derived from the "Yinxieling" prescription for the treatment of psoriasis, which has been clinically validated and was developed by Professor Guowei Xuan, a national TCM master. Over 10 years ago, Professor Chuanjian Lu's team improved PSORI-CM01, and it has since been validated through clinical trials and reliable experimentation.^{16,17} It is made up of seven herbs, including *Rhizoma Curcumae* (E Zhu, Chinese), *Sarcandra glabra* (Zhong-Jie Feng, Chinese), *Mume Fructus* (Wu Mei, Chinese), *Rhizoma Smilacis Glabrae* (Tu-Fu Ling, Chinese), *Radix Arnebiae* (Zi Cao, Chinese), *Radix Paeoniae Rubra* (Chi Shao, Chinese), and *Glycyrrhiza uralensis Fisch* (Gan Cao, Chinese), and their ratio is 3:5:5:5:2:3:2.^{16,18} These herbs have the ability to nourish blood, moisten dryness, detoxifying and resolving blood stasis.¹⁹ In our previous research on the PSORI-CM01 intervention for psoriasis patients, the PASI score was improved by 50% in 92.7% of patients. The combination of all the herbs together can promote blood circulation, dispel blood stasis and eliminate blemishes. The results of the study speculate that PSORI-CM01 may improve its pathological manifestations by activating blood circulation.²⁰

Our study utilized a network pharmacology approach to investigate the specific mechanism of PSORI-CM01 in psoriasis treatment and found differences in miRNA expression by miRNA microarray analysis. Additionally, we examined the miRNA expression profiles of peripheral blood mononuclear cells (PBMCs) from patients with psoriasis, as well as PBMCs from a control group before and after treatment with PSORI-CM01.

Materials and Methods

Materials and Reagents

PSORI-CM01 was obtained from the Tianjiang Pharmaceutical Co.Ltd. (Jiangsu province, China, Batch No. 20140815). Ficoll was purchased from Absin Science Co., Ltd (shanghai, China). TRIzol RNA reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, US). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Collecting Compounds of PSORI-CM01 and Their Targets

The Traditional Chinese Medicine Systems Pharmacology Database (TCMSP; <http://lsp.nwu.edu.cn/>) was used to identify the chemical components of PSORI-CM01; the candidate compounds with oral bioavailability (OB) 30% and drug-likeness (DL) 0.18 were identified as the active compounds, indicating the compounds had chemical stability and solubility.

Collecting Targets of Psoriasis and Venn Analysis

The targets of psoriasis were discovered using the Genecards Database (<https://www.genecards.org/>) and DisGeNET database (<https://www.disgenet.org/>), and a relevance score of at least 5 was necessary. Then, a Venn diagram was used to screen the intersection targets between psoriasis targets and targets of PSORI-CM01 compounds. The places where PSORI-CM01 chemicals might have an effect on psoriasis pharmacologically were identified as the intersecting targets.

Herbs-Compounds-Targets Networks Construction

In order to generate a network map that displays the distribution characteristics of PSORI-CM01 active compounds and their targets, the PSORI-CM01 herbs, active compounds, and pertinent targets were uploaded to the Cytoscape 3.1.1 program. The important active compounds were then screened by network topology analysis according to the connection degree value.

Protein-Protein Interaction Analysis

The Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING, <http://string-db.org>; version 11.0) was used to obtain the Protein-Protein Interaction (PPI) network for the species "Homo sapiens", and Cytoscape 3.1.1 software was then used for network topology analysis to screen the significant targets (Hub-Target).

Key Target-miRNA Regulatory Network Analysis

The core target-miRNA regulatory network was constructed using data imported into Cytoscape 3.1.1 software, with regulated miRNAs chosen based on the highest node degree. The MiRNet database (<https://www.mirnet.ca/>), which contains information about miRNAs and regulated genes, was used to collect the key target-miRNA regulatory network.

Gene Ontology Annotation and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analysis

DAVID Database (<https://david.ncifcrf.gov>) was used to undertake a Gene Ontology Annotation Enrichment Analysis (GO) of the screened hub targets, including cellular component (CC), molecular function (MF), and biological process (BP). The "org.Hs.eg.db" package in R software (Version 3.6.3) was used to perform the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The OMICSshare tool web platform (<http://www.omicshare.com/tools>) was used to create enrichment bubbles.

Molecular Docking on the Interaction Between Key Compounds and the Hub Targets

The protein structures of the screened targets were preprocessed using the Discovery studio software (Version 2.5) after being retrieved from the PDB Database (<https://www.rcsb.org/>). The structural data of the main active substances were downloaded and stored in *SDF format from the PubChem Database (<https://pubchem.ncbi.nlm.nih.gov/>). Hydrogens

were added to the aforementioned structures, and Discovery studio 2.5 software estimated the charges. Finally, after docking molecules and proteins using Discovery studio 2.5 software, the model with the greatest binding score was chosen, and Discovery studio 2.5 software carried out structural visualizations of the chosen model.

Preparation of PSORI-CM01

This study's PSORI-CM01 included seven ingredients of Chinese herbs (Table 1). All plant materials were obtained from the Tianjiang Pharmaceutical Co.Ltd. (Jiangyin, Jiangsu province, China). The Guangdong Provincial Laboratory approved the quality control of the PSORI-CM01 granule by coupling liquid chromatography with an LTQ Orbitrap mass spectrometer.¹⁸

Clinical Study

Blood samples were taken from psoriasis patients and healthy participants at the Guangdong Provincial Hospital of Chinese Medicine's Department of Dermatology. A clinical trial that was double-blind, randomized, and double dummy was used for the investigation. The previous study's findings were compatible with the diagnostic criteria for the psoriasis. Psoriatic patients' demographics and the PASI (Psoriasis Area and Severity Index) were covered in detail in Table 2. The human investigation was conducted in compliance with the Code of Ethics of the World Medical Association (Trial registration ChiCTR-TRC-14005185 was registered on August 8, 2014). The Clinical Research Ethics Committee of the Guangdong Provincial Hospital of Traditional Chinese Medicine's ethical guidelines were followed in all procedures involving human subjects (Ethical approval No. B2014-026-01). An informed consent form was initially needed of all patients who were enrolled. The aim of the study and available treatments were then discussed by the researchers. Following that, the samples were gathered.

Group Intervention

The previously stated treatments and procedures were described in detail.²⁰ Participants in the PSORI-CM01 treatment group consumed 5.5 g of PSORI-CM01 granules twice daily after meals, whereas those in the control group consumed 5.5 g of placebo granules three times daily for a period of 12 weeks.

Table 1 Constituents of PSORI-CM01

Linnean Classification	Botanical Origin	Ratio
<i>Rhizoma Curcumae</i>	<i>C. kwangsiensis</i> S. G. Lee et C. F. Liang	3
<i>Sarcandra glabra</i>	<i>Sarcandra glabra</i> (Thunb.) Nakai	5
<i>Fructus Mume</i>	<i>Prunus mume</i> (Sieh.) Sieb. et Zucc.	5
<i>Rhizoma Smilacis Glabrae</i>	<i>Smilax glabra</i> Roxb.	5
<i>Radix Arnebiae</i>	<i>Arnebia euchroma</i> (Royle) Johnst.	2
<i>Radix Paeoniae Rubra</i>	<i>Paeonia lactiflora</i> Pall.	3
<i>Glycyrrhiza uralensis Fisch</i>	<i>Glycyrrhiza uralensis</i> Fisch.	2

Table 2 Clinical Serum Samples for miRNA Analysis

Index	Healthy Controls (n=9)	PSORI-CM01 Group	
		0 Week (n=6)	12 Week (n=6)
Age	39.52±10.31	42.37±11.28	42.37±11.28
Male	5 (55.56)	3 (50.00)	3 (50.00)
Female	4 (44.44)	3 (50.00)	3 (50.00)
PASI	/	7.21±3.16	6.37±3.86

Sample Collection and Processing

10 mL of blood in heparin-anticoagulant tubes were drawn from each subject's cubital vein. Lymphocyte separation solution was used to isolate PBMCs using the Ficoll density gradient centrifugation method.

RNA Extraction

The total RNA was extracted using the TRIzol reagent per the manufacturer's instructions, and then purified using the miRNeasy Micro Kit and RNase-Free DNase Set. At each RNA extraction, total RNA sample was used as the positive control, and RNase-free water was used as the negative control. We evaluated the integrity of extracted total RNA using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

MicroRNA Chip Scanning

12 psoriasis patients and 9 healthy controls had their PBMCs samples taken. Prior to and following the PSORI-CM01 intervention, 6 blood samples were collected. 21 of the PBMCs samples were then collected and put through an Agilent microarray chip analysis (Human miRNA, Release 21.0). Total RNA was quantified via a spectrophotometer (NanoDrop ND-2000, Thermo Scientific, MA, USA). Following reverse transcription to cDNA, the samples were purified and labeled with cyanine 3-CTP. In the microarray chip, the samples were then hybridized. Finally, the Agilent Scanner G2505C was used to scan the microarray expression (Agilent Technologies, Santa Clara, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

According to the manufacturer's instructions, SuperScript IV Reverse Transcriptase (Thermo Fisher, Dalian, China) reverse transcribed the RNA samples into cDNA. The following PCR amplification settings were used: 40 cycles of 94 °C for 20s and 60 °C for 34s during the initial 15 min of denaturation at 95 °C. SYBR Green PCR Kits were used to look at the expression of microRNAs.

Cell Culture

The Cell Culture Unit of Shanghai Science Academy provided the HaCaT cell line with its source (Shanghai, China). The cells were cultivated in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA) at 37 °C in a humidified environment with 5% CO₂.

Cell Transfection

The mimics and inhibitor of MiR-20a-3p, besides their negative controls were designed and synthesized by RiboBio Inc. (Ribobio, Guangzhou, China). The sequences of miR-20a-3p mimics: sense strand: 5' ACUGCAUUAUGAGCACUAAAG 3' and antisense strand: 5' CUUUAAGUGCUCAUAAUGCAGU 3', miR-20a-3p inhibitor is single-strand: 5' CUUUAAGUGCUCAUAAUGCAGU 3'. The above-mentioned steps for cell transfection were carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. HaCaT cells were transfected with miR-20a-3p mimics, with the final concentration of its negative control being at 100 nM, or miR-20a-3p inhibitor, with the final concentration of its negative control being at 200 nM.

MTT Assay

The MTT assay was used to measure cell viability. Tumor necrosis factor (TNF) was added at a concentration of 20 ng/mL after the cells were seeded into 96-well plates with 10% FBS in MEM at a density of 5000 cells per well. MiR-20a-3p mimics (control mimics) or inhibitors (control inhibitors) were added to the wells after 12 hours of incubation, and then the wells were left to sit for 24 hours. The 96-well plates were incubated for 4 hours after the MTT solution had been applied in 10 L to each well. Finally, 0.04 N HCl in isopropyl alcohol was used to lyse the cells, and the absorbance in 96-well plates was measured at 570 nm.

Statistical Analysis

SPSS 19.0 software (Chicago, IL, USA) was applied to statistical analysis, which was visualized using GraphPad Prism 5.0 (La Jolla, CA, USA). Results were expressed as means \pm standard deviation (SD). For data comparisons between groups, one-way ANOVA was employed, then Duncan's test, $P < 0.05$ was deemed statistically significant.

Results

Collecting of Chemical Ingredients and Their Targets of PSORI-CM01

According to the TCMSP Database, 169 chemical ingredients of PSORI-CM01 were collected according to $OB \geq 30\%$ and $DL \geq 0.18$, including 29 in *Radix Padoniae Rubra*, 10 in *Herba Sarcandrae*, 3 in *Curcuma phaeocaulis Valeton*, 8 in *Dark Plum Fruit*, 12 in *Lithospermum erythrorhizon Sieb. et Zucc*, 15 in *Rhizoma Smilacis Glabrae*, and 92 in *Glycyrrhiza uralensis Fisch*. Then, 154 active compounds with 258 targets were selected after removing duplicate targets. In order to screen the hub active components of PSORI-CM01, a network of herb-compounds-targets was constructed. This network, which has 420 nodes and 2194 edges with an average degree of 10.45, is depicted in Figure 1A. Nine hub components in all were discovered, and their node degrees were three times higher than the typical node degree, including 139 in MOL000098, 65 in MOL000422, 40 in MOL004328, 40 in MOL003896, 39 in MOL000358, 35 in MOL004373, 34 in MOL002565 and 33 in MOL000392. In the network's findings, these 9 hub components were identified as potential compounds of PSORI-CM01 that could be used for the treatment of psoriasis.

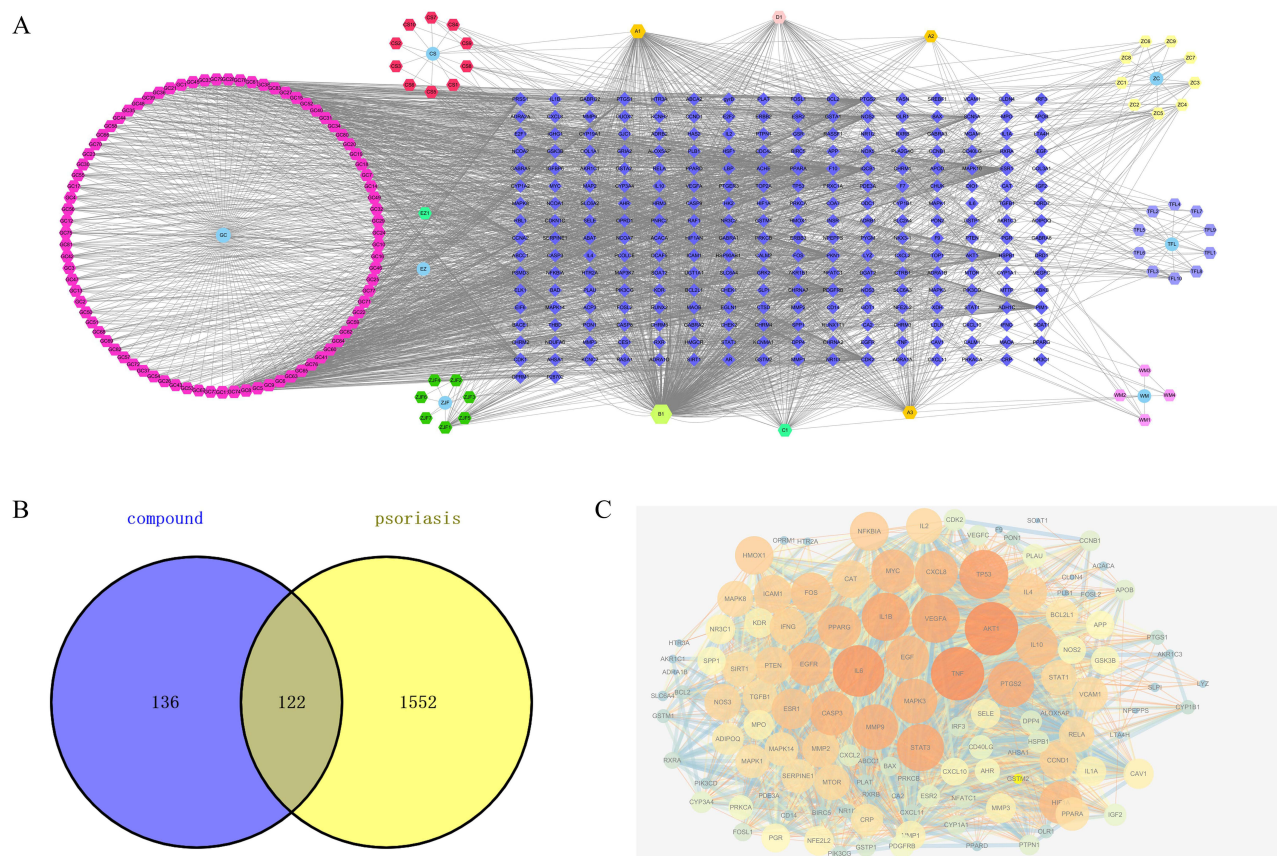


Figure 1 Activated Chemical Composition, targets of PSORI-CM01 and target of psoriasis. **(A)** Herb-compounds-targets network of PSORI-CM01; **(B)** Venn analysis of targets between targets of PSORI-CM01 compounds and target of psoriasis; **(C)** protein-protein interaction (PPI) network of intersection targets.

Identifying Targets of PSORI-CM01 Acting on Psoriasis

1308 targets of psoriasis and 906 targets of psoriasis were collected from DisGeNET database and GeneCards database with relevance scores no less than 5, respectively. A total of 1674 psoriasis diseases targets were selected after duplication. Subsequently, using Venn analysis, 122 intersection targets between PSORI-CM01 candidate targets and psoriasis targets were screened (Figure 1B). The intersecting targets were believed to be the targets operating on psoriasis from the PSORI-CM01 candidate targets.

Protein–Protein Interaction Analysis

As shown in Figure 1C, 122 intersection targets were analyzed by PPI network analysis to illustrate the interaction between them. There were 122 nodes and 2347 edges in this network. The shared targets had an average degree node of 38.46. Nine hub targets in all were found, and they had node degrees that were twice as high as the average node degree in this network (94 in TNF, 94 in AKT1, 90 in IL6, 85 in TP53, 83 in IL1B, 83 in VEGFA, 81 in STAT3, 79 in PTGS2 and 78 in MMP9).

GO and KEGG Enrichment Analysis of PSORI-CM01

The DAVID Database was used to conduct GO enrichment analysis to look at the enrichment of the related targets in biological processes (BP), cellular components (CC), and molecular function (MF). The primary functional modules in BP, as depicted in Figure 2A, included cell proliferation, apoptosis, aging, immunological response, autophagy, and

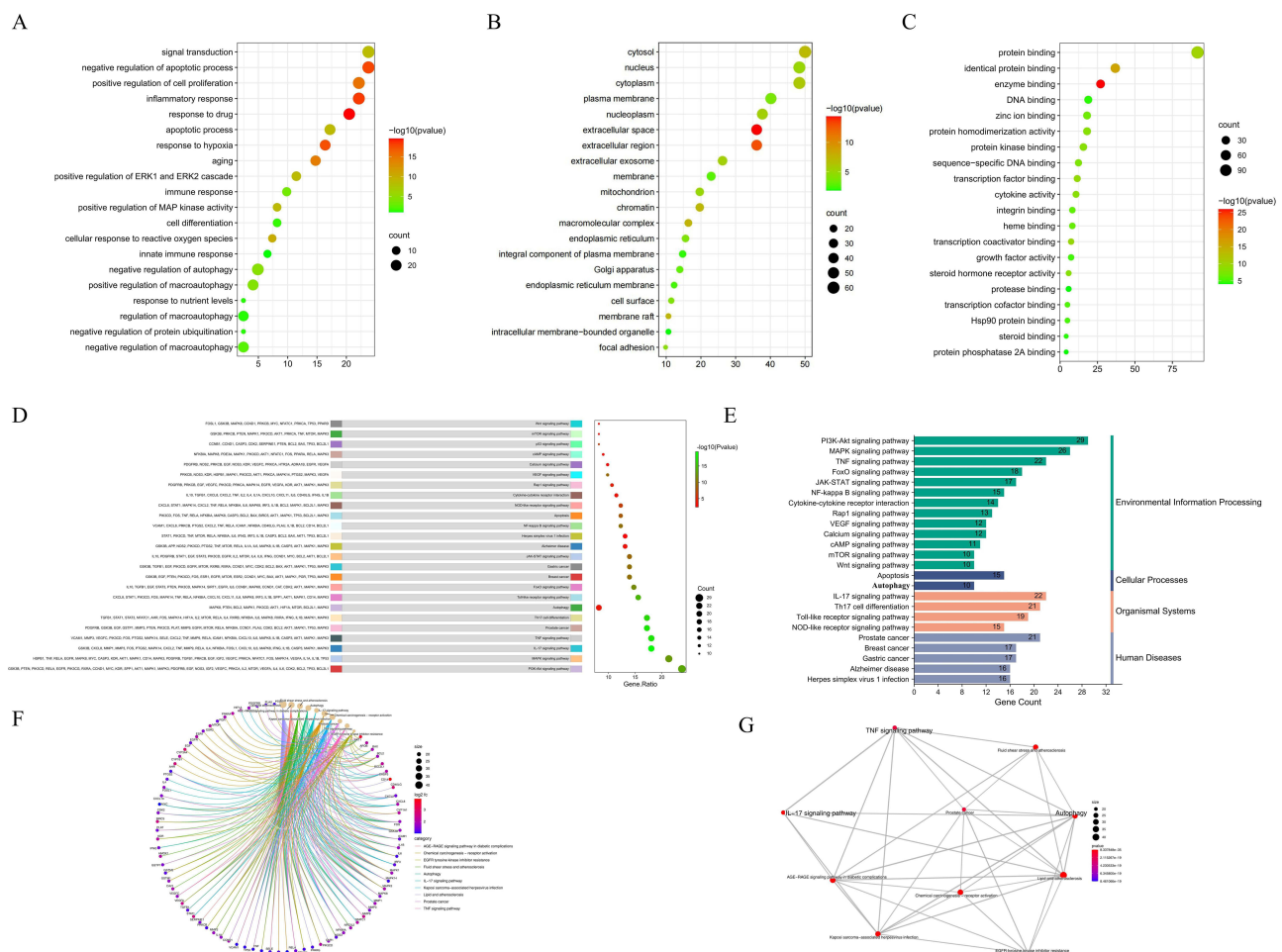


Figure 2 Go function and KEGG pathway enrichment analysis of PSORI-CM01 in the treatment of psoriasis. (A) Biological processes (BP); (B) cellular component (CC); (C) molecular function (MF); (D) KEGG pathway enrichment of the intersection targets; (E) Categorization of KEGG pathway enrichment; (F) gene-concept network analysis on KEGG enrichment; (G) enrichment map analysis on KEGG enrichment.

ERK1 and ERK2 cascade, among other processes. The related targets enriched in CC comprised the cell surface, extracellular space, mitochondria, plasma membrane, cytosol, nucleus, and cytoplasm (Figure 2B). The key components of MF enrichment were protein binding, enzyme binding, DNA binding, and receptor binding (Figure 2C). Previous studies have validated that cell proliferation, apoptotic process, aging, immune response and autophagy participates the pathological process of psoriasis, but there is no literature exploring whether PSORI-CM01 exert pharmacological effect via these biological processes.

To investigate the molecular mechanism of PSORI-CM01 involving psoriasis therapy, KEGG enrichment analysis was employed the mechanisms mainly included PI3K-Akt signaling pathway, MAPK signaling pathway, IL-17 signaling pathway, TNF signaling pathway, and Autophagy etc (Figure 2D). Figure 3E suggested that intersection targets mainly participated in the environmental information process, which was in consistent with the pathology of psoriasis. A study of the gene-concept network (Figure 2F) and enrichment map (Figure 2G) revealed that the PSORI-CM01 may have been caused by the autophagy signaling pathway.

Molecular Docking Results

According to network topology analysis, MOL000098, MOL000422, MOL004328, MOL003896, MOL000358, MOL004373, MOL002565 and MOL000392 were identified as the key active components of PSORI-CM01. The major targets of PSORI-CM01 for treating psoriasis include TNF, AKT1, IL6, TP53, IL1B, VEGFA, STAT3, PTGS2, and MMP9. To better understand the interactions between the key targets and active ingredient, these targets were docked with the discovered component. According to the findings (Figure 3A–C), the three medicines MOL000098, MOL000422, and MOL004328 may have a pharmacological effect on psoriasis by targeting IL6, TP53, and STAT3 respectively.

Key Target-miRNA Regulatory Network

As TNF, AKT1, IL6, TP53, IL1B, VEGFA, STAT3, PTGS2 and MMP9 were identified as the key target of PSORI-CM01 involving the treatment of psoriasis. Target-miRNA regulatory network showed that these key targets were mainly associated with regulation of hsa-mir-20a-3p, hsa-mir-155-5p, has-miR-3184-3p, hsa-mir-328-3p and hsa-mir-124-3p (Degree \geq 6), as showed in Figure 3D.

Differentially Expressed miRNAs Between Groups

Before treatment, compared with the control group by an FDR_{BH} < 0.05 and fold change > 2, we found that 184 miRNAs were differentially expressed in the PSORI-CM01 group (0 week), including 81 up-regulated and 103 down-regulated. After PSORI-CM01 treatment (12 weeks), 284 differentially expressed miRNAs were observed in comparison with healthy control. There were 96 up-regulated and 188 down-regulated. However, only 21 miRNAs were differentially expressed before and after treatment with PSORI-CM01, of which 5 were up-regulated, 16 were down-regulated (Table 3).

Differences in miRNAs Expression Profiles in the PSORI-CM01 and the Control Groups

The differences in miRNA expression between the PSORI-CM01 group and the control group were shown in heat maps and volcanic maps (Figure 4A–F) before and after treatment. In the volcano plot, red represented up-regulated genes, and green was the downregulation. The larger the Fold Change value, the more significant the difference in miRNA expression between groups. In the heatmap, each small square meant a miRNA, and its color indicated the level of miRNA expression. The darker the color, the higher the expression level (red for up-regulation, green for down-regulation). Each column represented the expression level of all miRNAs in each sample, whereas each row represented the expression level of each miRNA in various samples.

Pathway Enrichment Analysis for Differentially Expressed miRNAs

In order to identify the biological function or signalling pathway which may be influenced by the differentially expressed miRNAs, we set up a KEGG enrichment analysis through the David Online tools. We unearthed that the top 10 critical signal pathways targeted by PSORI-CM01 were as follows: pluripotency of stem cells signalling, axon guidance, MARK

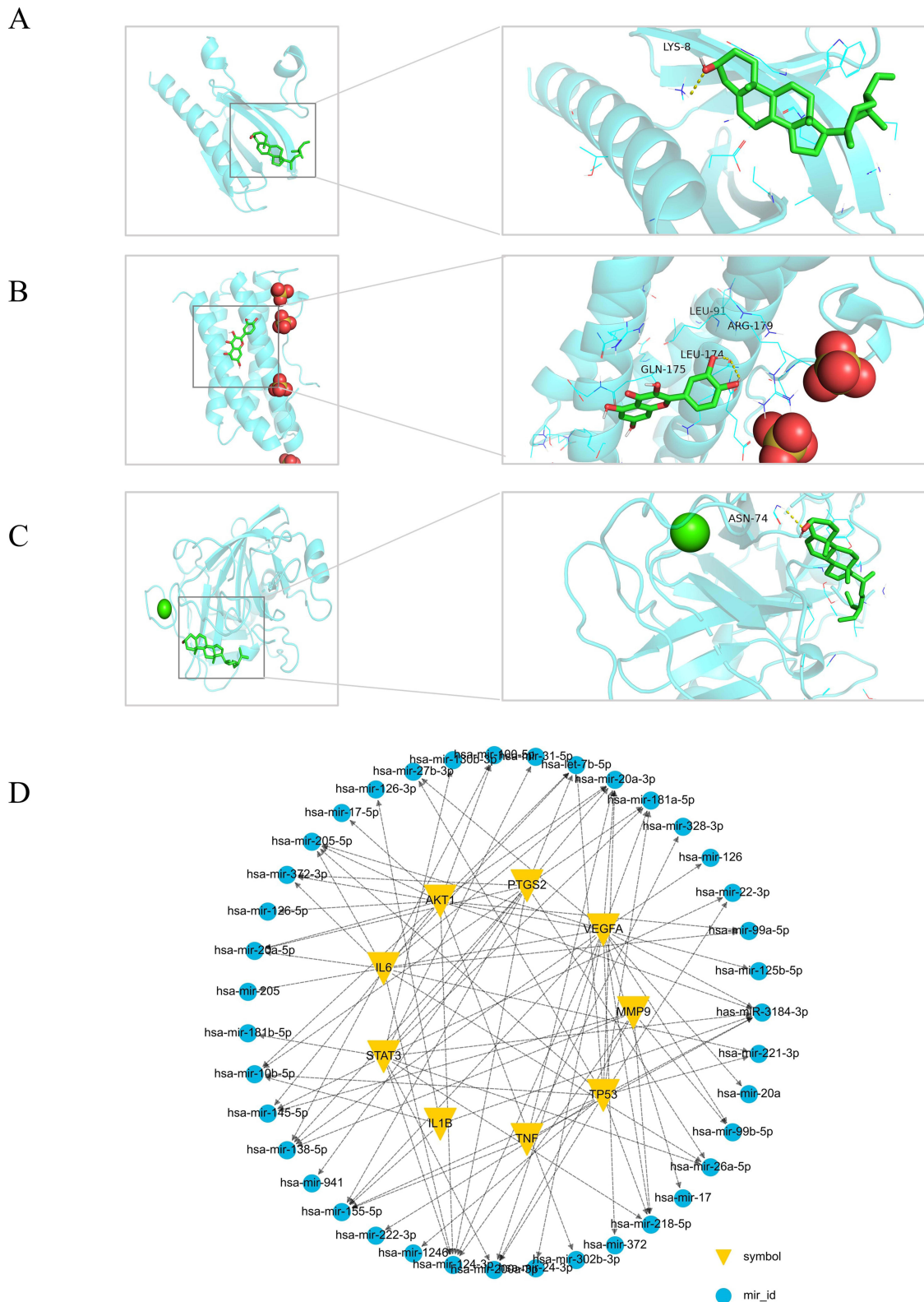


Figure 3 Molecular Docking PSORI-CM01 components docked with key targets. **(A)** MOL000098 docked with IL6; **(B)** MOL000422 docked with TP53; **(C)** MOL004328 docked with STAT3. **(D)** Key Target-miRNA Regulatory Network.

Table 3 Analysis of Differentially Expressed miRNAs Between Groups

Group	Sample Size	Differential miRNAs	Up-regulated miRNAs	Down-regulated miRNAs
A vs B	6 vs 9	184	81	103
B vs C	6 vs 9	284	96	188
A vs C	6 vs 6	21	5	16

pathway, proteoglycans in cancer, breast cancer, PI3K-Akt signalling, Wnt pathway, focal adhesion, mTOR signalling and gastric cancer (Figure 5A and B).

Verification of Selected microRNAs

Seven differentially expressed miRNAs including miR-20a-3p, miR-3184-3p, miR-487a-3p, miR-4793-3p, miR-3653-3p, miR-5681b and miR-26a-5p were found through microarray analysis, which were validated further by qRT-PCR (Figure 5C). In this study, we observed changes in the expression levels of specific microRNAs following a 12-week treatment with PSORI-CM01. Among these microRNAs, miR-20a-3p, miR-3184-3p, miR-487a-3p, miR-3653-3p, miR-5681b, and miR-26a-5p exhibited an upregulation, while miR-4793-3p showed a downregulation.

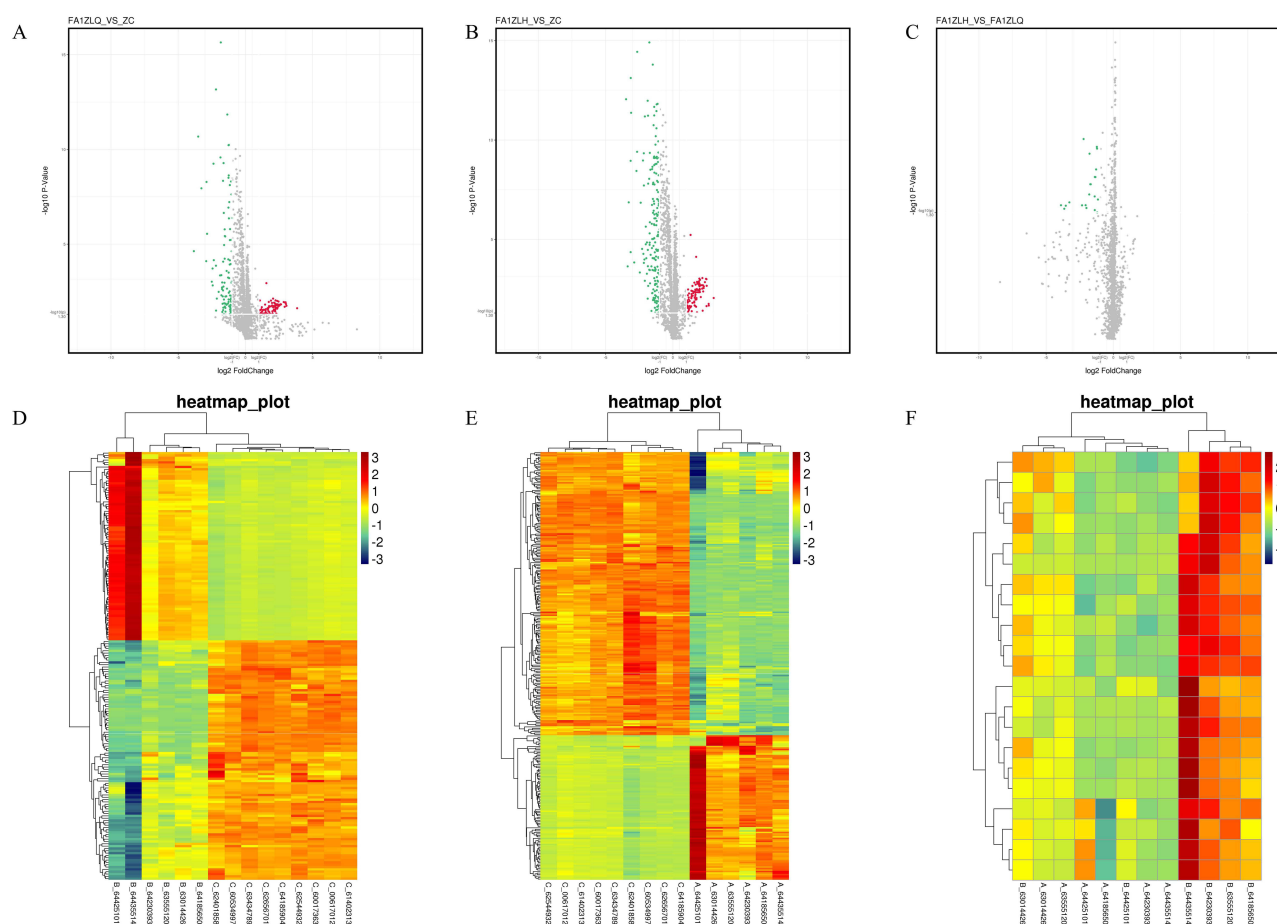


Figure 4 Unsupervised hierarchical cluster analysis of differentially expressed miRNAs before and after PSORI-CM01 treatment. (A) Volcanic map of PSORI-CM01 group before treatment versus the control group; (B) Volcanic map of PSORI-CM01 group after treatment versus the control group; (C) Volcanic map of PSORI-CM01 group after treatment versus PSORI-CM01 group before treatment; (D) Heat map of PSORI-CM01 group before treatment versus the control group; (E) Heat map of PSORI-CM01 group after treatment versus the control group; (F) Heat map of PSORI-CM01 group after treatment versus PSORI-CM01 group before treatment.

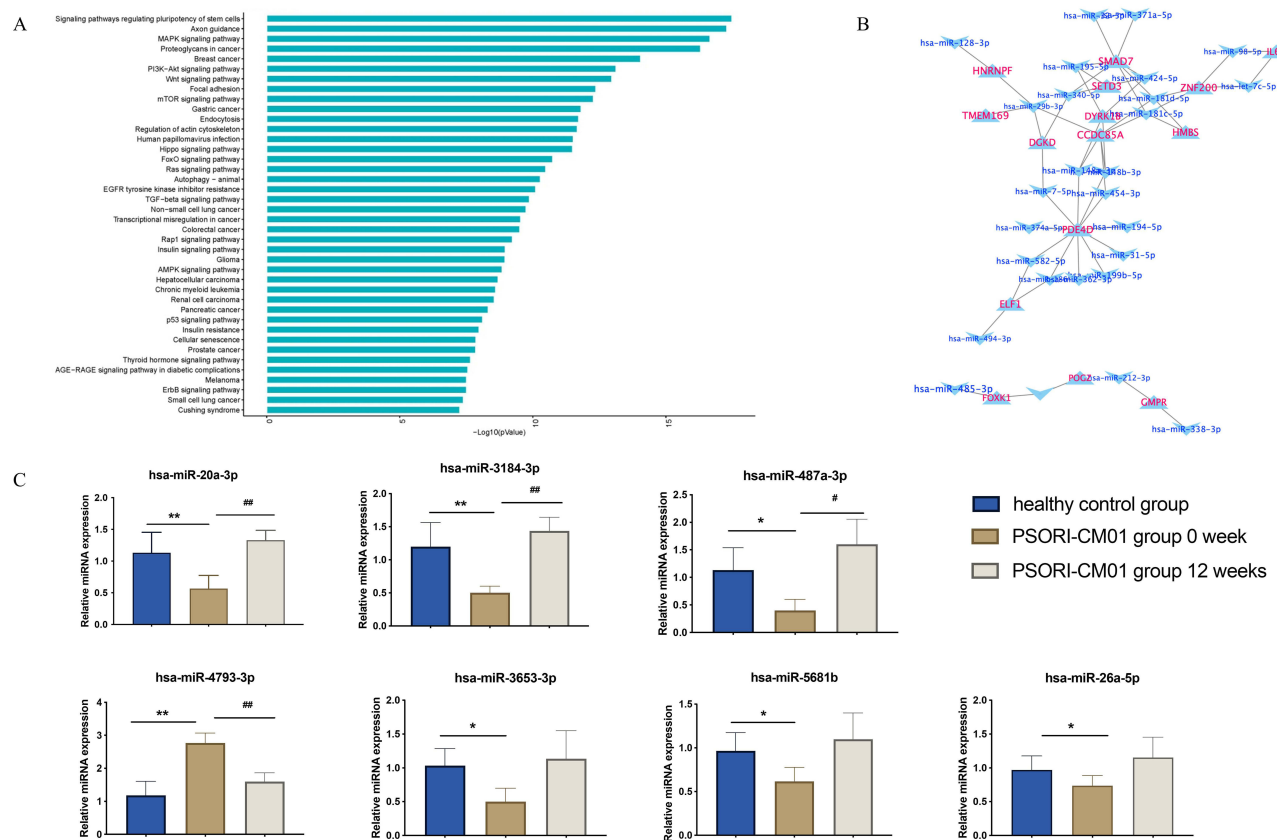


Figure 5 Pathway enrichment analysis for differentially expressed miRNAs and Verification of selected microRNAs. (A and B) Pathway enrichment analysis for differentially expressed miRNAs. (C) The miRNAs validation of miR-20a-3p, miR-3184-3p, miR-487a-3p, miR-4793-3p, miR-3653-3p, miR-5681b and miR-26a-5p by qRT-PCR. (* $P < 0.05$, ** $P < 0.01$ vs PSORI-CM01 group 0-week group; # $P < 0.05$, ## $P < 0.01$ vs PSORI-CM01 group 12 weeks group).

MiR-20a-3p Inhibits TNF- α Induced Keratinocyte Proliferation

In this study, we further investigated the induction of autophagy and the inhibition of cell proliferation by PSORI-CM01. To validate this effect, we conducted intervention experiments using inhibitors and mimics of miR-20a-3p and miR-3184-3p. The Results demonstrated that under the stimulation of miR-20a-3p and miR-3184-3p inhibitors, the autophagic process was suppressed, but when PSORI-CM01 was administered, no significant change in autophagy levels was observed. However, under the stimulation of miR-20a-3p and miR-3184-3p mimics and with the administration of PSORI-CM01, the autophagic process was promoted, and cell proliferation was effectively inhibited (Figure 6). Therefore, we propose that PSORI-CM01 treatment in psoriasis involves regulating the expression of miR-20a-3p and miR-3184-3p to promote the autophagic process while inhibiting cell proliferation.

Discussion

Psoriasis is a chronic autoimmune inflammatory disease caused by various factors that result in the excessive proliferation and abnormal differentiation of epidermal keratinocytes (KCs) and abnormal T-cell function. KCs have the ability to release a variety of cytokines that regulate growth, differentiation, and inflammation, thereby activating and regulating the skin's immune response.²¹ Although current treatment options such as topical agents, oral agents, biological agents, light therapy, and combination therapy can induce remission in most psoriasis patients, these therapeutic drugs cannot fully cure psoriasis, and the condition remains prone to relapse.²² Additionally, prolonged use of these drugs often leads to significant adverse reactions in patients. Currently, there is no curative treatment available for psoriasis, and the primary aim of treatment is to control the disease, minimize skin damage, prevent disease recurrence, and enhance the quality of life for patients. Therefore, it is urgent to develop a new

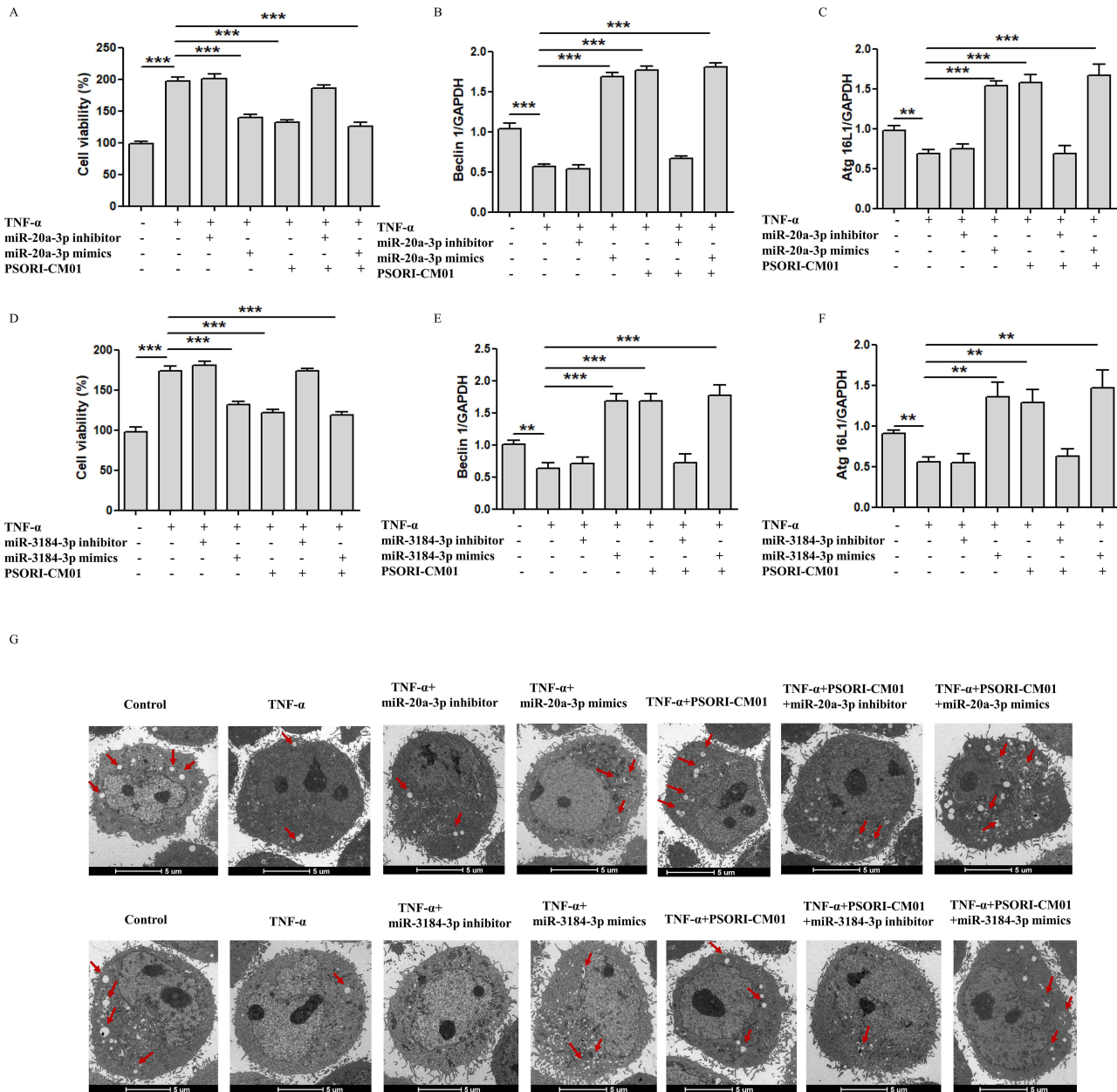


Figure 6 miR-20a-3p and miR-3184-3p induces autophagy and inhibits proliferation of TNF-α induced keratinocytes. (A) Inhibitors and mimics of miR-20a-3p on HaCaT cells were detected by MTT; (B and C) Expression of Beclin1 and ATG16L1 evaluated by qRT-PCR after inhibitors and mimics of miR-20a-3p; (D) Inhibitors and mimics of miR-3184-3p on HaCaT cells were detected by MTT; (E and F) Expression of Beclin1 and ATG16L1 evaluated by qRT-PCR after inhibitors and mimics of miR-3184-3p; (G) Autophagosomes in HaCaT was observed by TEM (Scale bar = 5 μm; red arrow: autophagosome); (**P < 0.01, ***P < 0.001 vs TNF-α).

therapeutic strategy with higher efficacy and fewer side effects to treat psoriasis, and Traditional Chinese medicine plays an important and active role in the treatment of the condition.²³

The PSORI-CM01 is a clinical treatment for psoriasis developed by Professor Xuan Guowei, a renowned medical expert. The preliminary findings of our research have also demonstrated the potential of PSORI-CM01 in ameliorating the symptoms of psoriasis, which includes significant reductions in PASI scores, BSA, VAS, and DLQI.²⁴ To gain deeper insights into the specific mechanisms through which PSORI-CM01 treats psoriasis, we employed network pharmacology, and our analysis revealed that PSORI-CM01 exerts its therapeutic effects by modulating autophagy, cellular apoptosis, cellular proliferation, and anti-inflammatory processes. Target-miRNA regulatory network showed that these key targets were mainly associated with regulation of hsa-miR-20a-3p, hsa-miR-155-5p, has-miR-3184-3p, hsa-miR-328-3p and

hsa-miR-124-3p. Forty-three patients with psoriasis provided randomized blood samples, and subsequently, a double-blind and double-dummy clinical trial was conducted to analyze microRNA expression profiles. The microRNA expression profile results indicated that, in comparison to the healthy control group, the PSORI-CM01 treatment group exhibited up-regulation of 5 genes and down-regulation of 16 genes. Among these genes, miR-20a-3p and miR-3184-3p were the primary differentially expressed microRNAs. Additionally, they were significantly enriched in the signaling pathways associated with autophagy, cellular apoptosis, cellular proliferation, and anti-inflammation processes.

Previous studies have shown that miR-20a is crucial for the development of several cancers, including lung cancer cells,²⁵ breast cancer cells,²⁶ and prostate cancer cells.²⁷ MiR-20a-3p is another mature microRNA product produced from the precursor miR-20a stem-loop structure. Traditionally, researchers believed it to be non-functional. However, growing evidence suggests that miR-20a-3p can indeed play functional roles in normal cellular behavior.²⁸ In psoriasis and IL-22-induced keratinocyte proliferation, Li R et al¹² investigated at the potential functional involvement of miR-20a-3p. They found that miR-20a-3p was down-regulated in both human keratinocyte cell line HaCaT and psoriatic lesions. MiR-20a-3p is essential for psoriasis, as shown by the possibility that keratinocyte hyperproliferation and aberrant apoptosis result from miR-20a-3p deletion in psoriasis.

Currently, majority of research have shown that miR-3184-3p is closely related to tumor^{29,30} and cardiovascular related aspects.³¹ Xu H et al²⁹ observed that miR-3184-3p was enriched in cerebrospinal fluid (CSF) exosomes of glioma patients but downregulated after tumor resection. They showed that miR-3184-3p inhibits apoptosis in glioma cells while directly promoting proliferation, migration, and invasion. These findings uncover a new mechanism and underscore the significant role of miR-3184-3p in glioma progression. Moreover, Liu et al³¹ explored the molecular mechanism underlying the therapeutic effects of total flavonoids from *Dracocephalum moldavica* L. (TFDM) in vascular dementia (VaD) through bioinformatics analysis and experimental validation. Their study revealed that TFDM and its active compounds, including tilianin, luteolin, and apigenin, significantly upregulated miR-3184-3p and downregulated miR-6875-5p in oxygen-glucose deprivation (OGD)-injured cells, leading to improved cell viability.

This study employed TNF- α stimulation on HaCaT cells for in vitro research, resulting in the stimulation of inflammatory cytokine production, thereby triggering the accumulation of inflammatory cells in the dermal and epidermal layers, leading to psoriasis lesions. Psoriasis is an inflammatory skin disease characterized by abnormal epidermal keratinocyte proliferation and infiltration of inflammatory cells.¹ Recent research has increasingly demonstrated the critical role of autophagy in regulating the pathological processes of psoriasis.⁸ Autophagy primarily functions by degrading excessive or aberrant keratinocytes and inflammatory mediators, which helps alleviate psoriatic skin lesions and inflammation.⁷ Consistent with our findings, our study also revealed that PSORI-CM01 significantly promotes autophagy to inhibit cell proliferation. To further validate the results of network pharmacology and miRNA microarray analysis, we employed mimics and inhibitors of miR-20a-3p and miR-3184-3p in our research, confirming that PSORI-CM01 exerts its therapeutic effects through the mediation of cell autophagy via miR-20a-3p and miR-3184-3p.

Although the number of available drugs is continuously growing, there still exist additional limitational such as predicting side-effect profile, immunogenicity, and primary lack or secondary loss of efficacy; hence, long-term safety and efficacy are sometimes impaired. Chinese medicine has demonstrated its role in treating psoriasis by regulating various miRNAs, making it highly promising to explore its genetic perspective for psoriasis treatment.³² Although the efficacy of PSORI-CM01 in the treatment of psoriasis is clear, it has the characteristics of multi-component, multi-target, multi-pathway and multi-function.¹⁶ By elucidating the intricate mechanisms through which PSORI-CM01 exerts its therapeutic effects, our research contributes to the development of personalized treatment strategies tailored to the genetic makeup and specific needs of individual patients. This personalized approach holds immense promise in optimizing treatment outcomes, minimizing adverse effects, and enhancing the overall quality of life for patients battling with psoriasis.³³ Moreover, our findings underscore the importance of integrating traditional Chinese medicine practices into mainstream treatment protocols, offering a comprehensive and integrative approach to managing psoriasis that addresses its multifactorial nature.³⁴

Our study acknowledges limitations, primarily due to the small sample size, constrained by the availability of clinical samples. While our findings provide valuable insights, further investigation with larger sample sizes is necessary to strengthen the robustness and generalizability of our conclusions. Additionally, it's noteworthy that autophagy, a pivotal cellular self-repair process, is governed by a network of signaling pathways, such as PI3K/AKT/mTOR, AMPK/mTOR,³⁵ and ROS pathways.³⁶

Although our study mainly focuses on the regulatory impact of PSORI-CM01 on miRNA in autophagy, there's a recognition of the necessity to broaden our exploration to include other autophagy-regulating signaling pathways. To comprehensively understand the regulatory mechanisms behind PSORI-CM01's therapeutic effects, future investigations should analyze its effects on these additional pathways, enabling the establishment of a more comprehensive theoretical framework and enhancing the clinical applicability of PSORI-CM01.

Conclusion

In this study, we thoroughly elucidated the therapeutic mechanism of PSORI-CM01 in treating psoriasis by employing a comprehensive approach involving network pharmacology, miRNA microarray analysis. Furthermore, we investigated the miRNA expression profiles of PBMCs in patients with psoriasis before and after treatment with PSORI-CM01 preparations, as well as in a control group. We experimentally demonstrated that PSORI-CM01 effectively regulates miR-20a-3p and miR-3184-3p, leading to increased autophagy and reduced keratinocyte proliferation. These molecular changes significantly contribute to the therapeutic effects of PSORI-CM01 against psoriasis. Overall, this study offers valuable insights into the therapeutic mechanism of PSORI-CM01 for psoriasis treatment, providing a solid foundation for further advancements in psoriasis therapy.

Abbreviations

BP, biological process; CC, including cellular component; DL, drug-likeness; OB, oral bioavailability; GO, Gene Ontology; KCs, keratinocytes; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; miRNAs, micro-RNAs; PBMCs, peripheral blood monocytes cells; PPI, Protein-Protein Interaction; PSORI-CM01, Optimized formula of Yinxielin; PASI, psoriasis area and severity index; TEM, Transmission Electron Microscopy. Tumor necrosis factor α , (TNF)- α .

Data Sharing Statement

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics Statement

All procedures were performed in accordance with the guidelines of the Guangdong Provincial Hospital of Traditional Chinese Medicine ethics committee (B2014-026-01).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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