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Review

Network Pharmacology in Research of Chinese Medicine Formula: Methodology, Application and Prospective*

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ABSTRACT Chinese medicine (CM) is usually prescribed as CM formula to treat disease. The lack of effective research approach makes it difficult to elucidate the molecular mechanisms of CM formula owing to its complicated chemical compounds. Network pharmacology is increasingly applied in CM formula research in recent years, which is identified suitable for the study of CM formula. In this review, we summarized the methodology of network pharmacology, including network construction, network analysis and network verification. The aim of constructing a network is to achieve the interaction between the bioactive compounds and targets and the interaction between various targets, and then find out and validate the key nodes via network analysis and network verification. Besides, we reviewed the application in CM formula research, mainly including targets discovery, bioactive compounds screening, toxicity evaluation, mechanism research and quality control research. Finally, we proposed prospective in the future and limitations of network pharmacology, expecting to provide new strategy and thinking on study for CM formula.

KEYWORDS network pharmacology, Chinese medicine formula, targets discovery, mechanism research, quality control research

Chinese medicine (CM) attracts worldwide attention in recent decades for its precise efficacy, relatively low-toxicity and low-cost.[1-3] Besides, a wealth of evidence has emerged to support that numerous widely used modern drugs are derived from CM.[4,5] In clinical practice, CM is usually used in form of compound formula, which often follows the principle of "sovereign-minister-assistant-envoy (Jun-Chen-Zuo-Shi in Chinese)" to achieve comprehensive therapeutic effect via the combination of various CM materials, and displays synergistic effect in CM to improve effect or decrease toxicity.[6,7] Wang, et al[8] demonstrated *Rhizoma chuanxiong-Radix Paeoniae rubra* combination synergistically promoted angiogenic activity via network pharmacological strategy. Ye, et al[9] found the potential synergism of herbal pair *Rhizoma chuanxiong-Radix Paeoniae rubra* on treatment for osteoarthritis by computational pharmacology approach. However, its characteristic of multi-compound, multi-target, and multi-pathway makes mechanism research of CM formula encounter a predicament.[10,11]

Molecular biology is most widely applied for Western drug pharmacological researches and achieves great progress from the phenotype of organisms into the molecular level of research. However, it may run into a dilemma when it is applied in CM formula research since it throws lights on single or limited bio-functional molecules or pathways in term of reductionist idea rather than considering the holistic theory of organisms.[12-15] It is urgent to seek a systemic methodology to study CM formula at the molecular level.

Systems biology helps us understand complicated interactions from a systemic view which can make up for the limitation of molecular biology.[16] Systems biology can easily produce a large amount of data via a series of omics technologies, including

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genomics, transcriptomics, metabolomics, lipidomics, proteomics, metallomics and so on, whereas, how to integrate these intricate data with life entities is really another challenge.[17] Network pharmacology can generate complicated interaction network based on target molecules, biological function, and bioactive compounds, which meets the natural feature of CM formula, and enable elucidate the action mechanism of CM formula at molecular level with systematic view point, expecting to be a promising holistic strategy for CM formula research.[18-20] Although the application in CM formula of network pharmacology is still under developing stage, some researches have achieved impressive accomplishments. Early in 1999, Li[21] discovered the relationship between CM syndrome and molecular networks and proposed the concept of network targets strategy, which can explain the complex relationship between biological systems and drugs, and screen synergistic effect of multiple drugs. When the strategy is applied in pharmacological research of CM formula, it provides a new horizon on the bioactive compounds discovery, mechanism research, quality control and many other fields.[22,23]

In this paper, we conclude the significance of network pharmacology technology for the development of CM formula by summarizing recent years' literature, mainly from the methodology of network pharmacology, the application in the research of CM formula and prospective in the future, expecting providing new guidances for the research of CM formula.

Methodology

In practice, network construction, network analysis and network verification are the general routing of network pharmacology research. Constructing a complicated biological network based on massive existing database is an easy beginning. Then find out the key nodes in the network and predict the key biological processes via network analysis. Finally, to ensure the reliability of predicted result, further network verification is necessary. The flow of the network pharmacology research is shown as Figure 1.

Network Construction

A biological network involves many topological parameters, such as node, edge, degree, etc. Node can represent gene, protein, CM compound, disease phenotype and so on. The connection between two nodes called edge, which can represent the interaction of protein-protein, compound-target, or transcription regulation. A special group composed by several nodes is called module, and the node connects two modules called bridge node. The degree shows multiple edges of a node with other else nodes, and the nodes with large degree value is generally regarded as center nodes. Betweenness is the ratio of the number of paths of all the shortest paths across through the node to the

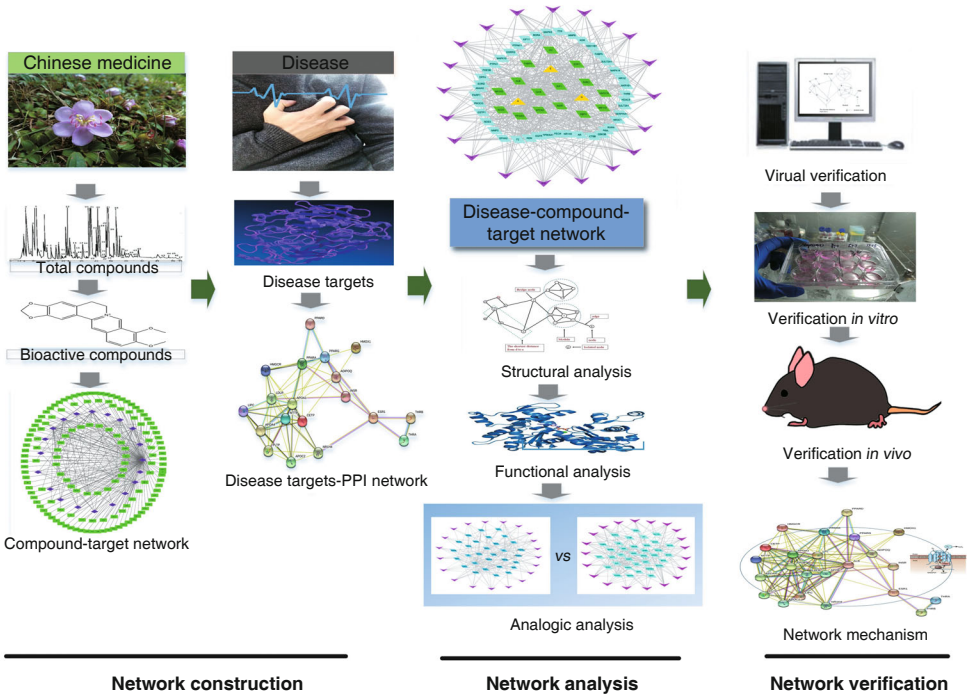

Figure 1. Basic Flow of Network Pharmacology Research

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total number of shortest paths. The nodes with large betweenness values are regarded as the key nodes in network.[24-26] The key nodes are found in different levels of networks, which can rapidly and systematically acquire illuminant for the study of complex CM formula. Network construction of CM research usually consists of bioactive compounds screening, targets discovery and network visualization.[27,28]

Chemical Compounds Screening

CM formula performs commendable effect on treating diseases, especially on complex polygene diseases, although almost medicinal compounds are unclear.[29] The main chemical compounds that could represent the holistic CM formula are usually screened via experimental data or database searching. Experimental data is got generally through the serum pharmacological experiments, or screened by "five principles of drug absorption".[30,31] High-frequency used method is database searching, such as TCMSP, DrugBank, TCM database@Taiwan, etc. Commonly used CM compounds database is shown in Appendix 1.

Targets Discovery

When the chemical compounds are determined, another pivotal node in the biological network is targets. Several approaches are available to predict targets, mainly including bioinformatics-based and pharmaceutical chemistry-based methods. Bioinformatics-based method is searching related disease or chemical compounds to get candidate targets through existing database. Niu, et al[32] collected 106 chemical compounds and 1,291 targets of *Angelica sinensis* via the TCMSP, TCM-Mesh, BATMAN-TCM, and PolySearch2 tools and 349 acute myocardial infarction-related targets by the Comparative Toxicogenomics Database (CTD), Therapeutic Target Database (TTD), Genetic Association Database (GAD), PharmGKB, and Online Mendelian Inheritance in Man (OMIM). Yang, et al[33] studied the molecular mechanisms of Lianxia Ningxin Formula (连夏宁心方) on coronary heart disease (CHD) by searching HIT6, CMID, PubMed and so on, and constructed protein-protein interaction (PPI) network and identified two related significant CHD related disease modules. Commonly used target databases are listed in Appendix 1. Pharmaceutical chemistry-based method screens out biological macromolecules of effective compounds as candidate targets via reverse pharmacophore matching. PharmMapper, a free server for potential drug target identification by reversed pharmacophore matching the query compound against an in-house pharmacophore model database is widely used to find targets.[34] In addition to the chemical compounds and targets, nodes in the biological network can also represent diseases, genes, pathways and so on. After obtaining the candidate targets, the corresponding genes, pathways can be searched through the relevant database.

Network Visualization

There are lots of open source webs and software to achieve visualization of complicated biological network. After a preparatory work, different biological networks can be obtained through visualization technology, such as compounds-target network, disease-gene network, PPI network and so on. Gao, et al[35] studied the efficacy of CM formula on cancers by screening bioactive compounds and targets, achieving the visualization of compound-target and drug-target and predicting the key targets. In order to study the relationship between adverse reaction of drugs and adjacent targets in the network, Brouwers, et al[36] constructed a drug-drug interaction network and found out that the similarity of adverse reaction of drugs is more related to adjacent targets rather than the shared drug targets. Network visualization simplifies and directly presents complex irregular data.

Network Analysis

Biological networks provide us abundant information. How to extract key information from the networks is the key point of complicate CM formula research. Network analysis aims at finding the crucial targets, bioactive compounds, and metabolic pathways by extracting targets or target combinations, drugs or drug combinations. Several analysis methods are used in network analysis, mainly including network structural analysis, network functional analysis and network analogical analysis.[37-39]

Network Structural Analysis

Network structural analysis equates to network topological analysis. By analyzing the basic topological attributes of biological network, we can find some key nodes in the network via the degree of connection, which may be the significant compounds, targets or genes. Most visualization software provides toolkit to do network topological analysis.[40,41] Alkan, et al[42] developed a new algorithm-RedNome based

on the network topology reconstruction problem, which provides a reliable analysis method for PPI. The network topological parameters are not only the basic attributes of biological network, but also an important entry of network analysis.

Network Functional Analysis

It has been found that the biological networks possess the modular feature and many effective compounds perform therapeutic effect through modulation of multiple proteins rather than single protein.[43] Through the topological analysis, some subnetworks with specific functions and topologies in the complex networks are exposed. Network modularization makes the primary disorderly network hierarchical and modular, and highlights the important group nodes speedily. The nodes inner the module connects densely to each other and the nodes outside the module connects sparsely to each other. The bridge nodes, bottleneck nodes and overlapping nodes between modules are significant to the connection between modules, network disturbance and network collaboration.[44-47] Li, et al[48] proposed a drug-gene-disease relationship "common module" theory, and developed a new method-comCIPHER for common module detection. The method was applied to 723 drugs, 275 diseases and 1,442 gene combinations, identified common modules, 8 drug modules and 2 disease modules. It suggests the special advantage of the new method by comparing the distance between the shortest drug target (DT) and the co-module gene (CG) of the smallest network, the disease gene (PG) and CG, DT and PG. Besides, many different module decomposition algorithms, including node density clustering-based algorithm and hierarchical clustering-based algorithm, etc. are applied in network functional analysis.

Network Analogic Analysis

The ultimate manifestation of the biological activity process lies in the spatial and temporal changes of networks. The stability or variability of a biological network can be fully differed through network analogic analysis, which may provide a viable strategy for the research of the disturbance mechanism.[49] The occurrence and development of disease owes to the imbalance of internal network and dynamic change of organism, so that it is generally accepted that network analogical analysis is expected to be used in disease diagnosis and prognosis.[50] In order to determine whether the difference between drug targets and non-drug targets is regulated by the miRNA-regulated protein, Wang, et al[51] set the targets into two groups-regulated by miRNA and non-miRNA, established networks of human PPIs and analyzed 3 different proteins in the network by network analogic analysis method. The results showed that miRNAs play a very important role in the network of PPIs and miRNA is proved to be a key target for the development of new drugs. With the development of network analogic algorithm, a large number of network analog software has been used by researchers. Network stratification and clustering analysis are important algorithms to simplify complex networks and some common algorithms, including splitting algorithm,[52] clustering algorithm,[53] RNSC algorithm,[54] MCODE algorithm, etc.[55,56] In addition, the development of different stages of a disease can be also described by network analogic analysis. The disturbance mechanism can be studied by constructing different disease networks of different stages with the key biomarkers which got through high-throughput techniques.

Network Verification

It is significant to validate the reasonability of predicted results by biological network analysis. Virtual verification and experimental verification are the common methods. Virtual verification is the calculation of network parameters through the specific function, algorithm, or reconstruction network under limited conditions, and helps to understand the network interaction relationship by optimizing the targets.[57] The results based model prediction need further experimental verification. Guo, et al[58] studied the mechanism of Wutou Decoction (乌头汤) in the treatment of rheumatoid arthritis, and then conducted the *in vivo* validation through animal experiment, with the results that Wutou Decoction played an important role in the inhibition of inflammatory response and was closely related to the regulation of macrophage CCR5 signaling pathway. Wu, et al[59] used bioinformatics analysis method to identify and validate potential treatment target gene of berberine-treated Zucker diabetic adipose rats, and finally confirmed 8 node genes through the PPI network and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Application

Complex chemical compounds of CM formula and its unclear mechanism greatly limit its development. Network pharmacology provides an

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opportunity to transform CM formula study strategy from the experience-based to evidence-based, which has been used in several aspects of CM formula research.[60,61] The paper mainly discusses the application in CM formula research from targets discovery, bioactive compounds discovery, toxicity evaluation, mechanism research and quality control research. Appendix 2 lists some typical application of network pharmacology in CM formula study in recent literatures. Most of these examples used the methodology described in the above, and bioinformatics-based methods and pharmaceutical chemistry-based methods are widely used in the discovery of targets. In these cases, bioactive compounds, action mechanism and new targets of CM formula were fully and systematically studied, even some new algorithms of network pharmacology were instituted and verified. Although it needs further verification, network pharmacology is undisputed a higher-efficiency technology for complicated CM formula compared to classical pharmacological methods.

Targets Discovery

The discovery of new targets has become a focus on CM formula research for drug target is a key breakthrough in the development of drugs.[62] Conventional technology of drug target discovery is biochemical method, with a small molecule compound as a probe to study its specific binding with protein macromolecules or related protein expression, thus locate to the specific binding site. Conventional methods possess the advantages of high specificity and selectivity, however, the defect is that it needs to mark small molecules,[63-65] so it is time consuming and inefficient. Besides, many effective compounds act via modulation of multiple proteins rather than single protein.[41] Network pharmacology is an innovative technology based on computer aided algorithm and construction of virtual models to achieve multi-targets prediction,[66] which is suitable for the discovery of new targets of complicate CM formula. Zhang, et al[67] studied Qixuehe Capsule (气血和胶囊) in the treatment of menstrual disorders by network pharmacology and identified 66 targets connected with several biological pathways, such as vascular endothelial growth factor (VEGF), chemokine signaling pathways, etc. Gao[35] studied the molecular targets of herbs prolonging survival time of patients with advanced hepatocellular carcinoma (HCC) based on network pharmacology. Network analysis revealed that the 8 herbs regulated multiple HCC relative genes, among which the genes affected proliferation (KRAS, AKT2, MAPK), metastasis (SRC, MMP), angiogenesis (PTGS2) and apoptosis (CASP3), etc.

Bioactive Compounds Screening

The unclear bioactive compounds of CM formula are one of the key issues leading to the bottleneck of CM formula research, so a comprehensive method that can identify multi-compound is urgently required. Network pharmacology provides an easy method by mapping chemical compounds into the disease-gene network to seek potential bioactive compounds.[68-71] Li, et al[72] studied Danshen Formula (丹参方) by network pharmacology, with the result of 9 bioactive ingredients and 30 cardiovascular-related targets, indicating that Danshen Formula possesses the new research prospects for clinical application. Wang, et al[73] studied the active compounds of Xuesaitong (血塞通, XST) via establishing cardiovascular disease-related PPI network and mapping the potential drug targets which got from text mining and model prediction into the network. With weighted calculation, the main active compounds of XST were identified, including notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd and ginsenoside Re.

Toxicity Evaluation

Some clinical news report the potential safe risk of CM formula although it makes a great contribution on treating complex diseases. So toxicity research of CM formula is payed more and more attention to. Traditional toxicity evaluation method is investigating the change of biomarkers by detecting the biochemical index of organism.[74,75] However, potential toxicity and chronic toxicity may not be reflected instantly on body, which may ignore the potential safety hazard of CM formula. Therefore, it is of great significance to seek a non-destructive, rapid and accurate method for assessing potential toxicity of CM formula. Network pharmacology can break the limitations of traditional methods to find the toxic compounds and toxic mechanism quickly. Toxicity mainly derives from the toxic compounds of CM formula itself, moreover the production of toxic effects may be caused by the same targets of multi-compound to the biological network disturbance from the point of view on network pharmacology, while the multi-target characteristic of CM formula is more promising to weaken adverse reactions than the single target-drug.[76] Li, et al[77] studied the efficacy-toxicity interaction of Sini Decoction (四逆汤) and regulation mechanism by network pharmacology. They found

5 genes related to neurotoxicity, cardiotoxicity and anti-heart failure function by constructing efficiency-toxicity network, which provides evidence for further attenuating toxicity research.

Mechanism Research

Mechanism research is the most challenging work for CM formula. Network pharmacology throws light on overall view and study the network mechanism with systemic notion, so as to predict the key metabolic pathways.[78] Shi, et al[79] studied the mechanism of CM formula Bushen Huoxue Recipe (补肾活血方) on curing chronic kidney disease. The network analysis results show that Bushen Huoxue Recipe regulates coagulation and fibrin dissolution balance and expression of inflammatory factors with multi-pathways. Zhou, et al[80] established a neuroendocrine immune regulation network, and the results show that Liuwei Dihuang Decoction (六味地黄汤) regulates neuroendocrine immune network balance through the regulation of neuroendocrine immune network communication and interaction. Zeng[81] studied the mechanism of Yanghe Decoction (阳和汤, YHD) against breast cancer. They found the YHD ingredients' synergistic effect for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer, and predicted the potential for HER2-positive breast cancer-related targets, clusters, biological process and pathways through 4 networks. All the results show that network pharmacology plays an important role in the process of illuminating complicated action mechanism of multi-compounds through constructing the drug-target interaction network and network analysis.

Quality Control Research

Quality control is vital to ensure the safety and efficacy of CM formula. However, the large amount of natural compounds makes it difficult to control the quality. Fingerprinting combined with chemo-metrics and representative components determination are the two conventional strategies of quality control.[82] Whereafter, quality marker (Q-marker) was proposed to apply in quality control of CM.[83] How to choose appropriate Q-markers has been another challenge for quality control research. Conventional chromatographic techniques can indeed achieve the quantification of single or multi-compounds in CM formula, so as to determine whether the raw materials of CM formula meet the requirements. Nevertheless, it more or less ignores combining with the clinical effect. Does the high content of compounds confirm the effective compounds? Does the low content of compound unable exert therapeutic effect? With the application of network pharmacology, the questions can be solved commendably. Network pharmacology approach is applied more and more to screen Q-markers as the quality evaluation index of CM formula. Xiang, et al[84] took Shenxian Shengmai Oral Liquid (参仙升脉口服液) as the research object, screened out 10 Q-markers via network pharmacology strategy and established an ultra performance liquid chromatography-diode array detection quality control method based on the 10 Q-markers. Compared with conventional methods, network pharmacology applied in quality control research takes both chemical analysis technology and clinical efficacy of CM formula compounds into count, which makes up for the defect of conventional analysis methods.

To sum up, the lack of exploratory approaches of CM formula research is the major obstacle.[85] Network pharmacology provides a new approach and strategy for the study of CM formula, which can not only maximize the advantages of multi-compound, multi-target and multi-pathway characteristics of CM formula, but also provide a reliable solution for CM formula research with a systemic approach from quality control research to clinical efficacy research. Based on the above characteristics, network pharmacology can be applied in all aspects of CM formula, providing new view and strategies for CM formula study.

Prospective

Network pharmacology's comprehensiveness, systematicness and holistic concept are consistent with CM formula's characteristic of multi-compound, multi-target and multi-pathway. It provides new horizons for CM formula research. Most of CM formula prescription were got from empiricism, which leads the question that although its efficacy has been validated for many years in clinical practice, it lacks of scientific and rational mechanism interpretation. The application of network pharmacology in CM formula is expected to achieve the change from experience-based medicine to evidence-based medicine. Network pharmacology is expected to become an important direction of CM formula research.

Despite the broad prospects, network pharmacology still has some limitations. First, the existing database is incomplete. Secondly, with the popularization of network pharmacology technology,

different model algorithms are developed, and different algorithms produce different prediction results, so choose an appropriate algorithm according to different purposes to ensure the accuracy of the results is necessary. Thirdly, the application of network pharmacology in CM formula research is mainly in qualitative stage, whether in the discovery of new targets, or drug mechanism research. However, there is a dose-efficacy relationship between the drugs and disease, and current network pharmacology technology is difficult to achieve the goal of quantizing. Lastly, most researches based on network pharmacology are still on static network analysis while body function is an on-going dynamic process, and the occurrence of disease and development and efficacy process of drugs are also dynamic changes. So a lot of experimental verification *in vivo* or *vitro* are needed.

Even some unavoidable limitations stand in the research of network pharmacology, it provides a multi-dimensional research strategy for complicated CM formula. In the future, with the popularization of network pharmacology technology and the development of instrumental analysis and data analysis, an ultrahigh-throughput, fast and non-destructive method can be find to effectively solve the above limitations. It is expected to promote the modernization process of CM formula a big step forward. Different dynamic network and quantitative network may be another tendency and more and more employ of network pharmacology technology makes the expenditure much less in the future.

Conflict of Interest

The authors have declared that there is no conflict of interest.

Author Contributions

Guo J and Yan SK conceived the idea. Luo TT drafted the manuscript. Lu Y, Xiao X and Rong XL revised and proofed the manuscript. All authors contributed to literature review and approved the final manuscript for publication.

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