Network Pharmacology and Molecular docking Analysis on Mechanisms and Molecular Targets of LongChaiJiangXue Formula for Treatment of Polycythemia Vera

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ABSTRACT

Background: LongChaiJiangXue formula (LCJX) has the effect of not only clearing up excessive erythrocytes but also relieving clinical symptoms of Polycythemia vera (PV).

Material/Methods: The chemical constitution of LCJX was identified from Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). Seven hundred and fifty-nine targets were identified and a total of 248 targets were screened out after discarding duplication and genes without any ID in databases. GeneCards database, OMIM database, and GEO database were searched for differential expression genes. The network was built by Cytoscape (3.7.2) software and the protein-protein interaction (PPI) networks of PV and LCJX were merged by https://string-db.org. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was processed by the R platform. The molecular docking technology was used to further analyze the intense of the association of the compounds and targets.

Results: 73 compounds of LCJX were chosen as the candidate active compounds. The compound-targets network contained 105 nods and 216 edges that presented the interaction of agents and targets. The PPI network of LCJX targets involved 59 nodes and 168 edges. The network showed that the key nodes were concentrated in signal transducer and activator of transcription-3(STAT-3), interleukin-6(IL-6), Janus kinase 2(JAK2), and vascular endothelial growth factor-A(VEGFA). The most enriched terms in the GO analysis in the GO biological processes(BP) were reactive oxygen species metabolic process, response to lipopolysaccharide, and response to oxidative stress. According to GO molecular functions(MF), the central nodes were generally enriched in cytokine receptor binding, cytokine activity, and heme binding. Regarding the GO cell components(CC), the terms included vesicle lumen, cytoplasmic vesicle lumen, and secretory granule lumen. In light of the KEGG enrichment analysis, the Hepatitis B, AGE-RAGE signaling pathway in diabetic complications, Kaposi sarcoma-associated herpesvirus infection, measles, Human cytomegalovirus infection, and JAK-STAT signaling pathway were significantly enriched. The molecular docking technology found that puerarin and saikosaponin A had relative stronger affinity with VEGFA, HIF-1A, JAK2 and STAT3 than other compounds in terms of the binding free energy.

Conclusions: The effect of LCJX on PV is achieved through a series of complex mechanisms. Network pharmacology and molecular docking are powerful tools to reveal the effect of compound Chinese medicine on the disease.
Keywords: Polycythemia vera, Network Pharmacology, Molecular Docking Simulation, Chinese Traditional Medicine

Background
Polycythemia vera (PV), which is characterized by erythrocyte overproduction, is one of the most common myeloproliferative neoplasm (MPN) [1]. It is now well established from a variety of studies that JAK2V617F mutation plays a crucial role in the disease[2]. In addition, it has a disposition to develop blood coagulation, myelofibrosis and even acute myeloid leukemia (AML)[3]. The treatment strategy of PV should focus on alleviating symptoms, preventing myelofibrosis and leukemia[4].

However, every form of medicine has its own limitations. For instance, hydroxyurea has a certain rate (15-24%) of resistance or intolerance[5]. Moreover, potential risks of hydroxyurea-induced-nonmelanoma skin cancer should be considered for safety estimation[6]. Phlebotomy could lead to infection and iron deficiency[7]. Aspirin may cause gastrointestinal ulcers and hemorrhage[8]. Interferon alfa may lead to fever, fatigue, arthralgia, and myelosuppression[9, 10]. Ruxolitinib also has a latent risk of infection[11]. On the whole, these therapies could lead to inevitably adverse effects that are gradually enhanced over time. Moreover, the expense that patients have to cost exert severe economic pressure on their lives. Therefore, it is imperative to develop and innovate more effective approaches for the treatment of PV.

As a complementary and alternative medicine, Chinese medicine plays an increasingly important role in the medical care of PV. Zang et al. [12] revealed that Huoxue Tongmai Granule could significantly relieve symptoms, improve the peripheral hemogram of PV patients and the blood coagulation function of PV patients by inducing the expression of platelet surface receptors. Xiao et al. [13] treated 28 patients with PV through Huoxuetongluo Formula and concluded that this treatment can significantly improve various hematological parameters, including the whole blood viscosity, shear rate, plasma specific viscosity, hematocrit, erythrocyte electrophoresis time, erythrocyte sedimentation rate. Hou et al. [14] reported that they used a self-made Chinese herbal formula, Long-Dan-Xie-Gan-Yin, to treat 42 cases of PV. The results showed that 22 patients were completely relieved, 12 were clinically relieved, 3 were improved, and 5 were ineffective.
LongChaiJiangXue formula (LCJX), is derived from XueFuZhuYu Decoction and LongDanXieGan Decoction, including 6 Chinese medicine (Gentianae Radix et Rhizoma (GRR), Radix Bupleuri (RB), Atractylodes Macrocephala Koidz (AMK), Indigo Naturalis (IN), Radix Paeoniae Rubra (RPR), Radix Rehmanniae Recens (RRR)) developed by our team for the treatment of PV. LCJX has the effect of not only clearing up excessive erythrocytes but also relieving clinical symptoms. The previous clinical practices have proven that LCJX matched our expectations. However, the underlying mechanisms and molecular targets of LCJX related to PV remain unclear. The anti-PV effect of LCJX may be concerned with the regulation of immune function through specific biological processes and related pathways.

The multi-component compound is a key feature of traditional Chinese medicine (TCM). The chance of interacting with therapeutic targets can be raised when multiple components provide more bioactive ingredients. This method of multi-component, multi-target and multi-way is an effective way to treat diseases with complex pathophysiological, but it is still difficult for us to understand its mechanism. The therapeutic effects of traditional Chinese herbal medicine compounds are consistent with the integrity and systematicness of the interaction between drugs, targets and diseases in network pharmacology. Therefore, the research of TCM based on network pharmacology helps comprehensively understand the molecular mechanism of the pharmacological action and can provide a new perspective for the treatment of PV. Network pharmacology, widely used in the research of TCM to predict drug target and improve drug discovery efficiency, is based on the multi-level network of disease-target-drug [15, 16]. In this study, network pharmacology was applied to evaluate a TCM compound LCJX for the management of PV and seeking out its molecular targets, to provide a basis for further research on the mechanism.

**Material and Methods**

**Active Components Identifying**

The chemical constitution of LCJX was screened out from Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php). Candidate compounds should comply with the standard: oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥0.18. Seventy-four eligible compounds were obtained, 10 in GRR, 17 in RB, 7 in AMK, 9 in IN, 29 in RPR, 2 in RRR. Additionally, seven compounds including atractylenolide I [17, 18],
Atractylenolide II[19], atractylenolide III[20] in AMK, isoliquiritigenin[21], saikosaponin A[22] in RB, puerarin[23] in RB and GRR, ursolic acid[24] in GRR, have shown similar pharmalogical effects with the disease and were also selected. After the duplications were discarded, 73 compounds were identified as listed below (Table 1).

| ID         | Name                                                      | OB     | DL  |
|------------|-----------------------------------------------------------|--------|-----|
| MOL001558  | sesamin                                                   | 56.55  | 0.83|
| MOL002322  | isovitexin                                                | 31.29  | 0.72|
| MOL003137  | Leucanthoside                                             | 32.12  | 0.78|
| MOL003143  | gentirigenic acid                                         | 38.78  | 0.78|
| MOL003152  | Gentisin                                                  | 64.06  | 0.21|
| MOL003155  | pranferin                                                 | 52.14  | 0.28|
| MOL003169  | Gentiopicroside tetraacetate                              | 32.44  | 0.75|
| MOL003170  | Gentisine                                                 | 67.57  | 0.19|
| MOL000359  | sitosterol                                                 | 36.91  | 0.75|
| MOL000422  | kaempferol                                                | 41.88  | 0.24|
| MOL001645  | Linoleyl acetate                                          | 42.1   | 0.2 |
| MOL027776  | Baicalin                                                  | 40.12  | 0.75|
| MOL000449  | Stigmasterol                                              | 43.83  | 0.76|
| MOL000354  |isorhamnetin                                              | 49.6   | 0.31|
| MOL004598  | 3,5,6,7-tetramethoxy-2-(3,4,5-trimethoxyphenyl)chromone   | 31.97  | 0.59|
| MOL004609  | Areapilll                                                | 48.96  | 0.41|
| MOL013187  | Cubebin                                                   | 57.13  | 0.64|
| MOL004624  | Longikaurin A                                            | 47.72  | 0.53|
| MOL004628  | Octalupine                                                | 47.82  | 0.28|
| MOL004644  | Sainfuran                                                 | 79.91  | 0.23|
| MOL004648  | Troxerutin                                                | 31.6   | 0.28|
| MOL004653  | (+)-Anomalin                                              | 46.06  | 0.66|
| MOL004702  | saikosaponin c qt                                        | 30.5   | 0.63|
| MOL   | Chemical Name                                      | Value  | Standard Error |
|-------|--------------------------------------------------|--------|----------------|
| MOL004718 | α-spinasterol                                      | 42.98  | 0.76           |
| MOL000490 | petunidin                                          | 30.05  | 0.31           |
| MOL000098 | quercetin                                         | 46.43  | 0.28           |
| MOL011100 | bisindigotin                                       | 41.66  | 0.39           |
| MOL011105 | indican                                           | 34.9   | 0.23           |
| MOL011332 | 10h-indolo[3,2-b],quinoline                       | 54.57  | 0.22           |
| MOL011335 | Isoindigo                                         | 94.3   | 0.26           |
| MOL001781 | Indigo                                            | 38.2   | 0.26           |
| MOL001810 | 6-(3-oxoindolin-2-ylidene)indolo[2,1-b]quinazolin-12-one | 45.28  | 0.89           |
| MOL002309 | indirubin                                         | 48.59  | 0.26           |
| MOL000358 | beta-sitosterol                                    | 36.91  | 0.75           |
| MOL000020 | 12-seneciol-2E,8E,10E-tractylentriol              | 62.4   | 0.22           |
| MOL000021 | 14-acetyl-12-seneciol-2E,8E,10E-tractylentriol    | 60.31  | 0.31           |
| MOL000022 | 14-acetyl-12-seneciol-2E,8Z,10E-tractylentriol    | 63.37  | 0.3            |
| MOL000028 | α-Amyrin                                          | 39.51  | 0.76           |
| MOL000033 | propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol | 36.23  | 0.78           |
| MOL000049 | 3β-acetoxyatractyline                              | 54.07  | 0.22           |
| MOL000072 | 8β-ethoxy atractylenolide III                     | 35.95  | 0.21           |
| MOL001002 | ellagic acid                                       | 43.06  | 0.43           |
| MOL001918 | paeoniflorgenone                                   | 87.59  | 0.37           |
| MOL001921 | Lactiflorin                                       | 49.12  | 0.8            |
| MOL001924 | paeoniflorin                                      | 53.87  | 0.79           |
| MOL001925 | paeoniflorin_qt                                   | 68.18  | 0.4            |
| MOL002714 | baicalein                                         | 33.52  | 0.21           |
| MOL004355 | Spinasterol                                       | 42.98  | 0.76           |
| MOL00492 | (±)-catechin                                      | 54.83  | 0.24           |
| MOL006990 | (1S,2S,4R)-trans-2-hydroxy-1,8-cineole-B-D-glucopyranoside | 30.25  | 0.27           |
MOL006992  (2R,3R)-4-methoxyl-distylin  59.98  0.3
MOL006994  1-o-beta-d-glucopyranosyl-8-o-benzoylpaeonisuffrnone_qt  36.01  0.3
MOL006996  1-o-beta-d-glucopyranosylpaeonisuffrnone_qt  65.08  0.35
MOL006999  stigmas 7-en-3-ol  37.42  0.75
MOL007003  benzoyl paeaniflorin  31.14  0.54
MOL007004  Albiflorin  30.25  0.77
MOL007005  Albiflorin_qt  48.7  0.33
MOL007008  4-ethyl-paeoniflorin_qt  56.87  0.44
MOL007012  4-o-methyl-paeoniflorin_qt  56.7  0.43
MOL007014  8-debenzoylpaeanidanin  31.74  0.45
MOL007016  Paeaniflorigenone  65.33  0.37
MOL007018  9-ethyl-neo-paeoniaflorin A_qt  64.42  0.3
MOL007022  Evofolin B  64.74  0.22
MOL007025  isobenzoylpaeaniflorin  31.14  0.54
MOL002883  Ethyl oleate (NF)  32.4  0.19
MOL005043  campest-5-en-3beta-ol  37.58  0.71
MOL000043  atractylenolide I  37.37  0.15
MOL000044  atractylenolide II  47.5  0.15
MOL000045  atractylenolide III  68.11  0.17
MOL004635  saikosaponin A  32.39  0.09
MOL001789  Isoliquiritigenin  85.32  0.15
MOL005043  Puerarin  24.03  0.69
MOL000511  ursolic acid  16.77  0.75

**Screening of Targets**

The 73 candidate ingredients were input to TCMSP (http://tcmspw.com/tcmsp.php) to screen the potential targets of LCJX. Forty-eight of them were finally identified after dismissing 25 compounds without relationship to any targets. Then the targets of 48 compounds were collected. Seven hundred and fifty-nine targets were identified. 158 in GRR, 228 in RB, 18 in AMK, 67 in IN, 102 in RPR, 31 in RRR. A total of 248 targets were chosen after discarding duplication and genes without any
ID in databases.

![Volcano Plot](image)

**Figure 1** The Volcano plot shows the relationship between the significance and the foldchange of differentially expressed genes. They were highlighted by the red and green dots (|log 2(fold change)| > 1).

**Gene Target Prediction for LCJX to Treat PV**

The following electronic databases were searched to identify the genes related to PV: GeneCards database ([http://www.genecards.org/](http://www.genecards.org/)), OMIM database ([http://www.ncbi.nlm.nih.gov/omim](http://www.ncbi.nlm.nih.gov/omim)) and GEO([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/), Series: GSE61629, GSE26049. Samples: GSM1388590-1388596, GSM1388598-1388601, GSM1388603-1388608, GSM1388614, GSM1388616, GSM1388623, GSM1388624, GSM1509517-1509537, GSM639630-639670, GSM639681-639701, GSM938789-938829, GSM938839-938859. Significantly differential expression and PV-related genes had the following characteristics: P-value < 0.005 and |log 2(fold change)| > 1 (Figure 1). The duplicate data and false-positive genes were deleted. Finally, the common gene targets of LCJX and PV, which may be the potential targets for LCJX to treat PV, were analyzed by Venny tool ([http://bioinfogp.cnb.csic.es/tools/venny/index.html](http://bioinfogp.cnb.csic.es/tools/venny/index.html), Version 2.1).

**Network Building**

The compound-target network of LCJX and PV was built and presented by Cytoscape (3.7.2) software.
Network Merge
The protein-protein interaction (PPI) networks of PV and LCJX were merged by https://string-db.org (version 11.0). The gene expression was correlated with others by minimum required interaction score thresholds of 0.9.

Functional Enrichment Analysis
Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were further performed to research the function of each interactional pathways involved in the pathway of PV. “ClusterProfiler” package in R platform (3.6.1) was used for executing GO and KEGG analysis of the functional characteristics of targets. “Pathview” package of R platform is used to visualize GO interactive network and KEGG pathways.

The Molecular Docking Processing
Although the relationship between the targets and compound was verified, the intensity of the interaction remained unclear. The computer-assistant molecular docking technology was used in this study to further confirm the reliability of the result above. First, the SDF file of structure of compounds was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Second, the protein data bank (PDB) database (http://www.rcsb.org/) was searched for obtaining the molecular structures of the target proteins. The ligand-receptor compound structures were priorly chosen and modified by removing the original ligands. The pocket of combination was preferred constructed in the slot. Third, after adding polar hydrogen atoms, the AutoDock Vina software (ver 1.1.2, the Scripps Research Institute, U.S.) was used to achieve docking of ligands and receptors[25]. Finally, the 3D-structures of the ligand-receptor were visualized by PyMol software.

After completing all the docking simulations, the strength of interaction between all active molecules and disease targets was sorted by binding free energy. If the value of the binding free energy was not higher than -5.0kcal mol⁻¹, it was regarded as a valid docking.

RESULTS
Analysis of Compound-Target Network
Finally, 248 compounds of LCJX were screened as the potential compounds. One thousand and eleven PV-related targets were identified from GEO(661), GENECARDS(386), OMIM(1) database(removing 37 duplication). The differentially expressed genes have been showed in Figure
1 as a volcano plot. The Venn diagram shows the overlapping of LCJX and PV related genes. (Figure 2)

**Figure 2** Venn diagram. The Venn diagram shows the overlapping of LCJX and PV related genes.

The Compound-target network of LCJX was built by the selected compounds and the potential targets displayed in Figure 3. The network included 105 nods (34 compounds in LCJX and 71 potential targets) and 216 edges. The edges presented the possibly interaction of compounds and targets.
Figure 3 Compound-target network of LCJX. The pink round rectangles represent targets; the green ellipses represent the compounds from LCJX.

PPI Networks Analysis

A PPI network of LCJX was built to visualize the relationship for the 64 candidate target genes of PV (Figure 4a). In the PPI network, potential targets of LCJX included 59 nodes and 168 edges, after removing disconnected nodes. The network showed that the key nodes were concentrated in signal transducer and activator of transcription-3(STAT-3), interleukin-6(IL-6), Janus kinase 2(JAK2), and vascular endothelial growth factor-A(VEGFA). The edges linked to these nodes are more than others (24 in STAT-3, 16 in IL-6, 13 in JAK2, 13 in VEGFA) (Figure 4b).
GO and KEGG enrichment

GO enrichment and KEGG pathway enrichment analyses were further performed to show the biological activity and possible mechanisms of the potential targets. GO and KEGG pathways enrichment analysis were performed to discover the biological functions of the 64 candidate target genes of LCJX working on PV. The most enriched functions in the GO analysis are shown in Figure 5a-5c. In detail, the top three terms in the GO biological processes (BP) were reactive oxygen species metabolic process (GO:0072593), response to lipopolysaccharide (GO:0032496), and response to oxidative stress (GO:0006979). In light of GO molecular functions (MF), they were mainly enriched in cytokine receptor binding (GO:0005126), cytokine activity (GO:0005125), and heme binding (GO:0020037). According to the GO cell components (CC), the terms included vesicle lumen (GO:0031983), cytoplasmic vesicle lumen (GO:0060205), and secretory granule lumen (GO:0034774). GO and KEGG pathways enrichment analysis were performed to further explore the possible functions of the targets (P<0.05). Regarding the KEGG enrichment analysis, the Hepatitis B (hsa05161), AGE-RAGE signaling pathway in diabetic complications (hsa04933), Kaposi sarcoma-associated herpesvirus infection (hsa05167), measles (hsa05162), Human cytomegalovirus infection (hsa05163) and JAK-STAT signaling pathway (hsa04630) were significantly enriched (Figure 5d).
Molecular Docking

The results indicated that puerarin and saikosaponin A had relative stronger affinity with VEGFA, HIF-1A, JAK2 and STAT3 than other compounds in terms of the binding free energy. Then ursolic acid, ellagic acid and quercetin could bind VEGFA easily. Baicalein was inclined to bind HIF1A.

Figure 5 GO enrichment analysis of potential targets of LCJX to PV. The top 20 GO functional pathway with p.adj < 0.05 were shown. GO and KEGG enrichment analysis of candidate targets of LCJX for treating PV. (a-c) Bar plot showing the top 20 terms of MF, BP, and CC in the GO: the horizontal coordinate stood for the quantity of genes included, and the different colors of the bars represented the –log2 (p-value). (d) Dot plot showing the top 20 terms of KEGG pathways: the size of the dots represented the quantity of genes involved, and the color of the dots linked to the corrected p-value.
Ellagic acid was prone to link JAK2. Ursolic acid preferred to combine STAT3. On the whole, the ligands and receptors were binding mainly by hydrogen bonds. The secondary effects were hydrophobic interaction of amino acids and π-conjugated effects.

Table 2 Targets and docking parameters

| Targets | PDB ID | Original ligands removed* | Size of cube(X×Y×Z)(nm³) | Center grid box(X,Y,Z) |
|---------|--------|--------------------------|--------------------------|------------------------|
| VEGFA   | 1BJ1   | Yes                      | 58×64×56                 | 14.48, -8.25, 20.03   |
| HIF1A   | 1L8C   | No                       | 120×108×98               | 0.31, 0.00, 1.93      |
| JAK2    | 6AAJ   | Yes                      | 22×18×22                 | 12.78, 4.36, 42.39   |
| STAT3   | 6NJS   | Yes                      | 30×62×34                 | 13.53, 53.72, 0.00   |

*If there exists original ligands in the model, the ligands should be replaced by the candidate compounds.

Table 3 Binding free energy of compounds and targets simulative calculation results (kcal/mol)

| Compounds      | VEGFA | HIF-1A | JAK2 | STAT3 |
|----------------|-------|--------|------|-------|
| Puerarin       | -7.5  | -6.9   | -9.0 | -6.8  |
| Isoliquiritigenin | -5.6  | -5.8   | -7.1 | -6.0  |
| Quercetin      | -7.2  | -6.5   | -8.4 | -6.4  |
| Ursolic acid   | -7.4  | -5.9   | -7.4 | -7.0  |
| Kaempferol     | -6.9  | -6.0   | -8.0 | -6.2  |
| Gentisin       | -6.6  | -6.2   | -8.5 | -6.1  |
| Beta坐osterol | -5.3  | -5.7   | -7.6 | -5.7  |
| Saikosaponin A | -11.3 | -10.8  | -13.8| -10   |
| Atractylenolide I | -5.3  | -5.6   | -8.3 | -5.9  |
| Indirubin      | -7.0  | -5.6   | -8.6 | -6.3**|
| Triptolide     | -6.6  | -6.5   | -8.4 | -6.5  |
| Elagic acid    | -7.4  | -6.6   | -9.0 | -6.6  |
| Baicalein      | -7.0  | -6.8*  | -8.0 | -6.3* |
* referred to that the results were switched to rank 2 model calculated by AutoDock Vina if the binding location biased from original pockets obviously. ** referred to that the results were switched to rank 3.

Figure 6 3D-structure of ligand-receptor interaction built by AutoDock Vina
DISCUSSION

LCJX, which is derived from XueFuZhuYu Decoction and LongDanXieGan Decoction, is composed of Gentianae Radix et Rhizoma (GRR), Radix Bupleuri (RB), Atractylodes Macrocephala Koidz (AMK), Indigo Naturalis (IN), Radix Paeoniae Rubra (RPR) and Radix Rehmanniae Recens (RRR). Promising therapeutic effects of LCJX on PV is observed clinically. However, complex composition limits precisely elucidation on the mechanism of Chinese medicine. Network pharmacology is a valuable approach based on bioinformatics and pharmacology. The relationship between targets and disease can be revealed integrally and systematically. This research was processed by network pharmacology approach to provide guidance.

Six Chinese herbals were analyzed. Seventy-three candidate compounds were screened by TCMSP database[26] which was based on big data analysis and provided links to DrugBank database.

To the best of our knowledge, the research is the original study to comprehensively investigate the mechanism of LCJX against PV by network pharmacology approach. LCJX includes multiple components effective for PV. For instance, isoliquiritigenin in RB inhibits the JAK2/STAT3 pathway to enhance apoptosis[27] since the JAK2/STAT3 pathway is the principal mechanism in PV[28]. Effect of anti-oxidation injury and controlling weight by puerarin through JAK2/STAT3 pathways were also discovered[29, 30]. Interestingly, quercetin had a potential effect on reducing polycythemia after anxiety[31]. Anti-tumor effect also has been explored in quercetin through JAK2/STAT3 pathway[32]. Kaempferol is a common component in hematology. It was found that kaempferol played an important role in anti-inflammatory and anti-tumor aspects through JAK2 signaling and NF-κB signaling pathway[33]. Saikosaponin A was not only an anti-stress regulator[34] but also an anti-cancer agent[35]. Moreover, ursolic acid in GRR had a strong effect on inducing apoptosis through JAK2/STAT3 in colorectal cancer cells[36]. Atractylenolide I could induce apoptosis in bladder cancer cells by apoptotic pathway, cell cycle progression and PI3K/Akt/mTOR signaling pathway[37], and reduce viability, induced apoptosis and inhibited migration of melanoma cells[17]. Indirubin in IN inhibited JAK/STAT3 to promote apoptosis in...
human pancreatic cancer cells[38]. Ellagic acid, another agent extracted from IN, had a protective function of ischemic-reperfusion injury caused by NOX4/JAK/STAT signaling pathway[39]. Baicalein in RPR could take effect on ROS production and JAK/STAT pathway to modulate inflammation[40].

On the base of the components–targets–pathways network, STAT3, IL-6, JAK2, and VEGFA have been verified as powerful targets of LCJX in the management of PV in previous study[28, 41]. Since over 90% of patients suffered from PV could be found JAK2V617F gene mutation[42], the JAK2/STAT3/HIF1A/VEGFA signaling pathway with a high degree, involving core targets such as JAK2 and STAT3, is supposed to be closely concerned with the pharmacological effect of LCJX act on PV. Previous research indicated that Ruxolitinib, the selective JAK1/2 inhibitors became potentially drugs against PV[43]. HIF1A/VEGFA signaling pathway is the downstream signaling pathway of JAK2/STAT3 pathway. Several pathological conditions might lead to HIF1A upregulation, to name a few, hypoxia, ischemia and high altitude[44]. IL-6 cytokine level is significantly upregulated accompanied by JAK2/STAT3 rising and could activate JAK2/STAT3 pathway[45].That implicated IL-6 served as a proinflammatory molecule in the pathological process.

In the PPI network, the key nodes were aggregated in STAT-3, IL-6, JAK2, and VEGFA. The edges linked to these nodes are more than others (24 in STAT-3, 16 in IL-6, 13 in JAK2, 13 in VEGFA). The results indicated that these core genes may be the pivotal target genes for LCJX to relieve erythrocytosis in PV and JAK2/STAT3/VEGFA might be the key pathway regarding PV.

In this study, the results of the functional enrichment showed several pathways involved in LCJX on PV treatment. On the question of biological processes, this study pointed out reactive oxygen species metabolic process (GO:0072593), response to lipopolysaccharide (GO:0032496), and response to oxidative stress (GO:0006979). A comparison of the findings with those of other studies confirms that oxidative stress and hypoxia acted a pivotal part in the pathological course[46]. Moreover, Lipopolysaccharide also has a close connection with oxidative stress[47]. In terms of signaling pathway, cytokine receptor binding (GO:0005126), cytokine activity (GO:0005125), and heme binding (GO:0020037) took the top places. It represents cytokine IL-6, VEGFA, IL-18, IL-2, and their binding conditions are in reference to the pathological process. Heme binding is related to globulin metabolism, which is vital in PV development. With regard to cell components, vesicle lumen (GO:0031983), cytoplasmic vesicle lumen (GO:0060205), and secretory granule lumen
(GO:0034774) came into sight. It showed that the location of cell components related to LCJX on PV treatment lay in cellular secretory apparatus. PV is associated with abnormal platelet activation. Activated platelets could release extracellular vesicles include procoagulant microvesicles and exosomes[48, 49].

The KEGG enrichment analysis shows that the Hepatitis B (hsa05161), AGE-RAGE signaling pathway in diabetic complications (hsa04933), Kaposi sarcoma-associated herpes virus infection (hsa05167), measles (hsa05162), Human cytomegalovirus infection (hsa05163) and JAK/STAT signaling pathway (hsa04630) were significantly enriched. LCJX is derived from XueFuZhuYu Decoction and LongDanXieGan Decoction. In Chinese traditional medicine, LongDanXieGan Decoction mainly treats liver disease and viral infective disease[50]. Viral infection related pathway, AGE-RAGE signaling pathway and JAK/STAT pathway share the fundamental targets of JAK2, IFNB1, BCL2, STAT1, VEGFA, STAT3, CASP8, CASP3, etc. These signaling pathways had highly homogeneity and was related to PV.

The molecular docking procedure was performed to validate the binding interactions of the candidate compounds and targets. As shown in our docking results, the most potential compounds were puerarin, saikosaponin A, ursolic acid, ellagic acid, quercetin, and baicalein. The affinity of saikosaponin A with almost all targets seemed high because the structure of saikosaponin A was 2D model. It must not to be neglected that the model could affect the calculation by software. Interestingly, puerarin could bind all of the four targets, while ursolic acid, ellagic acid, quercetin, and baicalein had an advantage of part of them. The results revealed that Chinese medicine had multi-target effect. In addition, these molecules may have strong attractive force on the targets for less binding free energy and appropriated spatial structure for combination. Hydrogen bonds are most important force between ligands and receptors. Hydrophobic interaction of amino acids and π-conjugated effects took secondary place.

Although the pharmacological mechanism of LCJX on PV was elucidated based on molecular and bioinformatic analysis in this study, there are several limitations should be regarded in the deciphering of our discoveries. First of all, further experiments in vitro and in vivo are needed to verify the deep mechanism of LCJX predicted by our finding. Secondly, overall metabolic changes in special tissue and organ, such as bone marrow, liver, and spleen should be disclosed in future studies to reveal the mechanism more accurately through effects of LCJX on PV. Additionally,
substances produced by metabolite process should be detected by innovative technologies such as high-throughput targeted metabonomics.

Conclusions

The effect of LCJX on PV is achieved through a series of complex mechanisms. The effect mainly involves in JAK2/STAT3/HIF1A/VEGFA signal pathway, oxidative stress, cytokine and cellular secretory apparatus. Network pharmacology and molecular docking are powerful tools to reveal the effect of compound Chinese medicine on the disease.

Abbreviations
LCJX: LongChaiJiangXue formula
PV: Polycythemia vera
TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform
PPI: protein-protein interaction
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
STAT-3: signal transducer and activator of transcription-3
IL-6: interleukin-6
JAK2: Janus kinase 2
VEGFA: vascular endothelial growth factor-A
MPN: myeloproliferative neoplasm
AML: acute myeloid leukemia
GRR: Gentianae Radix et Rhizoma
RB: Radix Bupleuri
AMK: Atractylodes Macrocephala Koidz
IN: Indigo Naturalis
RPR: Radix Paeoniea Rubra
RRR: Radix Rehmanniae Recens
TCM: traditional Chinese medicine
OB: oral bioavailability
DL: drug-likeness
PDB: protein data bank
BP: biological processes
MF: molecular functions
CC: cell components

Ethical approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of Xiyuan Hospital, Academy
of Chinese Medical Sciences, China (Ethics approval number: 2017XLA019-2, 2017XLA036-2).

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
Jing Ming and Xiaomei Hu drafted the manuscript. Shirong Zhu, Yujin Li and Weiyi Liu provided the literature. Xiaomei Hu and Yunyao Jiang designed the article.

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