Elucidation of the Mechanisms and Molecular Targets of Shuanglian Decoction for the Treatment of Hepatocellular Carcinoma Based on Network Pharmacology

Kun He, Hua Chen, Tianshou Cao, and Jiantao Lin

ABSTRACT: Shuanglian decoction (SLD) is traditionally used to treat hepatocellular carcinoma (HCC) in the clinical practice of traditional Chinese medicine. However, its mechanisms of action and molecular targets for the treatment of HCC are not clear. The active compounds of SLD were collected and their targets were identified. HCC-related targets were obtained by analyzing the differentially expressed genes between HCC patients and healthy individuals. Protein–protein interaction (PPI) data were then obtained and PPI networks of SLD putative targets and HCC-related targets were visualized and merged to identify the candidate targets for SLD against HCC. Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis were carried out. The gene-pathway network was constructed to screen the key target genes. In total, 35 active compounds and 31 targets of SLD were identified. Twenty-one pathways including cellular senescence, p53 signaling pathway, and cell cycle were significantly enriched. CYP3A4 was the core gene and other several genes including CYP1A2, PPP3CA, PTGS2, CCCNB1, and CDK1 were the key genes in the gene-pathway network of SLD for the treatment of HCC. The results indicated that SLD’s effects against HCC may relate to the regulation of an antioxidant function through specific biological processes and related pathways. This study demonstrates the application of network pharmacology in evaluating mechanisms of action and molecular targets of complex herbal formulations.

INTRODUCTION

Hepatic carcinoma is the most common cancer worldwide. The incidence rate of primary hepatic carcinoma ranks fourth in China. About 383 thousand people die of primary liver cancer every year, accounting for 51% of the total number of deaths in the world, and the 5 year survival rate is less than 5%. In primary liver cancer, hepatocellular carcinoma (HCC) is the most common type, accounting for 70%. At present, surgery is still the first choice for the treatment of liver cancer, but because most of the patients with hepatic carcinoma have cirrhosis or have reached the middle and late stage at the time of diagnosis, only about 20–30% of the patients get the opportunity of surgical resection. However, other treatment methods, such as interventional therapy, radiotherapy, and chemotherapy, have some effect but the effect is limited.

Traditional Chinese medicine (TCM) has its own unique advantages in controlling the development of patients’ condition, such as reducing recurrence, improving symptoms and signs, improving the quality of life, prolonging survival, etc. Therefore, the TCM compound, TCM extract, and effective components of TCM are widely used in the treatment of liver cancer, such as Huangqi Sijunzi decoction that can inhibit the proliferation and migration of HepG2 cells and induce apoptosis and Xiao cha hu decoction can significantly reduce the proliferation of Huh7 cells. The active groups of Chonglou extract and gecko extract from different parts in the Yiqi Huayu Jiedu formula can show antihepatoma effects; SLD extracts of three Chinese herbal medicines contain Scutellaria barbata (Ban Zhi Lian, BZL), Chinese lobelia (Ban Bian Lian, BBL), and selfheal (Xia Ku Cao, XKC). They have anti-inflammatory, analgesic, heat-clearing, and detoxification functions. It has been reported that BZL, BBL, and XKC in Shuanglian decoction show good inhibitory effects on tumor cells. Nevertheless, the mechanism of SLD on HCC is rarely reported, which greatly limits its application and promotion.

In the use of traditional Chinese medicine, many formulas are often not composed of a single component but a combination of multiple herbs. These prescriptions contain a large number of active ingredients, which can act on multiple targets. Perhaps, this network regulation through multiple targets, multiple
channels, and multiple components is a reasonable method for the treatment of complex diseases. However, to explaining the interaction between multiple targets and multiple components in this complex network is a key problem. Network pharmacology is a kind of novel drug research strategy based on the view of the network to analyze the mechanism of drug action, find the leading compound or new indication, identify the target of new drugs, etc., which corresponds to the idea of TCM regulating the body as a whole. At present, there are many studies using network pharmacology to study the mechanism of a single or compound Chinese medicine, and abundant research results have been achieved.13−15

In this research, network pharmacology was employed to understand the molecular mechanism and related targets of SLD in the treatment of hepatic carcinoma. The effective ingredients of SLD and their targets were assayed by the DrugBank database. The hepatic carcinoma-related targets accordingly analyzed the differentially expressed genes between hepatic carcinoma patients and healthy people. The mechanisms of SLD against hepatic carcinoma were assessed by gene ontology (GO) and pathway analysis.

■ RESULTS AND DISCUSSION

Results. Component−Target Network Assay. In total, 35 components of SLD (Table S1 in the Supporting Information) were chosen as alternative components, and 245 hepatic carcinoma-related targets were selected from the GEO data bank. A volcano map and a hot map were established to display the distribution of differentially expressed genes (Figure 1).

The component−target network of SLD was built with the selected components and their targets as shown in Figure 2. The network included 66 nodes (35 components in SLD and 31 component-related targets) and 108 edges, which represented the component−target interactions. Fifty alternative components had a median of 6°, which means most components of SLD acted on more than one target. Quercetin, kaempferol, luteolin, and baicalein affected 153, 63, 57, and 37 targets, respectively, and the OB of quercetin, kaempferol, luteolin, and baicalein was 46.43, 41.88, 36.16, and 33.52%, respectively. Because of their important position in the network, they may be the key active components of SLD.

PPI Network Assay. PPI controls massive biological processes and is considered as the chief aim of system biology by most researchers.16 The PPI network of SLD assumes targets including 1980 nodes and 42 605 edges, which means 1980 interacting proteins and 42 605 interactions.

To study the mechanisms of SLD’s effects on hepatic carcinoma, the PPI network of SLD-assumed targets and hepatic carcinoma-related targets were combined to pick the alternative targets for SLD against hepatic carcinoma. The system containing 1980 nodes and 42 605 edges is shown in Figure 3A. The nodes higher than 60° were regarded as significant targets by another reported research.17 A new network (DC > 60) for SLD against hepatic carcinoma was built, including 434 nodes and 15 250 edges (Figure 3B). The alternative targets were further sifted and the third network with BC > 600 was built (Figure 3C). In total, 68 target genes were selected finally for SLD against hepatic carcinoma.

GO and KEGG Assays. In total, 431 GO clauses were significantly enriched (FDR < 0.05): the biological process occupied 395, the cellular component occupied 19, and the molecular function occupied 17. The top 20 terms were chosen and are displayed in Figure 4. The highly enriched GO clauses contained response to a metal ion, response to a steroid hormone, cellular response to oxidative stress, and cellular response to an inorganic substance. The pathways that were notably affected by SLD in the process of treating hepatic carcinoma were chosen by the KEGG pathway assay. Twenty notably enriched pathways (FDR < 0.05) containing cellular senescence, p53 signaling pathway, cell cycle, and human immunodeficiency virus 1 infection were chosen and are displayed in Figure 5.

Gene-Pathway Network Assay. The gene-pathway network was built on account of the significantly enriched pathways and genes that controlled these pathways, as shown in Figure 6. The target genes and pathways are represented by squares and V shapes, respectively.

■ DISCUSSION

The theory of TCM treatment is a unique theoretical system formed after thousands of years of exploration and research in China. In the use of TCM prescriptions, many kinds of TCM are often used as compound preparations, through the network system of multiple components and multiple targets, to achieve the purpose of treating complex diseases.17 In TCM, hepatic carcinoma is considered to be a disease induced by the stagnation of liver Qi. Shuanglian prescription is composed of S. barbata and Prunella vulgaris. It has the effect of eliminating heat and detoxification, promoting blood circulation, and removing blood stasis.
In this research, a component–target network of SLD was built using 35 components and 31 component targets. We can draw a conclusion from the results that most components of SLD affected more than one target; for instance, quercetin, kaempferol, luteolin, and baicalein worked on 153, 63, 57, and 37 targets, respectively. So, they were probably key multi-effective ingredients for SLD, and we found that there are the same targets in different herbs, which means that multiple components of SLD may act on the same target exerting cooperative effects. Quercetin is a kind of flavonoid and is an important antioxidant. It has been reported that quercetin has many kinds of pharmacological actions, such as protection against aging, osteoporosis, and cancer.\textsuperscript{18} Li et al.\textsuperscript{19} reported that quercetin may cause severe apoptosis in HepG2 cells by arresting the cell cycle and destroying mitochondria membrane potential. Kaempferol is another representative flavonoid that shows many pharmacological actions like antioxidative, anti-inflammatory, and anticancer functions.\textsuperscript{20} Preclinical research reported that luteolin shows many pharmacological actions like antioxidative, anticancer, and antimicrobial functions.\textsuperscript{21} Baicalein also shows antibacterial, antiviral, anticancer, and anti-inflammatory functions and protects the liver and diuresis in clinical applications.\textsuperscript{22} The therapeutic effect of TCM is the result of the comprehensive action of various components. In this research, quercetin, kaempferol, luteolin, and baicalein controlled most of the targets related to hepatic carcinoma. All of these ingredients have antioxidative effects. Despite the fact that quercetin, kaempferol, luteolin, and baicalein are omnipresent components, there are some proof for their anticancer effects. Besides, the oral bioavailability of these components is high and so, these components may be confirmed as typical components for SLD. The PPI networks of SLD-assumed targets and HCC-related targets were built and combined to gain alternative targets for SLD against HCC. For obtaining more precise information, a new network has been built and two parameters containing DC and BC were set for picking out the new nodes.

Figure 2. Component–target network of SLD. Yellow triangles represent targets; and blue, green, violet, and red ovals represent the components from BZL, BBL, XKC, and multidrug, respectively.

Figure 3. Identification of alternative targets of SLD against hepatic carcinoma. (A) The interactive PPI network of SLD-assumed targets and hepatic carcinoma-related targets. (B) The PPI network of important proteins extracted from (A). (C) The PPI network of alternative SLD targets for hepatic carcinoma treatment extracted from (B).
Sixty-eight targets were ultimately chosen and applied to perform bioinformatics analysis to clarify the mechanisms of SLD against hepatic carcinoma. The targets of SLD against hepatic carcinoma were enriched in BP, CC, and MF by the GO assay. The results indicated that SLD controlled some biological processes, like response to a metal ion, response to a steroid hormone, cellular response to oxidative stress, and cellular response to an inorganic substance. Many diseases are caused by

Figure 4. Gene ontology terms of alternative targets of SLD against hepatic carcinoma. The top 20 GO functional items with FDR < 0.05 were chosen.
excessive production of free radicals in the body, especially in hepatic carcinoma.23 The occurrence and development of hepatic carcinoma can be divided into three stages: the initiation, the promotion, and the formation and development of hepatic carcinoma. In these three stages and in the treatment of hepatic carcinoma, free radicals are involved. The active oxygen-free radicals in the free radicals can be used as intracellular messenger molecules to change the structure of a protein in the oxidation of sulfydryl groups on the protein, thus affecting its function.24 Active oxygen-free radicals can activate the gene expression of hypoxia-inducible factor-1, angiogenesis, and cell metabolism, so as to enhance the survival of tumor, eliminate free radicals, and prevent and treat hepatic carcinoma.25 Therefore, SLD might contribute to adjusting antioxidative effects by disturbing these processes.

The pathogenesis of hepatic carcinoma is related to gene expression, apoptosis, cell proliferation, and nucleoplasm, and they are greatly enriched in this research. So, SLD might act on regulatory effects in the treatment of hepatic carcinoma and might also influence some cellular ingredients and effects of the molecules containing nucleoplasm, nucleus, and cytosol in the treatment process of hepatic carcinoma. TCM is characterized by multiple components, multiple targets, and multiple channels. SLD, as a traditional TCM prescription, possesses the same features as well. So, it can be concluded that SLD treats hepatic carcinoma by multiple pathways. In this research, altogether there are 21 KEGG pathways containing p53 and VEGF signaling pathways. p53 is one of the most important tumor suppressors;26,27 the p53 pathway can induce cell cycle arrest, repair, aging, and apoptosis by regulating p53 and other genes. Hypoxia is one of the basic characteristics of the tumor microenvironment, which can indirectly activate the expression of the angiogenic factor VEGF and then activate a series of downstream signal molecules, such as PLC-γ, PKC, MAPK, and PI3K, and finally, exerts a biological effect.28−30 Studies have reported that inhibition of protein kinase activation in the VEGF signaling pathway can prevent cell proliferation and migration. Flavonoids in S. barbata may affect the downstream factors of this pathway by controlling the expression level of VEGF and exert an antitumor effect by inhibiting angiogenesis.

In the process of tumor development, inflammation plays a promoting role, and the inflammatory response will be further stimulated by the tumor microenvironment.31 NF-κB is very important in the inflammatory response. It can not only enter the nucleus but also excite the increase of downstream inflammatory factors like IL-1 β, COX-2, IL-8, etc.32 Studies have shown that there is little overexpression of COX-2 in normal tissues, but it can be induced by LPS and other factors.33 Quercetin and luteolin can directly inhibit the expression of COX-2 through the NF-κB signal pathway and effectively affect the inflammatory response,34 which corresponds to the prediction results of this research. Therefore, S. barbata drug components regulate the growth of tumor by targeting the key signal pathway in the inflammatory microenvironment of the tumor site.

The gene-pathway network was built to find out key target genes for SLD against hepatic carcinoma. The results indicated that CYP1A2 owns the maximum BC and it may be the key one.
Cytochrome P450 (CYPs), belonging to the heme protein family, is a monooxygenase compound that can participate in the metabolism of endogenous substances, drugs, and other exogenous compounds. CYP450 family proteins not only participate in the metabolism of various drugs in the liver but also are closely related to various liver diseases including liver cancer. CYP1A2 is one of the main CYPs in the human liver, accounting for about 13% of the total CYP. CYP1A2 has substrate specificity, and the expression and activity of CYP1A2 vary greatly among individuals. It is found that about 35–75% of the variation in the CYP1A2 activity among individuals is caused by genetic factors, and the difference in the CYP1A2 activity will affect the susceptibility of individuals to cancer risk. A variety of carcinogens, including polycyclic aromatic hydrocarbons, heterocyclic amines, and aflatoxin B1, can be transformed into reactive electrophiles interacting with DNA and proteins. So far, more than 15 variant alleles of CYP1A2 gene have been found. The polymorphism of the CYP1A2 gene has been reported to be related to the generation of liver, lung, and ovarian cancer. Modak et al. found that CYP1A2 shows a low expression in HCC, and at the same time, the latest research shows that CYP1A2 can be used as an independent predictor of early postoperative recurrence in hepatitis C-related liver cancer. The network pharmacology seems to be an appropriate method for the research of complicated TCM prescriptions.

### CONCLUSIONS

The results suggested that SLD’s effects against HCC may relate to controlling the antioxidative effect according to given biological processes and relative pathways. This research indicated using network pharmacology in clarifying the mechanisms of the action of complex herbal prescriptions.

### EXPERIMENTAL SECTION

#### Sifting of Active Components

The components of SLD were selected from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php). The following conditions should be met by the candidate compounds: (1) oral bioavailability (OB) ≥ 30% and (2) drug-likeness (DL) ≥ 0.18. After screening, 57 compounds were obtained, 17 in BBL, 19 in BZL, and 11 in XKC. Eventually, 52 candidate components were selected in total because the duplicate ones were eliminated.

#### Identification of Potential Targets

In total, 52 alternative components were inputted into the DrugBank database (https://www.drugbank.ca/) to distinguish the related targets of SLD. Then, 35 components were finally selected after eliminating 17 components, which had no connection to any target. Finally, 35 components were obtained. In total, 1384 targets were distinguished, 417 in BBL, 591 in BZL, and 376 in XKC. A total of 245 targets were obtained after the duplicate ones were eliminated.

#### Hepatic Carcinoma Relevant Targets

The differentially expressed genes of hepatic carcinoma patients were selected from the GEO data bank (https://www.ncbi.nlm.nih.gov/geo/, Series: GSE101685, Samples: GSM2711996, GSM2711997, GSM2711998, GSM2712000, GSM2712001, GSM2712002, GSM2712003, GSM2712004, GSM2712005, GSM2712006, GSM2712007, GSM2712008, GSM2712009, GSM2712010, GSM2712011, GSM2712012, GSM2712013, GSM2712014, GSM2712015, GSM2712016, GSM2712017, GSM2712018, GSM2712019, GSM2712020, GSM2712021, GSM2712022, GSM2712023, GSM2712024, GSM2712025, GSM2712026, and GSM2712027). Genes with P values < 0.005 and log2 (fold change) > 1 were thought to be statistical differential expressions and hepatic carcinoma relevant targets.

#### Network Building

The component—target network of SLD was built by Cytoscape 3.5.2. The PPI networks of SLD-assumed targets and hepatic carcinoma relevant targets were visualized using Cytoscape.

#### Network Merge

The PPI networks of SLD speculative targets and hepatic carcinoma relevant targets were amalgamated by Cytoscape. The nodes with topological significance were further filtered according to two parameters, degree centrality (DC) and betweenness centrality (BC). DC and BC were used on behalf of topological significance and they were used in network pharmacology.

#### Bioinformatic Analysis

The OmicsBean platform (http://www.omicsbean.cn/) was used for the bioinformatics analysis of target proteins to explore the mechanism of target proteins in biological processes, cell components, and molecular functions. Functional classifications were enriched within genes (FDR < 0.05), and the top 20 GO functional classifications were chosen. The pathway assay was carried out by the Kyoto Encyclopedia of Genes and Genomes (KEGG) data bank. The network was built to select the key target genes so that SLD could treat hepatic carcinoma.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05550.

Final selected compounds in SLD for analysis (Table S1) and identification of alternative targets of SLD against hepatic carcinoma. (A) The interactive PPI network of SLD-assumed targets and hepatic carcinoma relevant targets. (B) The PPI network of important proteins extracted from (A). (C) The PPI network of alternative SLD targets for hepatic carcinoma treatment extracted from (B) (Figure S1) (PDF)

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The authors declare no competing financial interest.

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