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Interdisciplinary Knowledge

Systems-Based Interactome Analysis for Hematopoiesis Effect of *Angelicae sinensis Radix*: Regulated Network of Cell Proliferation towards Hemopoiesis

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ABSTRACT **Objective:** To explore the molecular-level mechanism on the hematopoiesis effect of *Angelicae sinensis Radix* (ASR) with systems-based interactome analysis. **Methods:** This systems-based interactome analysis was designed to enforce the workflow of "ASR (herb)→compound→target protein→internal protein actions→ending regulated protein for hematopoiesis". This workflow was deployed with restrictions on regulated proteins expresses in bone marrow and anemia disease and futher validated with experiments. **Results:** The hematopoiesis mechanism of ASR might be accomplished through regulating pathways of cell proliferation towards hemopoiesis with cross-talking agents of spleen tyrosine kinase (SYK), Janus kinase 2 (JAK2), and interleukin-2-inducible T-cell kinase (ITK). The hematopoietic function of ASR was also validated by colony-forming assay performed on mice bone marrow cells. As a result, SYK, JAK2 and ITK were activated. **Conclusion:** This study provides a new approach to systematically study and predict the therapeutic mechanism for ASR based on interactome analysis towards biological process with experimental validations.

KEYWORDS *Angelicae sinensis Radix*, interactome, cell proliferation, hemopoiesis, regulation, cross-talking agents

Angelicae sinensis Radix (ASR) is a widely used Chinese herbal medicine (CHM) of the dried roots of *Angelica sinensis* (Oliv.) Diels (Apiaceae). It is a fragrant and perennial herb found in China, Japan, and Korea. Chinese herbalists have used ASR over thousands of years to strengthen Xin (Heart), Fei (Lung), and Gan (Liver) meridians, as well as lubricate the bowel.[1] Described as the primary CHM with blood tonifying function,[2,3] it has gained much attention on the hematopoiesis therapeutic effects.[1,4-7] Among these studies, most were performed through chemical or biomedical experiment-based literature, immune support and hematopoiesis effects have been found to be associated with decreased targets of corticoliberin (CRH), adrenocorticotropic hormone (ACTH), 5-hydroxytryptamine (5-HT), and increased vascular endothelial growth factor (VEGF) and ghrelin.[1] Another study found that the hematopoietic effect of interleukin (IL)-6 stimulated by ASR water-soluble polysaccharides.[4] Some studies collected evidences of hematopoietic and myeloprotective activities on stimulated IL-3 and granulocyte-macrophage colony-stimulating factor through model of human $CD34^+$ stem cell;[3,4] and found the role of Janus kinase 2 (JAK2) and mothers against decapentaplegic protein (SMAD) 1/5/8 pathways in inhibiting hepcidin by ASR polysaccharides with hepcidin in mice;[6] meanwhile ASR polysaccharides can promote hematopoiesis and thrombopoiesis through the pathway of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT).[7]

However, limited with animal models and biochemical testing indexes, these individual studies cannot provide a broader picture of pathway networks on the therapeutic effects and block the deeper

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understanding of hematopoiesis mechanism based on systems level. On the other hand, with the rapid knowledge accumulation in different domain databases and web services worldwide, knowledge databases can be integrated to construct a universal interlinked knowledge background to demonstrate the whole picture even with scattered and discrete information.[8] These domain databases include: (1) herb to chemical compound: TCM@TaiWan[9] and traditional Chinese medicine integrated database (TCMID)[10] collect the CHM with their chemical compounds; (2) chemical compound to target proteins: chemical database of bioactive molecules with drug-like properties (ChEMBL),[11] DrugBank,[12,13] and search tool for interacting chemicals, a protein-chemical interaction database (STITCH);[14] (3) protein information: universal protein (UniProt),[15] and protein interaction databases of IntAct,[16] search tool for the retrieval of interacting genes/proteins (STRING),[17] and biological general repository for interaction datasets (BioGRID);[18] (4) metabolite database: human metabolome database (HMDB);[19,20] (5) gene associated database: gene ontology,[21] protein ANalysisTHrough evolutionary relationships (PANTHER),[22] database for annotation, visualization and integrated discovery (DAVID),[23] and online mendelian inheritance in man (OMIM);[24] (6) kinase network database: PhosphoSitePlus;[25] (7) protein complex database: comprehensive resource of mammalian protein complexes (CORUM);[26] and (8) pathway and reactions database: Reactome[27] and kyoto encyclopedia of genes and genomes (KEGG).[28] By integrating these bio-medical associated databases, the universal knowledge background can be constructed for the hematopoiesis therapeutic effect provided by ASR.

METHODS

The methods include 2 fundamental parts: (1) data warehouse for universal interactome network, and (2) systems-based interactome analysis.

Data Warehouse for Universal Interactome Network

The data warehouse for universal interactome network was constructed towards the designed workflow "ASR (herb)→compound→target protein→internal protein actions→regulated protein for hematopoiesis". Most of the source data were collected from FTP websites, then, heterogeneous data sets were transferred into relational database for primary understanding and further transactions. A small portion of data (e.g., herb compound) were manual curated from commercial database, books and Internet. In detail the data warehouse for universal interactome network includes following 10 parts are listed in Appendix 1.

Methods for Interactome Network

Methods applied to the interactome network for ASR's hematopoiesis effect including constructing the interactome network, system categorization on targeted protein, pathway enrichment, systems-based interactome network extraction, protein distribution of tissue-based protein-protein interaction (PPI), and online service of gene/protein overrepresentation.

Constructing the Universal Interactome Network for ASR

The universal interactome network was done mainly on the integrating of data sets of herb chemical compound (self-owned database, Appendix 2), compound to target data (ChEMBL, Appendix 3), PPI data (IntAct and BioGRID), metabolite database (HMDB), protein complex database (CORUM and Reactome), gene/disease data (GO and OMIM), kinase network data (PhosphoSitePlus), and reaction and pathway data (Reactome) are integrated (Appendix 4). The relationships among these knowledge databases of different domains are mainly built up on the ID mapping and small portion entity are matched through name comparison. Finally, the whole interactome was constructed with 29,723 entities (node) and 2,411,926 relationships (edge). Based on the universal interactome network, the interactome network for ASR was set up with restrictions on (a) herb name = "ASR", (b) at least one participant of PPI is targeted proteins or ending regulated proteins, and (c) the data flow of "ASR (herb)→compound→target protein→internal protein actions→ending regulated protein for hematopoiesis" are chained and interlinked in the form of "$(A \rightarrow B) \cap (B \rightarrow C) \Rightarrow (A \rightarrow C)$". As a result, the universal interactome for ASR contains 9,185 entities and 105,566 relationships (Appendix 4).

System Categorization on Targeted Proteins

Targeted proteins were extracted from the universal interactome network, their tissue-based distributions were calculated through hypergeometric distribution described above with *P* value (set to 0.05). With target protein's tissue-based distribution available, tissue locations of target proteins are further manual categorized into 11 biological systems, e.g., circulatory, integumentary, skeletal, reproductive, digestive, urinary, respiratory, endocrine, lymphatic, muscular, and nervous system.

Pathway Enrichment

In this study, the pathway enrichment analysis was done on hypergeometric distribution which can be expressed as: $P(X=k)=\frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$, and the enriched data are listed in Appendix 5.

Systems-Based Interactome Network Extraction

It is a data transaction on data flow of "target protein→internal protein actions→ending regulated protein for hematopoiesis" for kernel network extraction. This was performed in 2 complementary approaches: (1) constrain proteins with condition of being expressed only in tissue of bone marrow; (2) constrain interacted proteins under condition of being gene products of anemia OMIM genes.

Tissue Distribution of PPI Proteins

List all proteins exist in the interactome network for ASR and append proteins with tissue location data; and statistic the tissue distribution of PPI proteins.

Gene/Protein Classification System

This is done through online services of PANTHER.[29] PANTHER is a large curated biological database of gene/protein families and their functionally related subfamilies that can be used to classify and identify the function of gene products. It contains 1,424,953 total genes, 177 pathways, 257 biological process terms, and 550 terms in PANTHER™ GO slim. In this study, it provided classify proteins (and their genes) in order to analysis the overrepresentation on biological processes with *P* value calculated in binominal distribution.

Availability of Data and Materials

Available of data and materials are listed as follows: (1) herb to chemical compound data of TCMD is stored in Appendix 4; (2) Chemical compound to target proteins data in database of ChEMBL (https://www.ebi.ac.uk/chembl); (3) Protein information database of UniProt (http://www.uniprot.org); (4) PPI of IntAct (http://www.ebi.ac.uk/intact), and BioGRID (http://thebiogrid.org); (5) Metabolite database of HMDB (http://www.hmdb.ca); (6) Gene database of GO (http://geneontology.org), and OMIM (http://www.omim.org); (7) Kinase network database of PhosphoSitePlus (http://www.phosphosite.org); (8) Protein complex database of CORUM (http://mips.helmholtz-muenchen.de/genre/proj/corum); (9) Pathway and reactions database of Reactome (http://www.reactome.org); and (10) Ending regulated proteins in PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

Experimental Validation

Sample Preparation

ASR were purchased from Tong Ren Tang Group Co., Ltd. (Beijing, China., Lot No. 20160325). The herbal was prepared as the following procedure. Sliced roots of *Angelica sinensis* (AS, 40 g) was immersed in 2 L of distilled water for 2 h and then decocted to boil for 1 h. The residues were further filtered with filter. Next, the drug was boiled once again for 0.5 h, with 2 L water and filtered as the above method. Finally, the drug was dried under vacuum and stored at –20 ℃ for use.

Colony-Forming Assay

Cell suspensions prepared from bone marrow (BM) of BALB/c mice were used to measure colony-forming. BM cells (BMCs, 4×10^5) from BALB/c mice were plated in duplicates in complete methylcellulose medium (StemCell Technologies, Vancouver, Canada) with or without different concentrations (6.25, 12.5, 25.0, 50.0 μg/mL) of ASR. Granulocyte-macrophage colony-forming units (CFU-GM) and burst-forming unitserythroid (BFU-E) were counted on day 7. BMCs were plated in MethoCult M3234 medium (StemCell Technologies, Vancouver, Canada) in the presence of ASR at different concentrations (6.25, 12.5, 25.0, 50.0 μg/mL) for 3 days and the number of colony-forming units-erythroid (CFU-E) colony was measured. To detect the number of colony-forming units-megakaryocyte (CFU-Mk), BMCs were plated in collagen-based MegaCult medium (StemCell Technologies, Vancouver, Canada) in the presence of IL-6, thrombopoietin (TPO), IL-11 (PeproTech Inc., Rocky Hill, NJ, USA) and ASR for 8 days, then CFU-Mk colonies were counted according to manufacturer's instructions.

Western Blot Analysis

BMCs removed from BALB/c mice were cultured in 10-cm dishes containing α-minimum essential medium (α-MEM; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 2 mmol/L L-glutamine, streptomycin/penicillin, 10% fetal bovine serum at 37 ℃. After 24 h of incubation, adherent cells were removed, and the nonadherent cells (containing hematopoietic stem cells and hematopoietic progenitor cells) were collected and divided into 6-well plates with or without different concentrations (6.25, 12.5, 25.0, 50.0 μg/mL)

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of ASR. After 48 h, cells were collected for Western blot detection. Spleen tyrosine kinase (SYK) rabbit monoclonal antibody (dilution 1:500), JAK2 rabbit monoclonal antibody (dilution 1:1,000), IL-2 inducible T-cell kinase (ITK) rabbit monoclonal antibody (dilution 1:2,000) and β-actin rat monoclonal antibody (dilution 1:5000) were purchased from Abcam (Cambridge, UK).

Statistical Analysis

Data were analyzed with SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Multiple comparisons were made using ANOVA test followed by Dunnett's test. Results were expression as mean ± standard deviation ($\bar{x} \pm s$). $P<0.05$ was considered as statistically significant.

RESULTS

System Categorization on Targeted Proteins of ASR

As shown in Tables 1 and 2, the top 2 systems with the highest folds are skeletal and circulatory which are tightly associated with hematopoiesis process.

Table 1. Biology Systems Affected by Proteins Targeted by ASR

Biology system	Expected ratio	Actual ratio	Fold
Skeletal	0.000861	0.003285	3.81
Circulatory	0.010458	0.037882	3.62
Nervous	0.002551	0.008623	3.38
Urinary	0.003633	0.011397	3.13
Lymphatic	0.007852	0.023512	2.99
Endocrine	0.000116	0.000307	2.64
Respiratory	0.003214	0.008317	2.58
Reproductive	0.011877	0.026182	2.20
Digestive	0.000961	0.001950	2.02

Table 2. Top Level Reactome Pathway Enriched by PPI Proteins in Interactome Network of ASR

Pathway name	*P* value
Metabolism	9.7E-25
Signal transduction	9.9E-16
Gene expression	5.3E-08
Immune system	2.4E-07
Metabolism of proteins	2.5E-07
Disease	2.4E-06
Cell cycle	1.9E-05
Transmembrane transport of small molecules	4.9E-05
Extracellular matrix organization	5.1E-05

Systems-Based Interactome Network for ASR

To be visualized, the systems-based interactome network is demonstrated in Figure 1. The PPI network consists of 5 circles of interacted proteins in the right part. There are 26 targeted proteins, 22 anemia OMIM gene products, 7 proteins expressed in bone marrow,

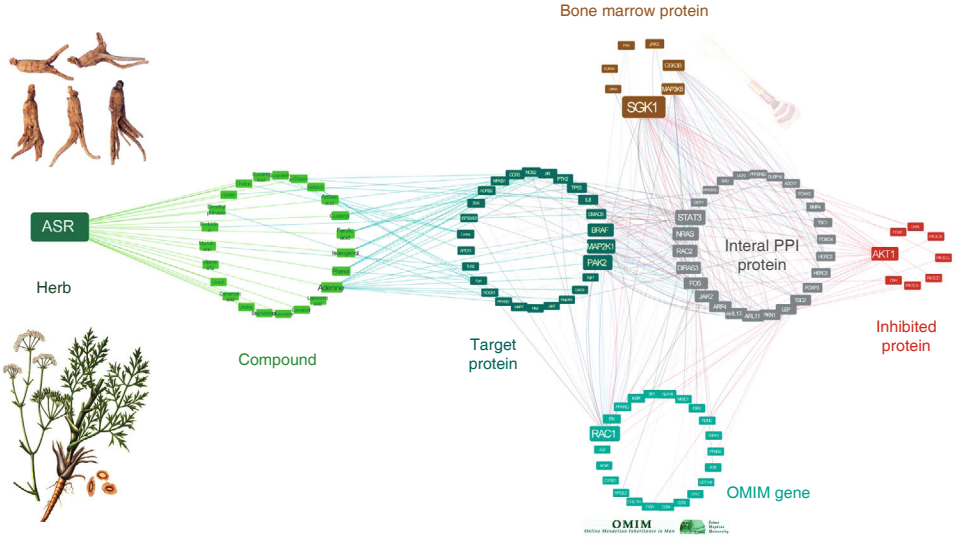

Figure 1. Systems-Based Interactome Network for ASR's Hematopoiesis Effect

Notes: It starts from herb of "ASR" in dark green node with light green lines linking to light green nodes of its chemical compounds. After that, these compounds are linked right to the dark blue nodes of target proteins. Right forward from target proteins, the complex protein-protein interaction networks are demonstrated with 5 densely connected sub-networks: apart from the target proteins in dark blue circle, the light blue nodes are proteins of anemia OMIM gene products, the brown nodes are proteins expressed in bone marrow, gray nodes of internal proteins linking proteins of bone marrow (brown nodes), target protein (dark blue nodes), anemia OMIM gene products (light blue nodes), and inhibited proteins (red nodes) reported in literature with experimental validated hematopoiesis effect of ASR. ASR: *Angelicae Sinensis Radix*; PPI: protein-protein interaction; OMIM: online Mendelian inheritance in man; SGK1: serine/threonine-protein kinase Sgk1; AKT1: RAC-alpha serine/threonine-protein kinase; PAK2: serine/threonine-protein kinase PAK 2; MAP2K1: dual specificity mitogen-activated protein kinase kinase 1; STAT3: signal transducer and activator of transcription 3; RAC1: Ras-related C3 botulinum toxin substrate 1

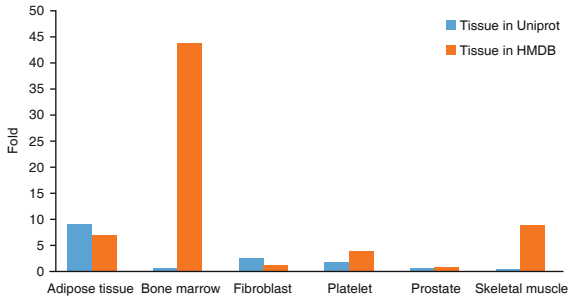

Figure 2. Tissue-Based Overrepresentation of PPI Proteins Targeted by ASR

Notes: The overrepresentation fold is calculated with formula R=(*n*/*N*)/(*k*/*K*), where *n* is the number of PPI proteins matched in UniProt or HMDB, *N*=89 is the total PPI protein number, *k* is the total protein number of one tissue in UniProt or HMDB, and *K* is the total protein number of tissue-expressed protein number in UniPrort or HMDB.

26 internal bridging proteins, 8 regulated ending proteins and 89 distinct proteins extracted from PPI network. In order to get an objective view of the tissue-based overrepresentation of PPI proteins targeted by ASR, 2 reference data sets, including UniProt and HMDB were adopted. With threshold set to 10, tissue named bone marrow is the outstanding one which is demonstrated in Figure 2.

Processes of Cell Proliferation and Hemopoiesis are Overrepresented by Proteins in Interactome Network of ASR

Two outstanding biological processes of cell proliferation and hemopoiesis ranked top 2 with overrepresentation fold over 10 is demonstrated in Figure 3.

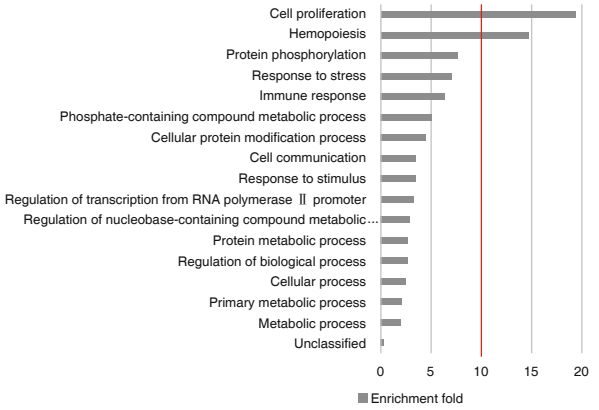

Figure 3. PANTHER Biological Process Overrepresentation Test of Targeted PPI Proteins from Interactome Network for ASR

Notes: Gray bars represent the overrepresentation fold of represented proteins compared with expected ones in each individual biological process. The *P* value of overrepresentation test was done by binominal distribution in web services of PANTHER on overrepresentation test.

Processes of Cell Proliferation and Hemopoiesis in Interactome Network of ASR

As biological processes of cell proliferation and hemopoiesis are heavily overrepresented, their participant proteins are downloaded from PANTHER web services with UniProt access keys. Then, these UniProt proteins are matched and displayed with highlighted background color in Figure 4. It is clear that cell proliferation is heavily targeted by ASR's compounds, directly. As for the hemopoiesis, most of its components are regulated indirectly by directly targeted proteins and anemia OMIM gene products. More interesting, these 2 processes share 3 cross-talking agents, e.g., SYK, JAK2, and ITK.

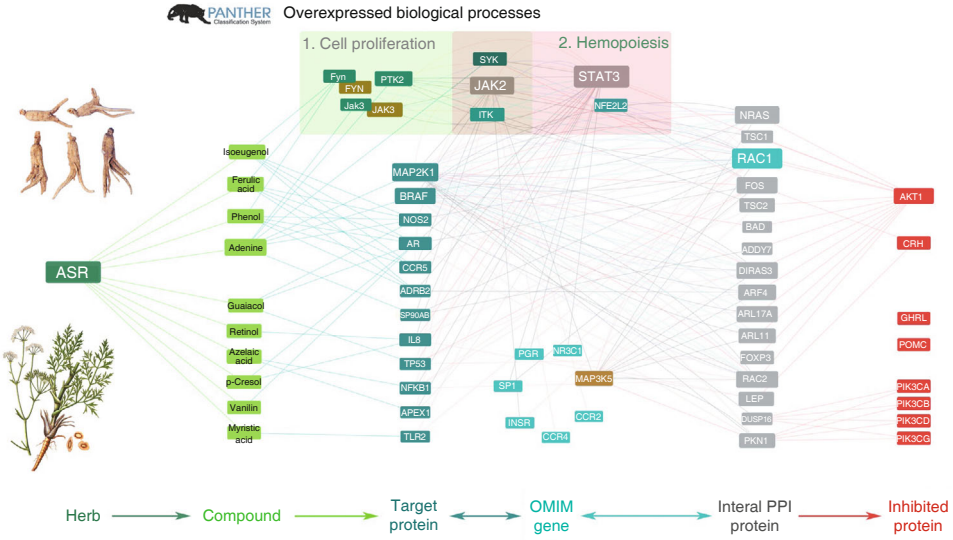

Figure 4. PANTHER Overrepresentation Biological Processes in Universal Interactome Network for ASR

Notes: From left to right, the sequence includes herb, compound PPI network from targeted proteins to regulated ending proteins with inhibited actions proposed mainly from internal PPI proteins. The top 2 heavily regulated outstanding biological processes of cell proliferation and hemopoiesis are demonstrated in the upper part with background of green and red, respectively.

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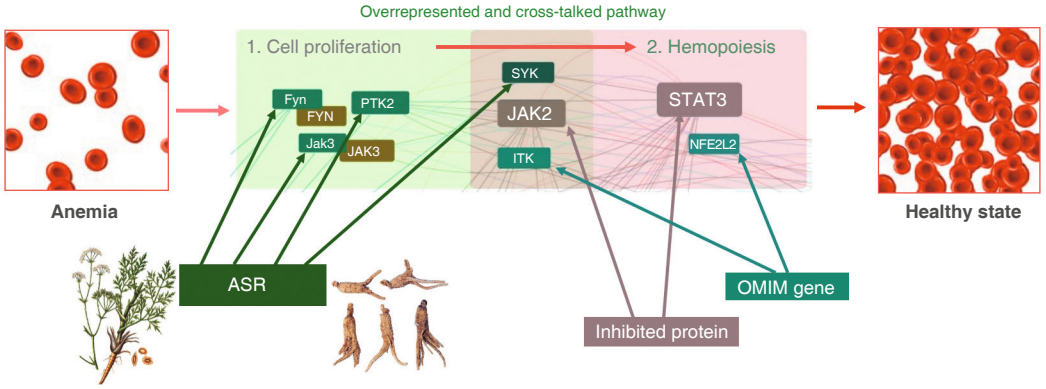

Figure 5. Regulated Cell Proliferation Towards Hemopoiesis by ASR

ASR: *Angelicae Sinensis Radix*; FYN: Proto-oncogene tyrosine-protein kinase; PTK2: protein tyrosine kinase 2; JAK3: Janus kinase 3; SYK: spleen tyrosine kinase; JAK2: Janus kinase 2; ITK: interleukin-2-inducible T-cell kinase; STAT3: signal transducer and activator of transcription 3; NFE2L2: nuclear factor (erythroid-derived 2)-like 2

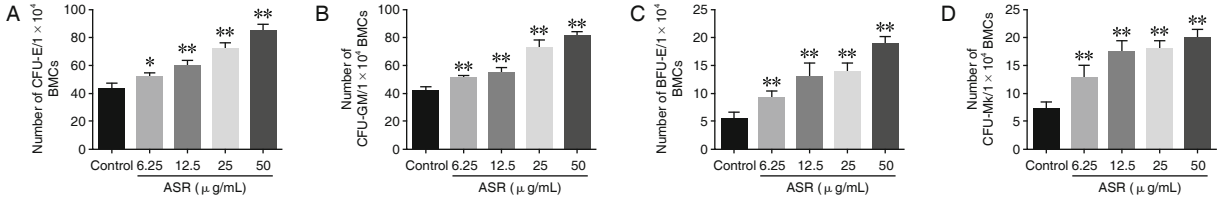

Figure 6. Effect of ASR on Colony Forming of Mice Bone Marrow Cells

Notes: BMCs: bone marrow cells: At different time, the colonies were counted under inverted microscope for CFU-E (A), CFU-GM (B), BFU-E (C) and CFU-Mk (D) , respectively. $^{*}P<0.05$, $^{**}P<0.01$, *vs*. control group.

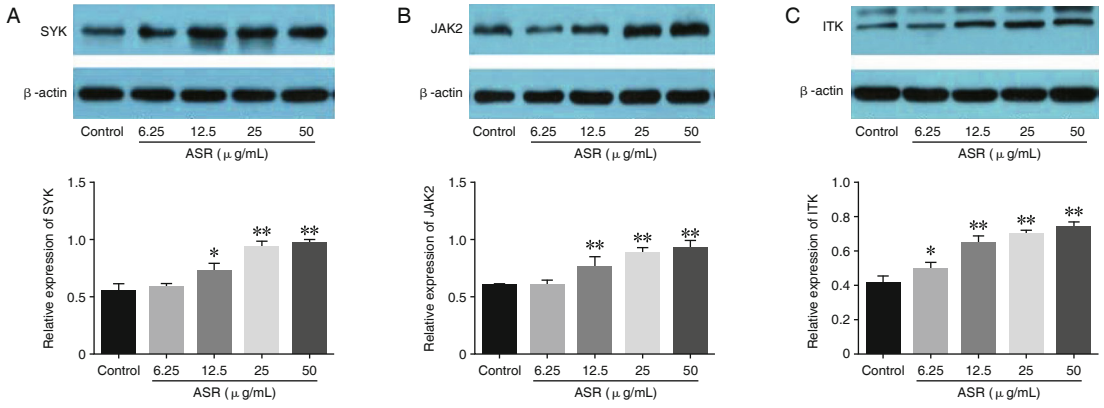

Figure 7. Effects of ASR on the Levels of SYK, JAK2, and ITK in BM Cells

Notes: Protein expression of SYK (A), JAK2 (B) and ITK (C) in the bone marrow cells with or without (control group) different concentrations of ASR. $^{*}P<0.05$, $^{**}P<0.01$, *vs*. control group.

Cross-Talking Agents of SYK, JAK2, and ITK Regulate Cell Proliferation and Hemopoiesis

The results from Figure 5 demonstrates that cross-talking agents of SYK, JAK2, and ITK participate either in process cell proliferation or hemopoiesis.

Effects of ASR on Colony Formation

To verify the effects of ASR on the hematopoietic function of mice BMCs, colony-forming assay was performed. As shown in Figures 6, ASR led to significant increase in formation of CFU-E, CFU-GM, BFU-E and CFU-Mk ($P<0.05$ or $P<0.01$).

Effects of ASR on Levels of SYK, JAK2 and ITK

In order to observe whether ASR accomplishes its hematopoiesis effect through regulating pathways of cell proliferation towards hemopoiesis, activation of SYK, JAK2, and ITK in BMCs from BALB/c mice were examined. Our results showed that ASR could increase the levels of SYK, JAK2 and ITK in BMCs ($P<0.05$ or $P<0.01$, Figure 7).

DISCUSSION

Literature review demonstrated that cross-talking agents of SYK, JAK2, and ITK participated either in

process cell proliferation or hemopoiesis at their most.[30-53]

SYK is vital in cell proliferation. It plays a critical role in the cell proliferation.[30] By activating signaling pathway with SYK/STAT axis, beta2 integrin-derived signals induce cell survival and proliferation of acute myeloid leukemia blasts.[31] With the presence of p60-c-Src tyrosine kinases, SYK may mediate sickle red blood cell adhesion to endothelium via intercellular adhesion molecule-4-alphavbeta3 and CD44-CD44 interactions.[32] Reactivation of SYK gene by azathioprine suppresses metastasis but not proliferation of breast cancer cells.[33] Besides, telomere elongation–SYK-induced APMF-type leukemia can be blocked on depletion of STAT5 with myelofibrosis and myelodysplasia in mice.[34] On the other hand, when inhibited, SYK protein tyrosine kinase induces apoptosis and blocks proliferation in T-cell non-Hodgkin's lymphoma cell lines.[35]

JAK2 deficiency can define an essential developmental checkpoint in biological process of hematopoiesis.[36] Autologous reconstitution leading to sustained JAK2-V617F negativity post allogeneic hematopoietic stem cell transplant in JAK2-V617F positive myelofibrosis.[37] Activating JAK2 mutants can cause hematological malignancies.[38]

ITK participates in proliferation of T cell, lymphocytes, and cytokines. Evidence collected that ITK had distinct roles in CD3 *vs*. CD28 signaling pathways. By negatively regulating the amplitude of signaling upon CD28 costimulation, ITK may provide a means for modulating the outcome of T cell activation during development and antigen-driven immune responses.[39] Another evidence is reported that ITK had an important immunomodulatory effect in mouse spleen lymphocytes proliferation and secretion of inflammatory cytokines.[40]

Previous research demonstrated that ASR could support the formation of BM CFU and promote hematopoiesis.[41] In our study, the results showed that not only CFU-Mk, but also CFU-E, CFU-GM and BFU-E increased by ASR in mouse primary BMCs, which suggest that ASR could promote the proliferation of BMCs.

SYK is widely expressed by haematopoietic cells and is necessary for the development of lymphocyte and signal transduction through immune receptors in non-lymphoid cells.[42,43] Meanwhile, previous researches demonstrated that SYK-deficient mice showed a lethal phenotype associated with severe petechiae, chylous ascites and diffuse haemorrhage.[44,45] JAK2 plays an important role in the maintenance and normal function of adult hematopoietic stem cells. JAK2 germ-line deletion causes impairment of fetal liver erythropoiesis leading to embryonic lethality in mice.[46] In the meantime, deletion of JAK2 at post-natal or adult stage results in anemia and thrombocytopenia in mice suggesting a role for JAK2 in the development of erythroid/megakaryocytic.[47] ITK is a kind of nonreceptor protein tyrosine kinase and expressed specifically in T cells, mast cells, human NK cell lines and myeloid cells.[48-51] A germline mutation in ITK was described in individuals manifesting hemophagocytic syndromes,[52] which demonstrated the indispensable effect of ITK in haematogenesis. In our research, the results showed that ASR could increase the expression of SYK, JAK2 and ITK in BMCs, demonstrating the hematopoiesis effect of ASR.

In brief, till now, no evidence-based literature declared that these 3 cross-talking agents of SYK, JAK2, and ITK participated in both processes of cell proliferation and hemopoiesis. However, in this study, according to the systems-based analysis on universal interactome network for ASR and experimental validation, they were identified to provide double-side function via cross-talking agents in both process of cell proliferation and hemopoiesis. To be more specified, cross-talking agents of SYK, JAK2, and ITK regulate cell proliferation functions towards hemopoiesis through which ASR accomplishes its hematopoiesis effect.

Taken together, the systems-based interactome analysis was deployed with workflow of "ASR (herb)→compound→target protein→internal protein actions→ending regulated protein for hematopoiesis" and then further validated with experiments. In order to make the interactome analysis more accurate, all gene and proteins were restricted on tissue bone marrow and disease anemia. The predicted results indicated that the hematopoiesis mechanism of ASR might be accomplished through regulating pathways of cell proliferation towards hemopoiesis with cross-talking agents of SYK, JAK2, and ITK. The results not only were validated by associated literatures collected in PubMed, 3 kernel proteins of SYK, JAK2, and ITK were also experimentally validated with BMCs. This study provides a new approach to systematically study and

predict the therapeutic mechanism for CHM based on interactome analysis towards biological process which might provide clues for further biomedical researches.

Conflict of Interest

The authors declare that they have no conflict of interests.

Author Contributions

Zheng G and Lu AP are responsible for the construction and analysis on interactome. He XJ and Fan DP are responsible for the experimental validation. Zhang H, Yang Y, Zhang YJ, Chen JP, and Hao T are responsible for data downloading, data format converting, data integrating, and double checking. Lu C, Guo HT, Sun YL and Zhang G are responsible for the biological analysis on interactome e.g., protein tissue expression, compound target protein, internal protein action, and overrepresented biological process. All authors read and approved the final manuscript.

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