



Research progress and prospect on the effective material basis and related mechanism of Traditional Chinese Medicine driven by systematic thinking

XIE Yuanyuan^{1,2,3}, LUO Jiayi^{1,2,3}, WANG Yiming⁴, LUO Guoan^{4*}, WANG Shumei^{1,2,3*}

¹Guangdong Pharmaceutical University, Guangzhou, 510006, China;

²Guangdong Research Center for Quality Engineering Technology of Traditional Chinese Medicine, Guangzhou, 510006, China;

³Key Laboratory of Digitalized Quality Evaluation Technology of Traditional Chinese Medicine, State Administration of Traditional Chinese Medicine, Guangzhou, 510006, China;

⁴Department of Chemistry, Tsinghua University, Beijing, 100084, China

[Abstract] Traditional Chinese medicine is the treasure of Chinese traditional culture. A series of questions should be answered on the base of systematic, comprehensive and in-depth research according to the TCM theory and its own characteristics. For example, how to construct a research framework with clear guidance and route, as well as reasonable plan for elucidating the effective material basis of Traditional Chinese medicine (TCM) formula? How to discover and excavate the effective material basis of TCM, clarify and interpret the related action mechanism? How to clarify the reasonableness of compatibility, the scientificity for usage of medication, as well as the scientific connotation of TCM in preventing and curing disease? Aforementioned questions had been considered as the important frontier scientific problems to be solved in the field of TCM. According to the practice progress and thinking on research of effective material basis and related mechanism in Naomaitong Granule and Dengzhan Shengmai Capsule, a systematic strategy from the perspective of interaction between drug system, intestinal microecosystem and host living system had been proposed and elucidated in three aspects: (1) Discovering the TCM regulated genes and proteins dated from functional metabolites, in order to characterize the biological response of host systematically. (2) Deciphering the effective material basis and related acting mechanism on the base of interaction between TCM formula and intestinal microecosystem. (3) The systematic characterization of material basis of TCM formula by Chemomics.

[Key words] Holistic Systems medicine; Systems biology; Ischemic stroke; Multi-omics; Traditional Chinese medicine

1 Introduction

Chinese herbal medical science had been run

a long history in China. Traditional Chinese medicine (TCM) is a treasure of traditional Chinese culture. TCM formula was considered as the main dosage form of TCM in clinic. According to the principle known as "monarch, minister, assistant and guide" in

[*Corresponding author] E-mail: luoga@tsinghua.edu.cn (LUO Guoan); 2395903468@qq.com (WANG Shumei).

TCM and the compatibility theory of "*Qiqinghehe*", which means the combination of two or more herbs, with 7 different interaction forms. Various ingredients in TCM prescriptions can complement and reinforce each other and play a role in integrating adjustment. It shows a unique advantage in the treatment of chronic, complex and multifactorial diseases caused by disorders of human body's own metabolism and regulation. However, how do the main components in the complex substance system of TCM treat diseases through "harmonious action"? How to construct a basic research framework with clear guidance and route, as well as reasonable scheme for elucidating the effective material basis of TCM formula according to TCM theory and its own characteristics? How to discover and excavate the effective material basis of TCM, clarify and interpret the related action mechanism? How to clarify the rationality of compatibility, the scientificity for usage of medication, as well as the scientific connotation of TCM in preventing and curing disease through systematic, comprehensive and in-depth research? Aforementioned questions had been considered as the important frontier scientific problems urgently to be solved in TCM^[1].

In recent years, the idea of solving complex biological problems with systematic thinking and multi-disciplinary technology has become the forefront and hot point of life science research, and also provides a new technical methods for the integration of TCM theory and modern medicine. In 2008, a team led by Academician Zhu Chen published a paper in the Proceedings of the National Academy of Sciences (PNAS), in which the molecular mechanism of Chinese medicine prescription Compound Huangdai tablet in the treatment of acute promyelocytic leukemia was systematically analyzed for the first time by using systems biology techniques. The mechanism of multiple components and multiple targets of Compound Huangdai tablet in the treatment of leukemia were clarified in molecular level. And

the compatibility principle of "monarch, minister, assistant and guide" in TCM prescription were explained with the modern medical language "data", which had become the "pioneer" of systematic biology research on TCM^[2]. At present, systems biology techniques such as metabolomics, proteomics, metagenomics and microbial metabolomics, as well as bioinformatics such as network pharmacology have become the supporting technologies for modern research of TCM, which have played a positive role in clarifying the scientific connotation of TCM theoretical system^[3-4]. Based on the practice and thinking in the research of Naomaitong granules and Dengzhanshengmai capsules, the author proposed a research strategy on the base of interaction between drug system, intestinal microecosystem and host living system, in order to explain the effective material basis and related action mechanism of TCM based on systematic thinking, in order to provide new ideas and methods for the modern basic research of TCM compound.

2 The interaction between "drug system-intestinal microecosystem-host living system" should be the main mode of oral administered TCM exerting its therapeutic effect.

TCM Formula was an ordered system formed by the compatibility of herbal medicines with the principle of "monarch, minister, assistant and guide". And components in TCM formula are transformed and metabolized under the influence of gastrointestinal acid and base, intestinal microecosystem and metabolic enzymes after oral administration. Then, the components are directly absorbed into the blood as prototype or metabolites and be sent to various organs of the host biological system, acting on the primary and secondary targets, disturbing different pathophysiological processes, and playing a role of integrate adjustment^[5]. Meanwhile, components in TCM formula can

regulate the components of intestinal flora, correct and improve the imbalance of intestinal flora. The changes in the structure of intestinal flora as well as the types and contents of intestinal microecological metabolites will affect the physiological and pathological state of the host life system, and then affect the occurrence and development of diseases. Therefore, the interaction between drug system, intestinal microecosystem and host biological system should be fully considered in identifying the effective material basis and explaining the action mechanism of TCM (Fig. 1). The drug system of TCM was characterized with step-by-step Chemomics strategy in integrated system biology, in order to discover and characterize the effective chemome (effective component groups) and effective compound mixtures (effective material basis). Classical pharmacology, genomics, proteomics, metabolomics, and microbiome were applied to characterize the intervention response of intestinal microecosystems and host living systems. The "system-system" model was established to clarify the interaction between TCM formula (drug system) and organism (living system), identify the primary and secondary targets and pathways, and provide a reference research model for explaining the effective material basis

and action mechanism of TCM through systematic biological analysis^[3-8].

3 Discover regulatory genes and proteins based on functional metabolites, and characterize host living system responses systematically

3.1 A multi-platform metabolomics research and biomarker discovery technology system should be built to improve the "explanatory power" of metabonomics results

Genes, proteins and small molecule endogenous metabolites are the basic substance in cellular living activity. Endogenous metabolites are located in the downstream of gene regulation network and protein action network, representing the terminal of biological information flow and phenotypic information. In the process of disease occurrence and development, small changes in gene and protein expression at the functional level will lead to corresponding changes in the types and concentrations of endogenous small molecule metabolites of the body. Compared with genome, transcriptome and proteome, metabolome can reflect the overall functional state of living system

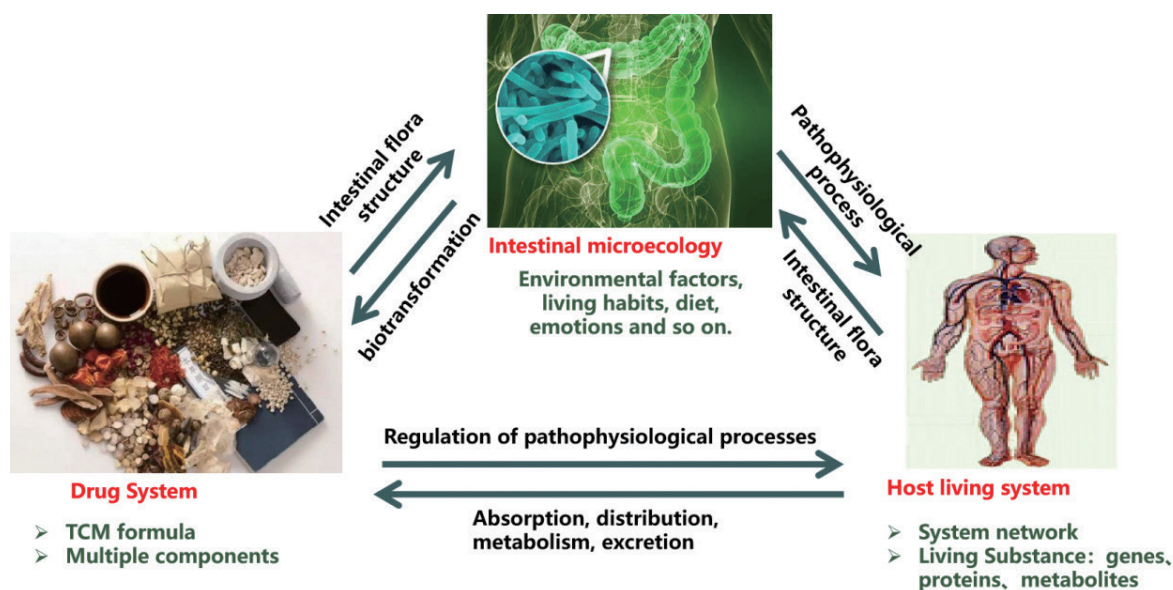


Fig. 1 The interaction between "drug system-intestinal microecosystem-host living system"

more instantly^[9]. Since endogenous small molecule metabolites can penetrate the blood-brain barrier and intestinal barrier, as biomarkers for the development of brain diseases, they are more advantageous than large molecules such as DNA, RNA, microRNA and protein^[10]. Therefore, it has been widely used in the basic research of TCM formula that using metabolomics as an entry point. The types and content changes of endogenous metabolite biomarkers would be measured to characterize the overall efficacy of TCM, and clarifying the pharmacodynamic substances basis and related mechanism^[11-14].

How to accurately identify biomarkers that representing the functional information of the pathological mechanism of diseases, accurately measure their content and change rules, and provide a reliable data basis for the integration of multi-omics studies and molecular biological verification, as well as the formation of "hypothesis" for in-depth research in the massive data obtained from high-throughput metabolomics studies, should be a key scientific problem to be solved in data driven metabolomics. One of the solutions is to improve the detection ability of analytical methods under the existing technology level. It is also necessary to optimize data processing methods and improve the efficiency and accuracy of data analysis.

Metabolic profiling analysis and target metabolomics analysis are two strategies in metabolomics research. However, due to the limitations of sample pretreatment methods and analytical techniques, biomarkers discovered by metabolic profiling was limited to characterize the overall effect, and the local characteristics are vague. By studying the changes of several endogenous metabolites in a specific cycle, however, it is difficult for target metabolomics to reflect the scientific connotation of the integrative regulation of TCM formula, which is one of the urgent scientific problems to be solved in TCM metabolomics. The holistic characterization of endogenous

metabolites derived from multi-platform metabolic profiling analysis should be combined with local characterization of key endogenous metabolites in key metabolic cycles derived from target metabolomics^[15]. It not only gives consideration to the breadth of metabolites that can be analyzed, but also gives consideration to the quantitative characterization of the disturbed key metabolic cycles, which can provide more detailed data for the metabolite network construction of disease phenotypic changes in living systems after drug intervention. In the systematic biology study of *Tangshen formula* in treating diabetic nephropathy, on the base of metabolomics research, a quantitative metabolomics platform was established by integrating metabolic profiling analysis, and target metabolomics analysis including mercaptan amino acid metabolism, purine and pyrimidine metabolism, fatty acid metabolism and phospholipid metabolism. Metabolic biomarkers closely related to the pathological stages of diabetic nephropathy in Western medicine and TCM syndrome differentiation were obtained. Neurofuzzy logic algorithm was used to integrate and focus pathological and biochemical indicators, evaluation indicators of TCM syndromes and symptoms, as well as metabolomics data, an integrated biomarker system was found for early diagnosis of diabetic nephropathy, TCM syndrome differentiation and clinical evaluation of *Tangshen formula*^[8,16].

Pathogenesis of disease should be related with complex, multi-level and non-linear network changes. However, multivariate statistical analysis based on linear computation is commonly applied to discover biomarkers at present, which certainly resulted in the loss of useful information. Biomarkers discovered in this way are scattered in multiple metabolic pathways with partially overlapping and Crosstalk effects. The correlation among biomarkers is weak, and the information obtained is too "fragmented", which makes it difficult to mine the biological information hidden behind

these phenotypic biomarkers. Neurofuzzy logic^[17] combined with adaptive learning ability of neural network and general expression ability of fuzzy logic, mainly screened metabolomic biomarkers according to the variation of variable content and the relationship between variables, and obtained phenotypic biomarkers with "cause-effect", which can effectively improve the "explanatory power" of metabolomics research results.

3.2 Constructing "functional metabolite-protein interaction network" to realize the organic fusion of "multi-omics" data

More and more research evidence indicates that endogenous small molecule metabolites are not only biomarkers of body phenotype, but also functional molecules (functional metabolites) that regulate physiological processes and phenotypes by regulating genome, transcriptome and proteome functions^[19]. On the base of biomarkers that characterize the overall effect of TCM formula, pathway enrichment analysis and functional interpretation with the help of pathway databases and bioinformatics tools, such as KEGG, Reactome, MetaboAnalyst and IMPaLA, had been applied to identify functional metabolites. However, due to the "fragmentation" of information acquired by different omics, the focused pathway is often limited to the enriched pathway with high abundance proteins and metabolites, which makes it difficult to explain the synergistic characteristics of TCM formula and may only provide one-sided conclusions. Pathway enrichment analysis is a key step in interpreting the biological significance of metabolomics and integrating it with other omics data. Over Representation Analysis (ORA) and Functional Class Scoring (FCS), as statistical tools, are commonly used for pathway enrichment analysis. R language and Cytoscape were used to construct a "functional metabolites-regulatory protein-pathway" network with wide coverage (holistic) and clear local characteristics. Some information such as metabolite content, chemical category,

biological correlation and positive and negative feedback regulation process could be reflected in the network structure. Thereby, the efficacy and related mechanism of TCM formula would be characterized in a more suitable way^[20-23].

Differential protein biomarkers reveal the dynamic changes of life activities under pathological conditions at the protein function level. Therefore, integrated metabolomics and proteomics studies have been applied to elucidate the pharmacodynamic substances basis and mechanism of TCM^[24-28]. Generally speaking, intersecting pathways were found by integrating metabolomics and proteomics studies, then specific targets and pathways were verified by molecular biology study.

Such as an integrated study on the active components and the related mechanism of Dengzhanshengmai capsule acting on ameliorating chronic cerebral ischemia in rats, metabolomics, proteomics, patch clamp technique, and other technology was applied. The results showed that scutellarin and 4,5-di-*O*-caffeoylquinic acid derived from *Erigeron breviscapus* were the pharmacodynamic substance basis of Dengzhan shengmai capsule to improve chronic cerebral ischemia, reduce neuronal injury and play a neuroprotective role in rats^[29]. In our previous studies, the metabolite and enzyme information associated with metabolomic and proteomics biomarkers were integrated for pathway enrichment analysis to elucidate the action mechanism of *Dengzhan shengmai formula*. Eight metabolic pathways closely related to the ischemic stroke were enriched, including glycolysis/gluconeogenesis, biosynthesis of unsaturated fatty acids, arginine and proline metabolism, glutathione metabolism, pentose phosphate pathway, aminoacyl-tRNA biosynthesis, butanoate metabolism, as well as and valine, leucine and isoleucine biosynthesis. However, there are also some problems in the existing pathway enrichment analysis methods, such as over-simplification of biological processes, lack of network characteristics of biological processes, and artificial "boundary"

of different pathways, which makes the results difficult to reflect the real biological mechanism^[21]. In order to present the biological correlation among biomarkers and the dynamic process of drug disturbance in different pathways intuitively and integrally, new breakthroughs in bioinformatics and artificial intelligence technology were urgently needed.

Small molecule metabolites play an important role in maintaining cell physiological function, by interacting with intracellular protein targets to provide energy, activate signal receptors and regulate cell communication. The activities of metabolic enzymes, transcription factors, transporters and membrane receptors are all mediated by protein-metabolites (PMIs), in order to link cellular metabolism with genetic/epigenetic regulation, environmental sensing and signal transduction^[30]. There are two strategies for establishing PMIs in natural cell matrices treated with specific metabolites or proteins. One is metabolomics studies based on proteins. Endogenous metabolites interacting with purified specific protein targets are captured and identified. Then the molecular structures of the metabolites were identified by metabolic profiling or target metabolomics analysis. The other one is proteomics research based on small molecule metabolites. Synthetic chemistry methods were applied to introduce photocrosslinkable groups and reporter groups into the small molecule probe (metabolites) in formation of bifunctional ligand. After the probe was incubated with cells for a period of time, the photocrosslinkable groups in the probe are excited by ultraviolet rays, then it is linked to its target protein with covalent bond. The labeled protein was enriched by reporter group, then, the protein (target protein) interacting with ligand compounds (metabolites) in cell lysate was found by stable isotope labeling with amino acids in cell culture (SILAC) or label-free proteomics analysis. The specific binding of proteins to ligands (small molecule probes) was analyzed through

the thermodynamics of molecular docking, drug affinity related target stabilization technology (DARTS), thermal proteome profiling (TPP) and so on. The interaction between small molecule metabolites and protein could be proved through immunoprecipitation or siRNA transfection of affinity protein. It has been successfully applied in the analysis of cholesterol, phospholipid and sphingomyelin^[30]. In addition, Moellering's team in Germany had reported a whole-cell method for the analysis of protein-metabolites and protein-protein complexes, molecular exclusion chromatography was used for separation in this method, the obtained components were then subjected to quantitative metabolomics and proteomic analysis, the putative interaction is defined by co-elution. This approach has been successfully used to identify putative feedback and feedforward regulation of pantothenate and methylthioadenosine metabolic pathways in *Arabidopsis thaliana* cell cultures^[33]. Therefore, using functional metabolites as probes, constructing "protein-metabolite interaction network" by "hooking" key target proteins closely related to functional metabolites by proteomics, is one of the approaches to realize the organic fusion of "multi-omics" data.

3.3 The interaction between TCM formula and intestinal microecosystem

Intestinal flora plays an important role in various pathophysiological functions such as energy uptake, drug metabolism, mucosal barrier, immune regulation and resistance to infection^[34]. Ischemic stroke is a cerebrovascular disease. It is caused by cerebral ischemia and hypoxia cascade reaction induced by cerebral ischemia, and then leads to necrosis, softening and formation of infarction^[35]. Small molecule metabolites can immediately reflect the pathological state of ischemic stroke through the blood-brain barrier. Therefore, metabolomics has become an ideal tool for understanding the pathogenesis of ischemic stroke, discovering

biomarkers for disease risk assessment, clinical diagnosis, identification of complications, prediction of recurrence and evaluation of drug efficacy. Amino acids, lipids (LPCs, PCs and fatty acids) and energy metabolism intermediates were found to be biomarkers of ischemic stroke disease progression in clinical practice, involving abnormal regulation of metabolic pathways such as neuroexcitatory toxicity, inflammation, apoptosis, oxidative stress, neuroprotection, disorder of energy and the increase of Ca^{2+} in cells^[11]. In the brain tissue of model rats with ischemia-reperfusion injury, nucleic acid metabolites and neurotransmitters were also significantly altered^[12]. TCM has shown good effect in the treatment of ischemic stroke, mainly reflected in the regulation of fatty acid metabolism, amino acid metabolism, lipid metabolism, energy metabolism and nucleic acid metabolic cycle^[13,32]. Intestinal flora and nervous system regulate each other through neuroimmunity, neuroendocrine, vagus nerve pathway, intestinal immunity, intestinal mucosal barrier and blood-brain barrier, also affect the occurrence and development of ischemic stroke^[36]. In clinical samples and animal models of ischemic stroke, significant changes in intestinal flora and its metabolites were found, including a significant decrease in the diversity of intestinal flora, excessive growth of bacteroidetes, structural disorder of intestinal flora, organism immune stress, and these changes affect the inflammatory response and prognosis of stroke^[37]. Conversely, dysregulation of the intestinal flora can also impair the functions of the intestinal barrier and blood-brain barrier^[38]. When some intestinal flora metabolites such as short chain fatty acids (SCFAs), trimethylamine-*N*-oxide (TMAO) and amino acids entered the circulation system, the "intestinal flora-gut-brain axis", together with immune system and inflammatory system would be regulated, the blood-brain barrier and nervous system functions would also be affected^[39]. SCFAs were found to be effective in providing energy for the host, regulating blood circulation, maintaining the balance of body

fluids and electrolytes, affecting the integrity of the blood-brain barrier, regulating neuroinflammation and nerve regeneration, maintaining the stability of the *vivo* environment in the central nervous system and regulating neuroinflammation^[40]. By activating platelet activity, TMAO promotes the formation of atherosclerotic plaque and thrombosis, as well as the occurrence of inflammatory reactions *in vivo*, and increases the risk of ischemic stroke and recurrence^[41]. The types and contents of intestinal flora metabolites would be changed due to the affected intestinal flora structure with TCM administered orally, and the nerve damage induced by cerebral ischemia-reperfusion would be improved^[42]. As signal molecules and substrates of host metabolic reactions, metabolites of intestinal flora also affect host physiological and pathological processes. Therefore, it is necessary to pay attention to changes of endogenous metabolites and intestinal microflora metabolites in the study of metabolomics.

Previously, intestinal flora changes together with metabolomics study was integrated to discuss the related mechanism of Naomaitong granule acting on ischemic stroke in view of "intestinal flora-gut-brain axis". As a result, Naomaitong granule could improve the neurological function and decrease infarct size in middle cerebral artery occlusion (MCAO) model rats, and reverse the damage of intestinal barrier of model rats. The improvement effect of Naomaitong granule on the prognosis of stroke was found to be associated with the adjustment of intestinal flora structure, and the increase of beneficial bacteria. The metabolic profiling of intestinal microbiota also changed significantly. Naomaitong can intervene the prognosis process of MCAO rats by reducing the content of ricinoleic acid, phospholipid choline, sphinophospholipid and creatine *in vivo*, as well as increasing the content of galacturonic acid and cystine.

These metabolites exhibited key effects on the intervention of Naomaitong in MCAO rats, such as stimulating colonic mucosa and reduce

bacterial migration by alleviating ricinolic acid, protecting neurons by increasing the content of genistein, increasing the content of galacturonic acid as the metabolic substrate of bifidobacterium and lactobacillus, and increasing the content of cystine and threonine for protecting astrocytes^[43].

At the same time, the intestinal flora is one of the bio-converters of TCM components, it can enhance the absorption of the active ingredients, improve the bioavailability, many TCM components would be metabolized by intestinal flora before being absorbed and exerting pharmacological effects. For example, ginsenosides play a role in the body with the form of its intestinal microecological metabolites rather than prototype compounds^[44]. Therefore, drug metabolism based on intestinal flora is expected to reveal the pharmacodynamic basis of brain-targeted neuroprotective effects of TCM formula.

4 Chemomics strategy applied for systematic characterization of effective material basis of TCM formula

TCM formula was considered as an "orderly" system, which was composed of herbal medicines following the principle of "monarch, minister, assistant and guide". Therefore, the Chemomics with the guidance of systematic thinking reveals the compatibility relationship of TCM formula in a hierarchical and progressive manner, and clarifies the pharmacodynamic substance group of TCM step-by-step. With the guidance of TCM theory, biological activity was taken as orientation, and modern and multi-disciplinary advanced technology was integrated in Chemomics study, including phytochemistry, analytical chemistry, molecular imaging, systems biology, chemical biology, information science and computational science^[6].

It is still a scientific problem need to be solved urgently that how to associate the biological response of living system with the pharmacodynamic substance basis of TCM, so as to reveal the effective components, and clarify their acting targets and

pathways respectively. Although components in TCM could be associated with biomarkers through correlation analysis such as Chinmedomics^[32] and pharmacokinetic integrated with metabolomics studies^[33]. However, it is still unclear how the effective substances regulate the genes, proteins and endogenous metabolites exactly. The combination of network pharmacology and omics technology, virtual screening and experimental verification has made some achievements in explaining the mechanism of *Tribulus terrestris*, Erzhi pill, Bushen-tiansui formula, and so on^[20,45-46]. Due to network pharmacology and molecular docking technologies are limited by databases and algorithms, they can only play an auxiliary role of prediction and inference in the discovery of effective substances basis and mechanism interpretation of TCM formula. On the one hand, the components used for target prediction are not the necessary direct effect components *in vivo*, which cannot reflect the real action process. On the other hand, the research results need to be verified, and the verification of regulatory relationship between components and pathways one by one usually cuts off the interaction among components. Recently, deep learning artificial intelligence and other technologies have played an important role in the structural identification of active ingredients in TCM^[47], prediction of action targets^[48] and interpretation of biological mechanisms^[49]. Applying artificial intelligence technology in omics research for in-depth analysis and mining of massive biological data, obtaining key information and realizing organic fusion of "multi-omics" should be a key issue in research of TCM systems biology. How to use artificial intelligence technology to realize the intelligent analysis of effective material basis and action mechanism of TCM is also a key challenge.

5 Summary and Prospect

Aforementioned, it has become the consensus in the field of TCM research to carry out the pharmacodynamic substances basis research under

the guidance of the holistic view and systematic theory of TCM. Recently, based on some technical methods and hypotheses, including systems biology, network pharmacology, serum pharmacochemistry, Chinmedomics, intestinal flora microbiomics, biological affinity chromatography, high-throughput screening, spectrum efficiency, as well as traditional pharmacokinetic (PK) and pharmacodynamics (PD) related model, have promoted the development and progress of TCM research to a certain extent^[50-52]. According to the practice progress and thinking on research of effective material basis and related mechanism in Naomaitong granule and Dengzhan Shengmai capsules, a systematic strategy from the perspective of interaction between drug system, intestinal microecosystem and host living system had been proposed here. The research progresses and existing problems during the integrated application of Chemomics, systems biology, intestinal flora microbiomics and bioinformatics in TCM research had been elucidated systematically. There is no doubt that modern research on TCM should adhere to the guidance of traditional Chinese medicine theory. Meanwhile, with the development of science and technology, there will be more and more new technologies to help the modernization of TCM. It has become an irresistible trend for multi-disciplinary integration to solve the key scientific problems in traditional Chinese medicine research.

6 Conflicts of Interest

These authors have no conflict of interest to declare.

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