Front. Med. 2020, 14(5): 651–663
https://doi.org/10.1007/s11684-019-0709-5

RESEARCH ARTICLE

High-throughput metabolomics reveals the perturbed metabolic pathways and biomarkers of Yang Huang syndrome as potential targets for evaluating the therapeutic effects and mechanism of geniposide

Heng Fang[1], Aihua Zhang[1], Xiaohang Zhou[1], Jingbo Yu[1], Qi Song[1], Xijun Wang (✉)[1,2]

[1]*National Chinmedomics Research Center, Sino-America Chinmedomics Technology Collaboration Center, National TCM Key Laboratory of Serum Pharmacochemistry, Laboratory of Metabolomics, Department of Pharmaceutical Analysis, Heilongjiang University of Chinese Medicine, Harbin 150040, China;* [2]*State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau 519020, China*

Abstract **High-throughput metabolomics can clarify the underlying molecular mechanism of diseases via the qualitative and quantitative analysis of metabolites. This study used the established Yang Huang syndrome (YHS) mouse model to evaluate the efficacy of geniposide (GEN). Urine metabolic data were quantified by ultra-performance liquid chromatography–tandem mass spectrometry. The non-target screening of the massive biological information dataset was performed, and a total of 33 metabolites, including tyramine glucuronide, aurine, and L-cysteine, were identified relating to YHS. These differential metabolites directly participated in the disturbance of phase I reaction and hydrophilic transformation of bilirubin. Interestingly, they were completely reversed by GEN. While, as the auxiliary technical means, we also focused on the molecular prediction and docking results in network pharmacological and integrated analysis part. We used integrated analysis to communicate the multiple results of metabolomics and network pharmacology. This study is the first to report that GEN indirectly regulates the metabolite "tyramine glucuronide" through its direct effect on the target heme oxygenase 1 *in vivo*. Meanwhile, heme oxygenase-1, a prediction of network pharmacology, was the confirmed metabolic enzyme of phase I reaction in hepatocytes. Our study indicated that the combination of high-throughput metabolomics and network pharmacology is a robust combination for deciphering the pathogenesis of the traditional Chinese medicine (TCM) syndrome.**

Keywords metabolomics; liquid chromatography-mass spectrometry; metabolites; metabolic pathways

Introduction

Metabolomics is a precise and comprehensive method for understanding and anticipating the mechanisms of a complex system [1,2]. It involves essential information about the dynamic metabolic responses of an organism to comprehensive factors and is applied in the biosciences. Recently, the development of robust analytical techniques has made the global detection of entire biomolecules of an organism possible. Modern tools with high resolution and sensitivity ensure that useful information about a disease can be found. Among them, nuclear magnetic resonance spectroscopy or ultra-performance liquid chromatography–tandem mass spectrometry (UPLC/MS) technology has been widely used for metabolomics given its remarkable performance and advanced data processing capabilities. Nowadays, these methods have been recognized as optimal tools for the analysis of complicated systems, especially for the clarification of traditional Chinese medicine (TCM). TCM has existed for thousands of years as the unique field of life science. However, the precise mechanisms of action of many of its agents remain unclear and severely limit the generalization of TCM. Fortunately, metabolomics, through holistic and systematic studies of metabolic pathways and biomarkers, coincides

Received March 16, 2019; accepted June 25, 2019
Correspondence: Xijun Wang, xijunwangls@126.com

with the theory of TCM and provides new directions for the basic research, clinical application, and new development of TCM [3–9].

Yang Huang syndrome (YHS), as indicated in the authoritative ancient codes and records of TCM "Shang Han Lun," has been reported for more than a thousand years. YHS is similar to the initial stage of jaundice characteristics associated with yellowing of the skin and pupils. The phenotypic characteristic of YHS differs from that of any other disease due to canary sclera, which is probably the primary distinguishing facet of clinical presentation between YHS and Yin Huang syndrome [10]. Pharmacological research shows that patients with YHS usually suffer from liver disease [11–22], which is one of the most threatening diseases in the world. Thus, unambiguous and exhaustive cognition is urgently needed to respond to this common fatal disease. We previously conducted urine metabolomics investigation for the biomarker discovery of patients with YHS [23]. These biomarkers deciphered the potential pathogenesis and metabolic network of YHS, which is characterized as the bridge for the construction of a YHS mouse model. Our previous work investigated the serum biomarkers of YHS mice, and 22 serum biomarkers were confirmed with the metabolomics method [24].

Collectively, we applied the triplet factors of Rhizoma Zingiberis, ethanol, and α-naphthylisothiocyanate (ANIT) to construct the YHS mouse model on the basis of the theoretical direction of TCM combined with our former investigation. Geniposide (GEN), used for the treatment of YHS for more than 1000 years, exerts synergistic effect with the guidance of TCM practitioners. TCM practitioners believe that the interaction among multiple components plays a synergistic effect. Therefore, the present investigation focused on a metabolomics and network pharmacology technology concerning the mechanism of GEN on the basis of massive biological datasets derived from advanced instrumentation and software (Fig. 1).

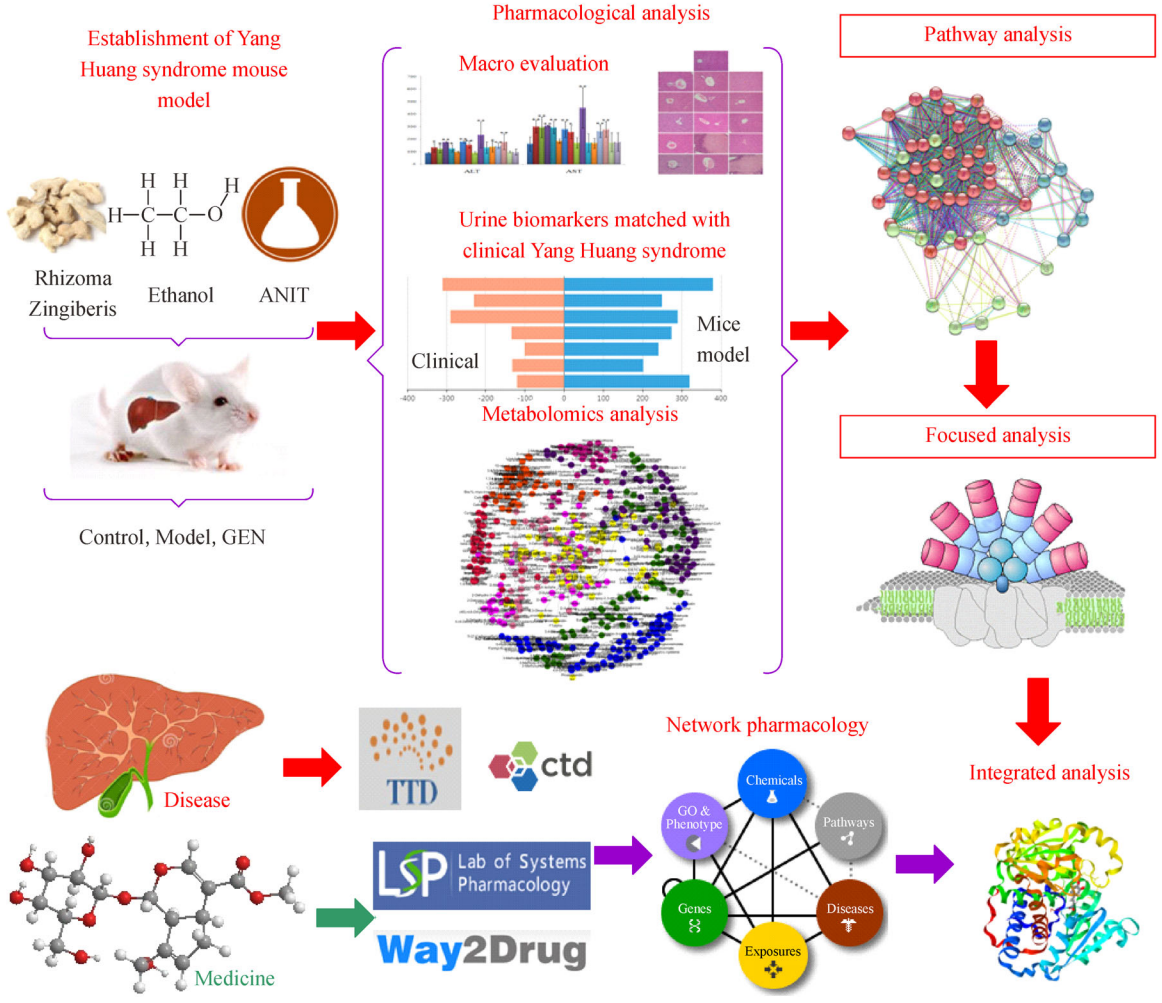

Fig. 1 Comprehensive workflow of the metabolomics investigation for the perturbed metabolic analysis of YHS in a mouse model by using liquid chromatography-mass spectrometry.

Materials and methods

Chemicals and reagents

Analytical formic acid and UPLC-grade acetonitrile were from the Merck Group (Darmstadt, Germany). Deionized water was from Watsons (A.S. Watson Group (Hong Kong) Ltd.). ANIT was from Sigma-Aldrich (Shanghai, China). Rhizoma Zingiberis was from Shi Yi Tang Pharmacy (Harbin, China); GEN (batch number: 110749-200511) was from the China Biological Products Testing Institute. The assay kits of aspartate aminotransferase (AST), alkaline phosphatase (ALP), direct bilirubin (D-Bil), total bilirubin (T-Bil), malondialdehyde (MDA), glutamyl transpeptidase (GGT), glutathioneglutathione peroxidase, total bile acid (TBA), superoxide dismutase (SOD) were from BioSino Bio-Technology and Science Inc. (Beijing, China).

Sample collection and preparation

BALB/c mice (15 ± 5 g) were from Xipuer-Bikai Laboratory Animal Co., Ltd. (Shanghai, China, Qualified number 20180006000662). They were given standardized animal feeding environment [9]. Mice were randomly divided into the following three groups after 7 days of adaptation experiment in a metabolic cage in accordance with body weight ($n = 6$): control, YHS, and GEN. The YHS model was prepared following the established and accepted procedures [8]. The YHS and GEN groups were orally administered with *Zingiber officinale* Rosc. extracted solution (0.013 g/mL) in alcohol (3.125% (v/v)) at 8 a.m. and 2 p.m. at a dose of 0.1 mL/10 g for 14 days. Mice from the control group were orally administered with distilled water of the same dose per day. The YHS group was given different concentrations of ANIT solution (1.5 mg/mL, 1 mg/mL, dissolved in olive oil) from 15 to 16 days once daily at 2 p.m. with a dose of 0.1 mL/10 g. At day 17, the GEN group was treated with 50 mg/kg for 7 days [24]. The control group was orally administered with olive oil of the same dose once a day.

Fresh urine samples were transferred from the metabolism cage and centrifuged immediately (4 °C, 13 000 rpm, 15 min). Then, the supernatants were passed through 0.22 μm filter membrane for metabolomics analysis. All mice were sacrificed for liver tissue and blood samples on day 25 to detect liver histopathology and liver metabolic enzymes.

Chemical ingredient database building and target prediction

GEN maintains high blood concentration and possesses significant pharmacological activity *in vivo* after oral administration. In recent years, GEN has been used in numerous applications in the life science field. In the present study, we first theoretically predicted the target of GEN from molecular docking point of view by using network pharmacological techniques and methods. We obtained the standard mol file from Chemspider and transformed it into mol2 file by using Bio3D Ultra 14.0 of ChemBioOffice 2014. Disease target prediction was carried out from two aspects: drug target and disease target correlation analyses. The top 10 target proteins with matching values were screened from numerous predictive results. Finally, a ligand–receptor docking process was simulated using Autoduck Tools 1.5.6, and the best software and docking orientation was determined.

Clinical chemistry and histopathology analysis

Histopathological observation was performed by separating the serum samples from fresh blood samples for the characterization of the following clinical chemistry parameters: ALT, AST, ALP, D-Bil, T-Bil, GGT, and TBA. Liver homogenate was used for detecting MDA, GSH-Px, and SOD. Intergroup statistical significance was based on P value of < 0.05. The liver sections were also stained with hematoxylin and eosin (H&E) to observe the changes in hepatic tissue and accomplished by the Pathological Center of Heilongjiang University of Chinese Medicine.

Chromatography experiments

Bioinformatics data acquisition section was employed with a Waters ACQUITY UPLC system. The effective separation of components was performed as previously described [8].

Mass spectrometry

A G2Si (Waters Q-TOF SYNAPT™, Waters Corp, Manchester, UK) instrument with electrospray ion source was used in the continuum mode. The detailed parameters were previously described [8].

Multivariate data analysis

Data dimension reduction processing in metabolomics is a key procedure for biological information visualization. This multivariate analysis can be also used to visualize inherent clustering behavior for organ-specific substances in accordance with their mechanism of action. An Advanced Progenesis QI platform (Waters Corp, Version: 2.2) was used to acquire the potential and important notes from raw centroid-data files obtained from SYNAPT G2Si-HDMS. Massive amounts of raw data were disposed through automatic data preprocessing with Progenesis QI. Multidimensional bioinformatics data were transformed after dimensionality reduction into a dataset containing a

3D matrix, which was generated as a temporary result for multivariate data analysis with Simca 14.1 (Umetrics, Sweden). The metabolic data completed through the dimensionality reduction of principal component analysis (PCA) had the most substantial influence on health and disease state. Potential differential metabolites were labeled from the Orthogonal Partial Least Squares Discriminant Analysis-Variable Important Projection (OPLS-DA-VIP-plot), whose components with a considerable influence on grouping were screened out by objective numerical values. The Student's *t*-test was less than 0.05.

Biomarker identification and metabolic pathway analysis

Identifying YHS biomarkers and the relationship with TCM syndrome (or disease) is the core purpose of metabolomics and directly affects the application and objective knowledge on the bioactivity of TCM. Therefore, we used the advanced metabolomics analysis platform Progenesis QI and IPA (Qiagen, Redwood City) for the structural identification of biomarkers on the basis of the accurate detection of MS and MS/MS signals from a G2Si high-definition MS. The qualitative description of low molecular weight metabolites involved a comparison of their chromatographic retention time and MS/MS fragmentation on the basis of commercial database established from literature. The interpretation of the full scan mass spectra was combined with the results from Human Metabolome Database, Metline, and Kyoto Encyclopedia of Genes and Genomes that match the standard MS/MS structure.

Results

Biochemical analysis and histopathological observations

Massive metabolic data interpretation was first based on macroscopic evaluation index validated assessments, including metabolic indicators of liver function and histopathology. AST, ALP, ALT, D-Bil, T-Bil, MDA, GGT, GSH-Px, TBA, and SOD were used to characterize hepatic function as summarized in the Supplementary Table S1 and Fig. 2. D-Bil and T-Bil are the most common indices for jaundice in clinical practice and directly lead to

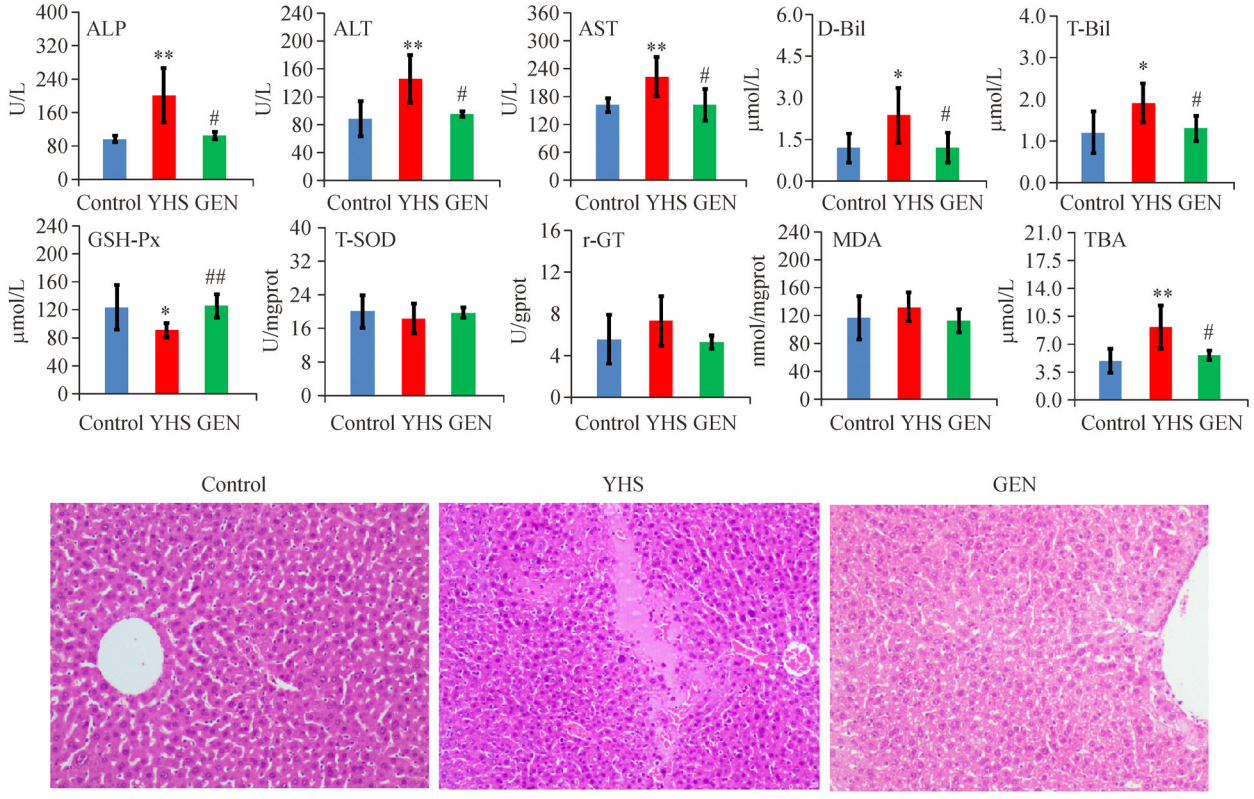

Fig. 2 Biochemical characteristics and H&E staining for liver histological evaluation. *Significant difference from control at $P < 0.05$; **Significant difference from control at $P < 0.01$; $^{\#}$Significant difference from model at $P < 0.05$; $^{\#\#}$Significant difference from model at $P < 0.01$. The corresponding markers are indicated in the Supplementary Table S1.

the exclusive phenomenon of jaundice. The Student's *t*-test results between the control and YHS groups show a significant elevation of the biochemical indicators, such as ALT, AST, T-Bil, and TBA. Meanwhile, significant trends show that the function and metabolism of YHS mouse liver has already been damaged and disordered in terms of ALP, D-Bil, MDA, GGT, GSH-Px, and SOD. Furthermore, we established an H&E staining of liver tissue to obtain a straightforward sense of nidus (Fig. 2). Evident changes were observed in the microscopic analyses of H&E-stained liver sections compared with those of the control group. The liver cells of model group mice showed regional laminar necrosis, and the liver lobules showed edema around the central vein and inflammatory cell infiltration. Thus, YHS was successfully established, and the prevention of GEN by recovering the baseline levels of the control group occurred simultaneously.

Urine metabolic profiles

Massive amounts of raw files from the overall quantitative G2Si-HDMS were analyzed using the Masslynx and Progenesis QI software for data pre-processing, such as peak picking, denoising, and normalization for the generation of a bioinformation matrix. This dataset was then exported to Simca 14.1 software for chemometric analysis. Further visual comparison of complex giant system data was impossible due to the multidimensional elements present in the biological samples. Principal components analysis, as a commonly used multivariate statistical method, was performed to reduce the multiple variables to few sets of factors for the investigation of this complex system. Consequently, the principal component 1 versus principal component 2 plots showed that the profiles of each group from the mass spectrum signal present a regular distribution, with significant intragroup aggregation and distinct intergroup segregation (Fig. 3).

Metabolite structural identification

A UPLC-G2Si-HDMS high throughput analyzer coupled with Progenesis QI showed the retention time, precise molecular mass, and MS/MS data for the structural identification of biomarkers. All of the potential endogenous molecules were identified on the basis of the database embedded in the Progenesis QI software. Finally, 33 potential biomarkers (VIP > 1.0, $P < 0.05$) were statistically discovered (Table S2), which revealed the maximum difference between the control group and YHS group. A total of 17/33 potential biomarkers were downregulated

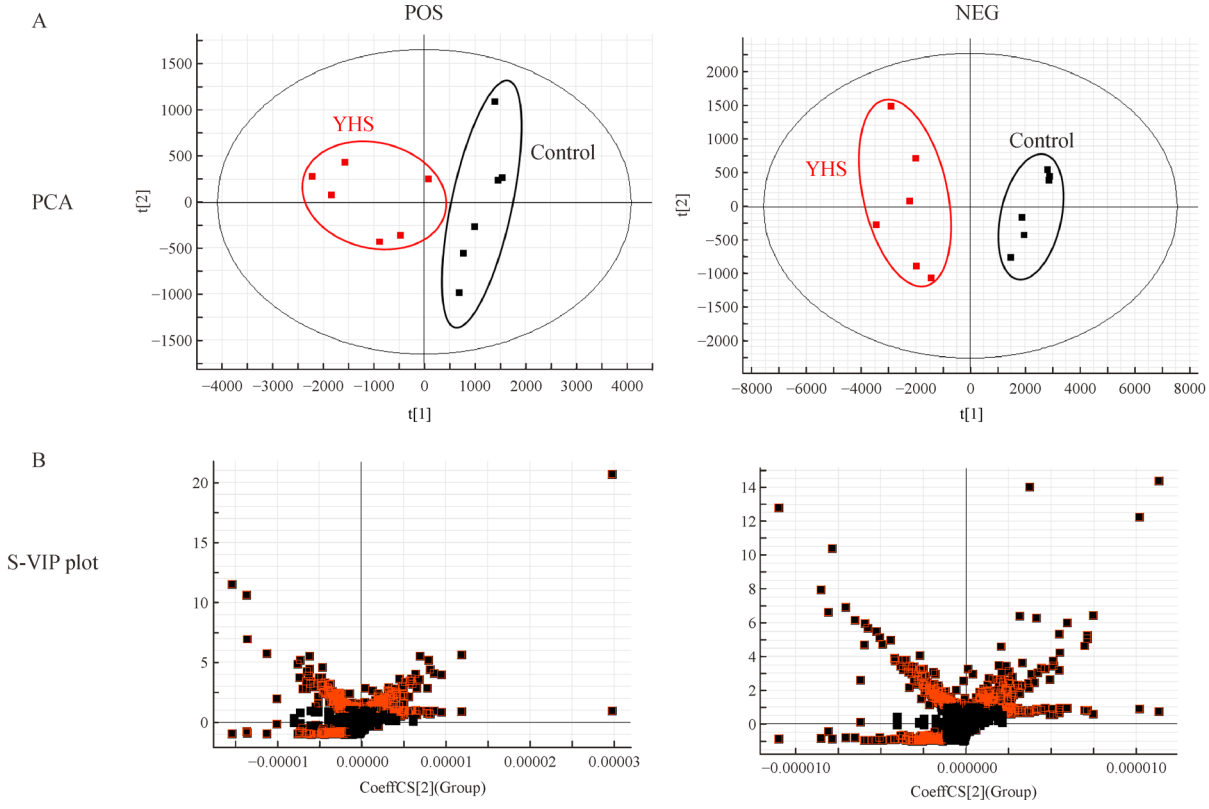

Fig. 3 Pattern recognition analysis of the urine profiling of YHS by using UPLC-Q/TOF-G2Si-HDMS. (A) Trajectory analysis of PCA score plots of YHS in positive and negative modes; (B) S-VIP plot from the supervised OPLS-DA analysis of YHS in positive and negative modes.

from the YHS group, and 16/33 were upregulated (Fig. 4). Fig.5 shows an example of the identification process.

Protection and verification of GEN

The box-plot of the potential biomarkers of YHS showed that the biomarkers in the GEN group gravitated toward the control group compared with those of the YHS group. Thus, the significant changes in metabolism occurred as a result of GEN. GEN, as an effective therapeutic medicine, had a verified probe effect on YHS; thus, the specificity of the YHS model was established. These low molecular mass metabolites may respond to the key mechanism of cellular regulatory and metabolic processes in YHS mice. Our results represented the intuitive information from the key enzymes and those reactions, such as pentose and glucuronate interconversions and primary bile acid biosynthesis, which were involved in the metabolic network built with MetaboAnalyst platform (Fig. 6). The relevant metabolic pathways were analyzed, and a clear metabolic network of YHS pathogenesis and GEN protection of all metabolic pathways involved in this experiment were established and summarized (Fig. 7).

Network pharmacological and integrated analysis

Fig. 8 shows the interaction between the active components with GEN and potential target protein in liver disease. A total of 536 nodes were screened for the network structure. GEN (green circle) hits multiple potential targets, which are involved in a variety of physiologic activities and disease directions. Moreover, the 22 predictions of liver disease involved complex pathways and metabolic mechanisms. A common connection exists between the prediction of GEN and liver disease. Heme oxygenase 1, as the potential target of GEN, possesses the core physiologic activities in the phase I reaction of hepatocytes.

Figs. S1 and S2 reveal the connection between metabolomics and network pharmacology, which involves the bilirubin metabolism and activation of key metabolic enzymes by GEN. The molecular docking results showed that the binding energy of GEN and heme oxygenase 1 was greater than 5 eV, indicating a stable binding capacity and molecular docking state. Analysis of numerous network pharmacological predictive results showed that heme oxygenase 1 is a regulatory factor of tyramine glucuronide,

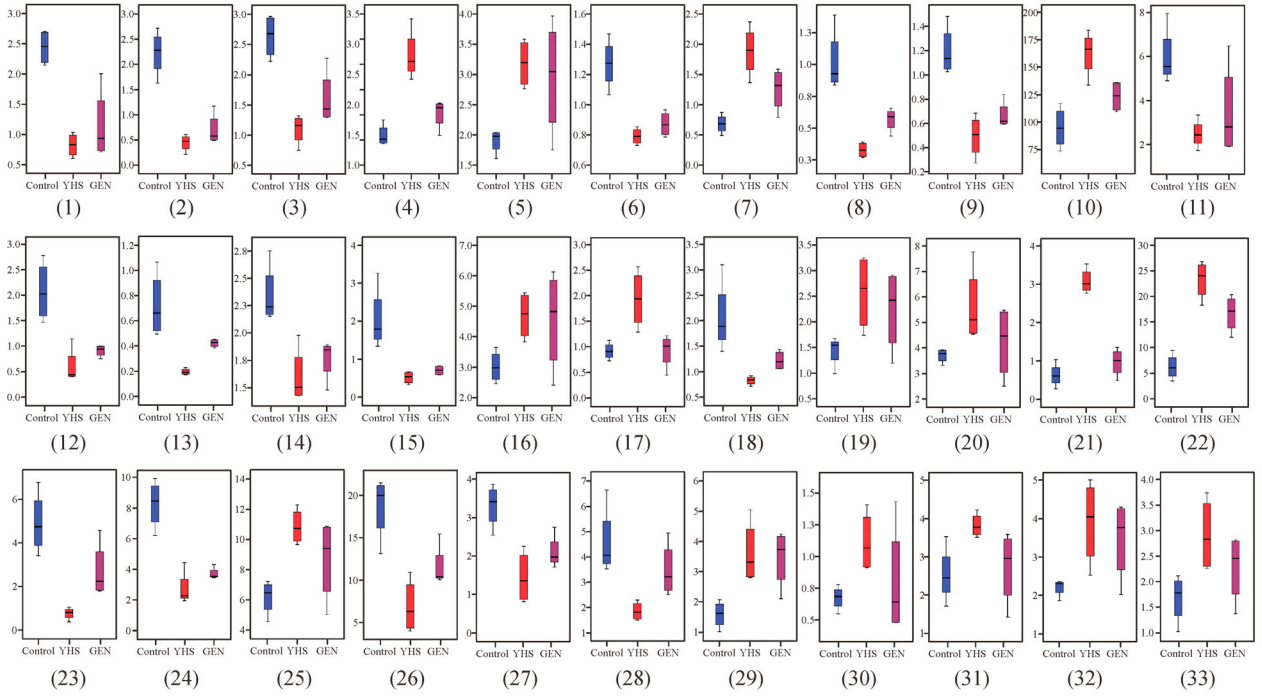

Fig. 4 Dynamic changes of the urine metabolic biomarkers from YHS. (1) pyridoxal; (2) hydroxyphenyl acetylglycine; (3) 7,8-dihydropteroic acid; (4) 1,2-benzoquinone; (5) 5-methoxytryptophan; (6) kynurenic acid; (7) melatonin; (8) L-β-aspartyl-L-leucine; (9) 8-hydroxyguanosine; (10) epinephrine; (11) taurine; (12) 3b,16a-dihydroxyandrostenone sulfate; (13) spermidine; (14) L-cysteine; (15) 2-keto-6-acetamidocaproate; (16) L-β-aspartyl-L-phenylalanine; (17) 3-methylene-indolenine; (18) 5,6-dihydrouridine; (19) 10-formyltetrahydrofolate; (20) tyramine glucuronide; (21) riboflavin reduced; (22) phenylpyruvic acid; (23) isovaleryl alanine; (24) N-formyl-L-methionine; (25) L-γ-glutamyl-L-leucine; (26) L-gulonolactone; (27) gluconic acid; (28) vanillactic acid; (29) homogentisic acid; (30) dopaquinone; (31) N-acetylvanilalanine; (32) 2-isopropylmalic acid; (33) tyrosol.

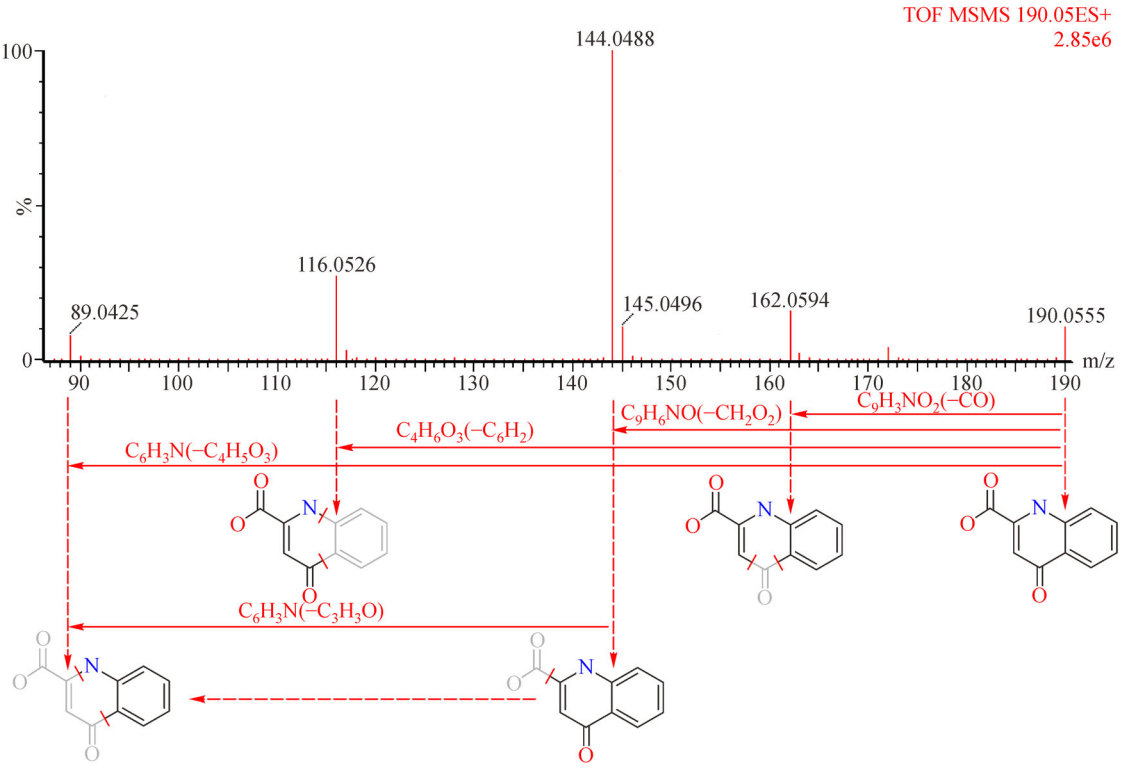

Fig. 5 Chemical structure and mass fragment information of kynurenic acid in the positive ionization mode for the visualization of metabolite identification.

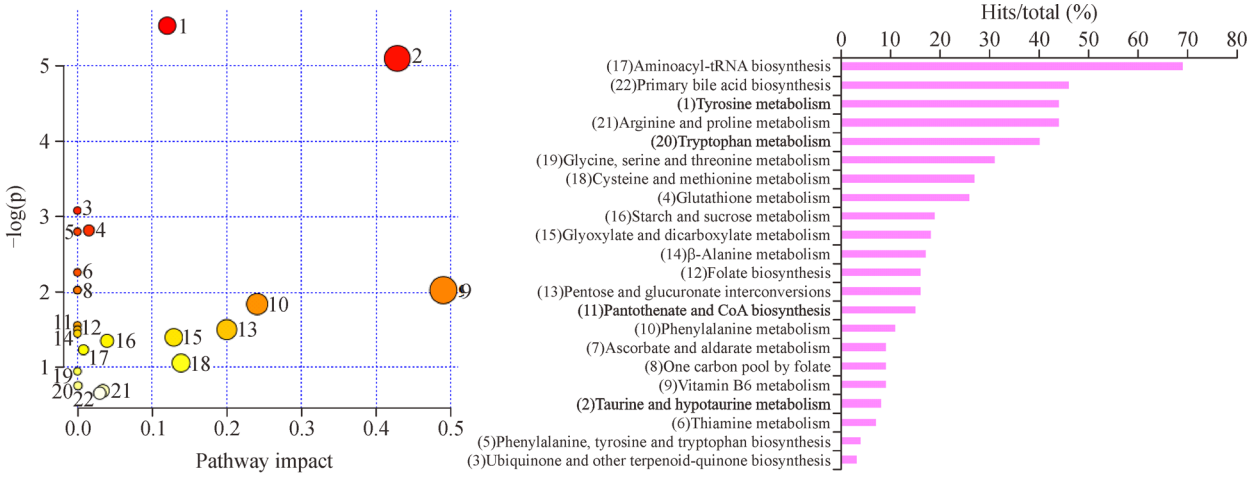

Fig. 6 Summary of pathway analysis with the MetaboAnalyst tool. Note: (1) Tyrosine metabolism; (2) taurine and hypotaurine metabolism; (3) ubiquinone and other terpenoid-quinone biosynthesis; (4) glutathione metabolism; (5) phenylalanine, tyrosine, and tryptophan biosynthesis; (6) thiamine metabolism; (7) ascobate and aldarate metabolism; (8) one carbon pool by folate; (9) vitamin B6 metabolism; (10) phenylalanine metabolism; (11) pantothenate and CoA biosynthesis; (12) folate biosynthesis; (13) pentose and glucuronate interconversions; (14) β-alanine metabolism; (15) glyoxylate and dicarboxylate metabolism; (16) starch and sucrose metabolism; (17) aminoacyl-tRNA biosynthesis; (18) cysteine and methionine metabolism; (19) glycine, serine, and threonine metabolism; (20) tryptophan metabolism; (21) arginine and proline metabolism; and (22) primary bile acid biosynthesis.

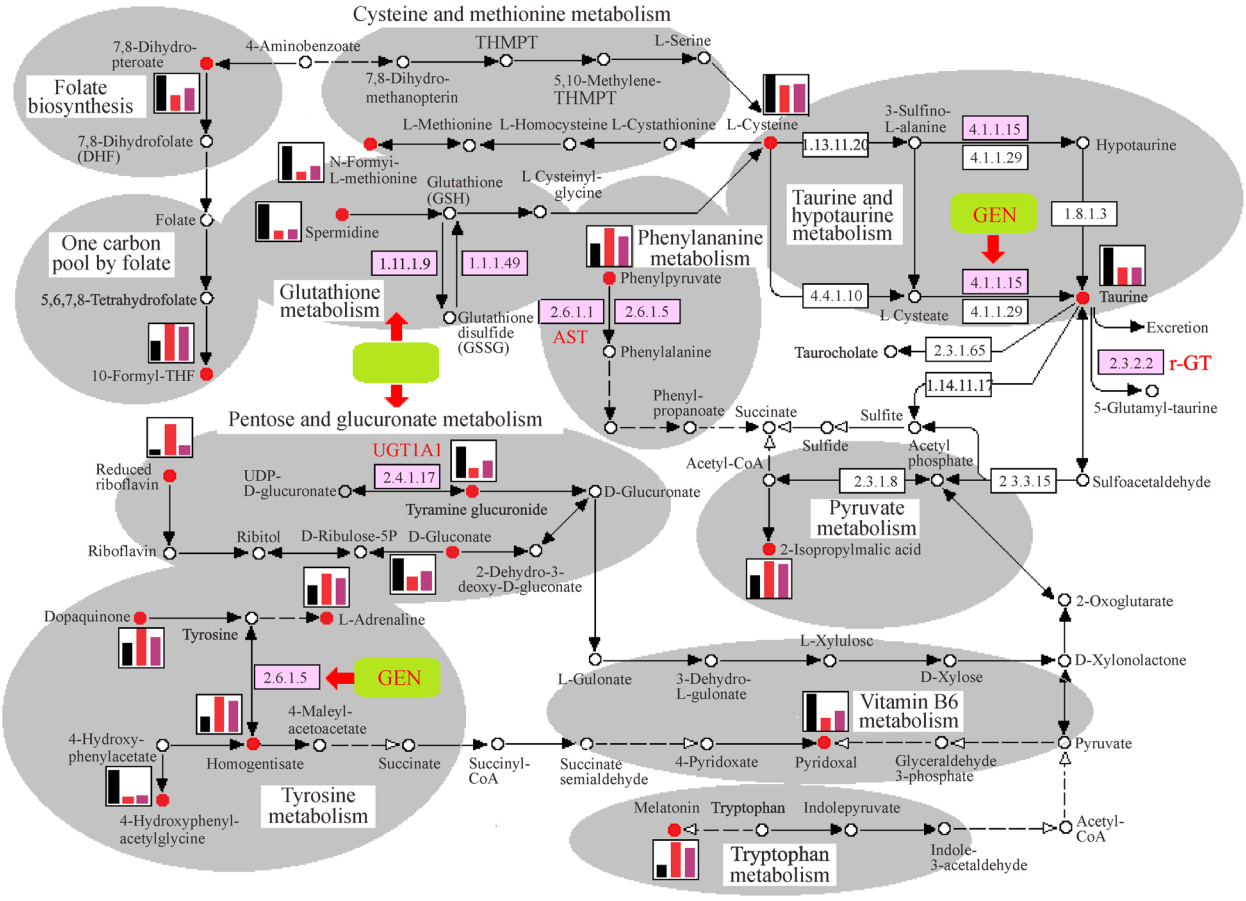

Fig. 7 Perturbed metabolic network of GEN protection against YHS via the Ingenuity Pathway Analysis. Figure in red: potential biomarker of YHS mouse model influenced by GEN, and the relative content is represented by the column graph next to it.

which is a urine biomarker of YHS in mice. Tyramine glucuronide should be synthesized with the modification of heme oxygenase 1. Moreover, heme oxygenase 1 is an important metabolic enzyme of bilirubin, that is, hemoglobin should be metabolized into bilirubin under the heme oxygenase 1 modification. Bilirubin is a direct factor in the pathogenesis and the most intuitive manifestation of jaundice. Hence, heme oxygenase 1 metabolism in patients with YHS is abnormal. Through objective urine metabolic signal data from patients with YHS and network pharmacological analysis of GEN, the therapeutic drug has been validated two times. Thus, GEN may play an irreplaceable role in activating the life activity of heme oxygenase 1.

Correlation analysis of biomarkers between clinical samples and the YHS mice

YHS cannot be diagnosed using the traditional biochemical index. The emerging metabolomics methods used in this study shows a comprehensive and dynamic viewpoint, which is a suitable approach for characterizing YHS. Focusing on biomarkers which have potential roles in the regulation of metabolism may reveal the underlying biological mechanism. We have previously reported [2,8,23,24] urine metabolomics analysis for biomarker discovery and detection of jaundice syndromes in patients and relative mouse model. We compared the time-course changes and metabolic pathways of biomarkers and found the bilirubin metabolism and liver injury indicators related to YHS [8, 23].

Discussion

YHS is a unique disease in terms of the combination of its pathology and specific constitution. Therefore, investigating YHS pathogenesis has a considerable role in promoting drug discovery or effective treatments. We used urine metabolomics approach with a platform combined with UPLC-G2Si-HDMS and advanced data processing software to explore the interactions of GEN and YHS. The YHS mouse model was established to test the actual

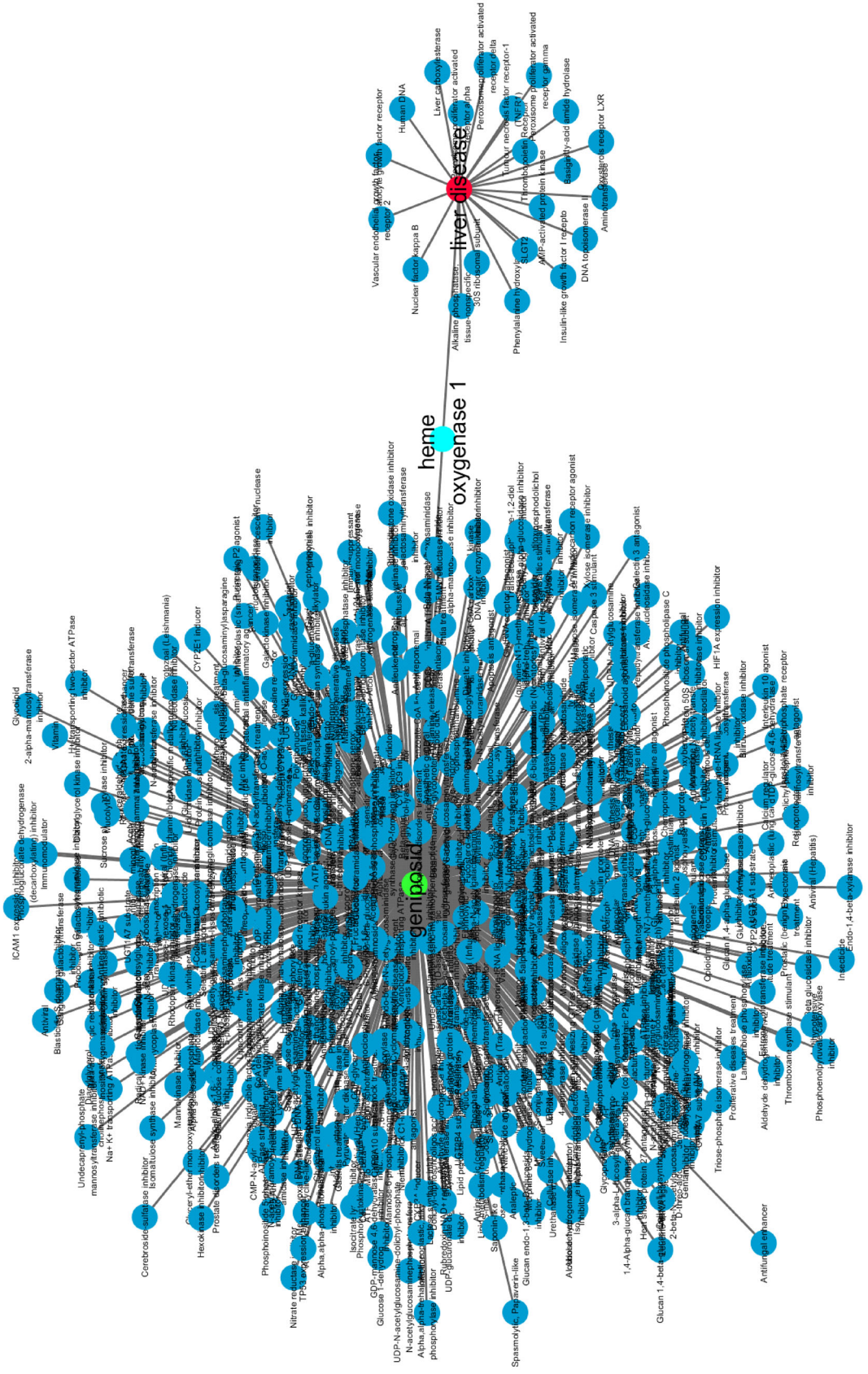

Fig. 8 Prediction network of GEN for potential protein targets. The green circles represent the compounds, red circles delineate the disease, and blue circles are the protein targets.

application of GEN to a considerable extent by using Rhizoma Zingiberis, ethanol, and ANIT on the basis of our understanding of TCM. The response to the treatment of GEN and the biomarkers in clinical samples were used to construct the YHS mouse model. The biochemical parameters of clinical liver function and histopathology are shown in Table S1. The concentrations of ALT, AST, T-Bil, and TBA were significantly elevated in YHS mice, and ALP, D-Bil, MDA, γ-GT, GSH-Px, and T-SOD exhibited a prominent trend, suggesting that the YHS mouse liver was already damaged. T-Bil and D-Bil are specifically diagnostic indicators of jaundice. All of the biological parameters were significantly regulated to their normal levels after treatment with GEN.

Then, a comprehensive and non-invasive metabolomics study combined with pattern recognition technology was used to explore the pathogenesis of YHS and the protective effect of GEN. PCA was conducted, and a complete separation of control and YHS groups was presented. OPLS-DA analysis was conducted, and a total of 33 different metabolites, which were related to pentose and glucuronate interconversions and primary bile acid biosynthesis, were recognized as major contributors to grouping. Interestingly, most of these biomarkers recovered to normal levels with GEN treatment, thereby proving the effectiveness of GEN and the specialization of the biomarkers.

Tyramine glucuronide is the terminal metabolite of tyramine and is derived from a wealth of sources, including protein degradation or intestinal bacterial action. Its redundant accumulation is excreted via the glucuronidation pathway by UDP-glucuronosyltransferase 1A1, which is also the key enzyme dominating hepatic detoxification [25]. The liver has an irreplaceable function for metabolism and detoxification. Bilirubin shows a certain degree of neurotoxicity due to the terminal metabolite of erythrocyte. The turbulence of bilirubin metabolism increases serum bilirubin concentration, and the clinical manifestation of jaundice finally forms. Modern pharmacological studies demonstrate that the exclusion of bilirubin is solely controlled by UDP-glucuronosyltransferase 1A1 through glucuronidation. The levels of tyramine glucuronide in YHS mice increased markedly compared with that of the control group. This phenomenon may contribute to the decrease of UDP-glucuronosyltransferase 1A1 activities, which influence the metabolism and transport of UDP-glucuronosyltransferase 1A1 activities. Finally, YHS and liver disorders are induced. In recent years, a series of reports on GEN or its active constituents indicates its activation function with UDP-glucuronosyltransferase [26 – 29]. In our research, the increasing level of tyramine glucuronide in GEN group suggested a better knowledge of the preventive effects of YHS via regulating the disturbed metabolism pathways.

Glutathione metabolism is the fundamental reaction in every cell of an organism. It involves many key metabolites and enzymes, such as glutathione disulfide, glutathione, glucose-6-phosphate dehydrogenase, and glutathione peroxidase. Liver disorder and damage occur when the normal operation of the above compounds is disrupted. The present study found that the intensities of spermidine and L-cysteine were significantly reduced in the YHS group. The correlation between the reactions proved that GSH and GSH-Px concentrations are lower than normal levels. The latter has a broad spectrum of applications as a hepatoprotective, anti-inflammatory, antioxygenation, and detoxication agent and is also used as a common biochemical indicator of liver function. Glucose-6-phosphate dehydrogenase is also a vital enzyme in GSH transformation, and its deficiency is directly related to neonatal jaundice [30]. Combining clinical chemistry with metabolomic data, we found that spermidine and L-cysteine content decreased in YHS mice, thereby verifying the potential pathogenesis of YHS.

Taurine is a key component of bile acid [31] and plays an important role in liver metabolism. In addition, there are several putative medicinal effects used for the therapeutic category of liver disease agents. Fig. 7 shows that the content of taurine is significantly lower than in the control group. Thus, the activity of glutamate decarboxylase had been inhibited. A disorder in this reaction may cause cerebral palsy [32,33] whose clinical manifestation is hyperbilirubinemia (also known as jaundice) [34].

As one of the definite active ingredients of Yinchenhao Decoction, GEN has a good effect on lung injury and jaundice and plays a protective role through the synergistic mechanism of multiple pathways. The metabolic pathways involved in GEN in the course of treatment are shown in Fig. 7, and a connection exists between them. For example, L-cysteine is the link between glutathione metabolism and the taurine metabolism pathway and is also consistent with the overall view of symptoms. In Fig.7, the different metabolic pathways are linked; thus, GEN may play a role in the treatment of YHS by regulating the above targets or pathways.

Metabolomics and network pharmacology are high-throughput and large-data technologies that have emerged in recent years. With the vigorous development of analytical technology and electronic information industry, promoting these two technologies is possible [35–43]. Importantly, TCM has considerable consistency with its holistic view and the mode of network interaction and synergy [44–46]. Therefore, it has been widely used to understand the mechanism of action of complex diseases and drugs with complex components [47–49]. Network pharmacology predicts protein structures that may possibly dock with drugs from the perspective of drug-specific spatial structure and chemical bond stability [50–52]. Through the identifies of metabolites, characterizes the changes of metabolites' content and species in objective

signal intensity and category form, and traces the upstream metabolic mechanism through the acknowledged database network. The combination of the two may result in the rapid focusing and targeting of the pathogenesis and drug targets of diseases, especially in the cognitive stage of treating complex diseases by using TCM. In the present study, metabolomics and network pharmacology techniques were used to study the mechanism of GEN as intervention for YHS and provide experimental and theoretical basis for the pathogenesis of YHS and the development of effective monomer drugs.

Conclusions

We established a YHS mouse model on the basis of theoretical direction from TCM combined with a previous investigation and response to GEN. With the successful verification of clinical chemistry and histopathology analyses, a high-throughput, comprehensive, urinary metabolomics platform was established for investigating the metabolic typing of YHS, and 33 differential metabolites were detected and confirmed. After the global pathway analysis of the perturbed metabolism, we constructed a map of the metabolic network of YHS, which was directly involved in pentose and glucuronate interconversions and primary bile acid biosynthesis. The key enzymes and metabolites were significantly associated with YHS to improve our understanding of the precise pathogenesis of YHS. Thus, urine metabolomics is suitable for evaluating the disease/syndrome and provides a promising strategy for the use of TCM in treating YHS.

Acknowledgements

This work was supported by grants from the National Key Research and Development Program of China (No. 2018YFC1706103), Key Program of National Natural Science Foundation of China (Nos. 81830110, 8181101160, 81430093, 81673586, 81703685, 81302905, 81503386, and 81373930), National Key Subject of Drug Innovation (Nos. 2015ZX09101043-005 and 2015ZX-09101043-011), TCM State Administration Subject of Public Welfare (No. 2015468004), Major Projects of Application Technology Research and Development Plan in Heilongjiang Province (No. GX16C003), Young Talent Lift Engineering Project of China Association of Traditional Chinese Medicine (No. QNRC2-B06), Natural Science Foundation of Heilongjiang Province (Nos. YQ2019H030 and H2016056), and Foundation of Heilongjiang University of Chinese Medicine (Nos. 2018jc01, 2018bs02, and 201809).

Compliance with ethics guidelines

Heng Fang, Aihua Zhang, Xiaohang Zhou, Jingbo Yu, Qi Song, and Xijun Wang declare that they have no potential financial conflict of interests related to this manuscript. The research program was approved by the Ethics Committee of Heilongjiang University of Chinese Medicine, and the study was conducted in accordance with the *Declaration of Helsinki*.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-019-0709-5 and is accessible for authorized users.

References

1. Zhang A, Liu Q, Zhao H, Zhou X, Sun H, Nan Y, Zou S, Ma CW, Wang X. Phenotypic characterization of nanshi oral liquid alters metabolic signatures during disease prevention. Sci Rep 2016; 6(1): 19333
2. Zhang A, Yan G, Sun H, Cheng W, Meng X, Liu L, Xie N, Wang X. Deciphering the biological effects of acupuncture treatment modulating multiple metabolism pathways. Sci Rep 2016; 6(1): 19942
3. Wang X, Zhang A, Sun H, Han Y, Yan G. Discovery and development of innovative drug from traditional medicine by integrated chinmedomics strategies in the post-genomic era. Trends Analyt Chem 2016; 76: 86–94
4. Zhang A, Sun H, Wang X. Mass spectrometry-driven drug discovery for development of herbal medicine. Mass Spectrom Rev 2018; 37(3): 307–320
5. Zhang AH, Ma ZM, Sun H, Zhang Y, Liu JH, Wu FF, Wang XJ. High-throughput metabolomics evaluate the efficacy of total lignans from acanthophanax senticosus stem against ovariectomized osteoporosis rat. Front Pharmacol 2019; 10: 553
6. Zhang A, Sun H, Yan G, Wang P, Wang X. Mass spectrometry-based metabolomics: applications to biomarker and metabolic pathway research. Biomed Chromatogr 2016; 30(1): 7–12
7. Zhang A, Wang H, Sun H, Zhang Y, An N, Yan G, Meng X, Wang X. Metabolomics strategy reveals therapeutical assessment of limonin on nonbacterial prostatitis. Food Funct 2015; 6(11): 3540–3549
8. Fang H, Zhang A, Yu J, Wang L, Liu C, Zhou X, Sun H, Song Q, Wang X. Insight into the metabolic mechanism of scoparone on biomarkers for inhibiting Yanghuang syndrome. Sci Rep 2016; 6(1): 37519
9. Sun H, Wang M, Zhang A, Ni B, Dong H, Wang X. UPLC-Q-TOF-HDMS analysis of constituents in the root of two kinds of Aconitum using a metabolomics approach. Phytochem Anal 2013; 24(3): 263–276
10. Wu YB, Zeng CC, Peng QH, Deng X, Nie G. Study on differences of colorimetric between Yin Zheng and Yang Zheng jaundice in patients with hepatogenic jaundice. Chin J Integr Tradit West Med Liver Dis (Zhong Xi Yi Jie He Gan Bing Za Zhi) 2013; 23(3):139–141 (in Chinese)
11. Kim HJ, Lee SK, Ryu CH, Park DH, Lee SS, Seo DW, Kim MH. The clinical usefulness of simultaneous placement of double endoscopic nasobiliary biliary drainage. Clin Endosc 2015; 48(6): 542–548
12. Okamura S, Mikami K, Murata K, Nushijima Y, Okada K, Yanagisawa T, Fukuchi N, Ebisui C, Yokouchi H, Kinuta M.

Long-term multidisciplinary therapy for multiple liver metastases from colorectal cancer with biliary drainage for occlusive jaundice—a case report. Gan To Kagaku Ryoho 2015; 42(12): 1740–1742 (in Japanese)
13. Lammert C, Bjornsson E, Niklasson A, Chalasani N. Oral medications with significant hepatic metabolism at higher risk for hepatic adverse events. Hepatology 2010; 51(2): 615–620
14. Mendizabal M, Silva MO. Liver transplantation in acute liver failure: a challenging scenario. World J Gastroenterol 2016; 22(4): 1523–1531
15. Muttaqillah N A S, Abdul Wahab A, Ding CH, Mohammad M, Biswas S, Rahman MM. Presence of anti-mitochondrial antibodies and elevated serum immunoglobulin G levels: is this primary biliary cirrhosis-autoimmune hepatitis overlap syndrome? EXCLI J 2015; 14: 175–178
16. Yeluru A, Cuthbert JA, Casey L, Mitchell MC. Alcoholic hepatitis: risk factors, pathogenesis, and approach to treatment. Alcohol Clin Exp Res 2016; 40(2): 246–255
17. Issa D, Eghtesad B, Zein NN, Yerian L, Cruise M, Alkhouri N, Adams R, Hanouneh IA. Sofosbuvir and simeprevir for the treatment of recurrent hepatitis C with fibrosing cholestatic hepatitis after liver transplantation. Int J Organ Transplant Med 2016; 7(1): 38–45
18. Tang X, Yang C, Gu Y, Song C, Zhang X, Wang Y, Zhang J, Hew CL, Li S, Xia N, Sivaraman J. Structural basis for the neutralization and genotype specificity of hepatitis E virus. Proc Natl Acad Sci USA 2011; 108(25): 10266–10271
19. Friedman LS, Gee MS, Misdraji J. Case records of the Massachusetts General Hospital. Case 39-2010. A 19-year-old woman with nausea, jaundice, and pruritus. N Engl J Med 2010; 363(26): 2548–2557
20. Zhu J, Li XW, Li L, Zhong GY, Xiong HZ, Liu CH, Luo GM. Therapeutic effects of Zhizi Baipi Decoction and its separated prescriptions on relieving jaundice of jaundice rat with Yang-jaundice syndrome. Tradit Chin Drug Res Clin Pharmacol (Zhong Yao Xin Yao Yu Lin Chuang Yao Li) 2015; 26(1): 25–30 (in Chinese)
21. Mu CS. Factors of Yin and Yang transformation of neonates with breast milk jaundice. J Liaoning Univ Tradit Chin Med (Liao Ning Zhong Yi Yao Da Xue Xue Bao) 2013; 15(11): 200–202 (in Chinese)
22. Suthar PP, Bumiya RG, Patel K, Patel AB. Incidental diagnosis of liver tuberculosis in a patient with jaundice. BMJ Case Rep 2015; 2015: pii: bcr2014206866
23. Wang X, Zhang A, Han Y, Wang P, Sun H, Song G, Dong T, Yuan Y, Yuan X, Zhang M, Xie N, Zhang H, Dong H, Dong W. Urine metabolomics analysis for biomarker discovery and detection of jaundice syndrome in patients with liver disease. Mol Cell Proteomics 2012; 11(8): 370–380
24. Liu XY, Zhang AH, Fang H, Li MX, Song Q, Su J, Wang XJ. Serum metabolomics strategy for understanding the therapeutic effects of Yin-Chen-Hao-Tang against Yang Huang syndrome. RSC Adv 2018; 8(14): 7403–7413
25. Fujiwara R, Maruo Y, Chen S, Tukey RH. Role of extrahepatic UDP-glucuronosyltransferase 1A1: advances in understanding breast milk-induced neonatal hyperbilirubinemia. Toxicol Appl Pharmacol 2015; 289(1): 124–132
26. Yin J, Wennberg RP, Miller M. Induction of hepatic bilirubin and drug metabolizing enzymes by individual herbs present in the traditional Chinese medicine, yin zhi huang. Dev Pharmacol Ther 1993; 20(3-4): 186–194
27. Huang W, Zhang J, Moore DD. A traditional herbal medicine enhances bilirubin clearance by activating the nuclear receptor CAR. J Clin Invest 2004; 113(1): 137–143
28. Wang H, Yan G, Zhang A, Li Y, Wang Y, Sun H, Wu X, Wang X. Rapid discovery and global characterization of chemical constituents and rats metabolites of Phellodendri amurensis cortex by ultra-performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry coupled with pattern recognition approach. Analyst (Lond) 2013; 138(11): 3303–3312
29. Yang D, Yang J, Shi D, Deng R, Yan B. Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. Br J Pharmacol 2011; 164(5): 1547–1557
30. Wu S, Wu G, Wu H. Hemolytic jaundice induced by pharmacological dose ascorbic acid in glucose-6-phosphate dehydrogenase deficiency: a case report. Medicine (Baltimore) 2018; 97(51): e13588
31. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap—bile acids in metabolic control. Nat Rev Endocrinol 2014; 10(8): 488–498
32. Heidari R, Jamshidzadeh A, Keshavarz N, Azarpira N. Mitigation of methimazole-induced hepatic injury by taurine in mice. Sci Pharm 2015; 83(1): 143–158
33. Fujiya K, Ganno H, Ando M, Chong JM. Clonorchis sinensis ova in bile juice cytology from a patient with severe hyperbilirubinemia and portal vein thrombosis. Diagn Cytopathol 2016; 44(3): 223–225
34. Malla T, Singh S, Poudyal P, Sathian B, Bk G, Malla KK. A prospective study on exchange transfusion in neonatal unconjugated hyperbilirubinemia—in a tertiary care hospital, Nepal. Kathmandu Univ Med J (KUMJ) 2015; 13(50): 102–108
35. Song Q, Zhang A, Yan G, Liu L, Wang X. Technological advances in current metabolomics and its application in tradition Chinese medicine. RSC Adv 2017; 7(84): 53516–53524
36. Xie J, Zhang A, Sun H, Yan G, Wang X. Recent advances and effective strategies in the discovery and applications of natural products. RSC Adv 2018; 8(2): 812–824
37. Zhang A, Fang H, Wang Y, Yan G, Sun H, Zhou X, Wang Y, Liu L, Wang X. Discovery and verification of the potential targets from bioactive molecules by network pharmacology-based target prediction combined with high-throughput metabolomics. RSC Advances 2017; 7(81): 51069–51078
38. Qiu S, Zhang A, Zhang T, Sun H, Guan Y, Yan G, Wang X. Dissect new mechanistic insights for geniposide efficacy on the hepatoprotection using multiomics approach. Oncotarget 2017; 8(65): 108760–108770
39. Yu J, Kong L, Zhang A, Han Y, Liu Z, Sun H, Liu L, Wang X. High-throughput metabolomics for discovering potential metabolite biomarkers and metabolic mechanism from APPswe/PS1dE9 transgenic model of Alzheimer's disease. J Proteome Res 2017; 16(9): 3219–3228
40. Zhang A, Sun H, Wang X. Emerging role and recent applications of metabolomics biomarkers in obesity diseases research. RSC Adv

2017; 7(25): 14966–14973

41. Li X, Zhang A, Sun H, Liu Z, Zhang T, Qiu S, Liu L, Wang X. Metabolic characterization and pathway analysis of berberine protects against prostate cancer. Oncotarget 2017; 8(39): 65022–65041
42. Zhang A, Sun H, Yan G, Wang X. Recent developments and emerging trends of mass spectrometry for herbal ingredients analysis. Trends Analyt Chem 2017; 94: 70–76
43. Xie J, Zhang A, Wang X. Metabolomic applications in hepatocellular carcinoma: toward the exploration of therapeutics and diagnosis through small molecules. RSC Adv 2017; 7(28): 17217–17226
44. Wang X, Zhang A, Zhou X, Liu Q, Nan Y, Guan Y, Kong L, Han Y, Sun H, Yan G. An integrated chinmedomics strategy for discovery of effective constituents from traditional herbal medicine. Sci Rep 2016; 6(1): 18997
45. Dong W, Wang P, Meng X, Sun H, Zhang A, Wang W, Dong H, Wang X. Ultra-performance liquid chromatography-high-definition mass spectrometry analysis of constituents in the root of Radix Stemonae and those absorbed in blood after oral administration of the extract of the crude drug. Phytochem Anal 2012; 23(6): 657–667
46. Wang X, Wang Q, Zhang A, Zhang F, Zhang H, Sun H, Cao H, Zhang H. Metabolomics study of intervention effects of Wen-Xin-Formula using ultra high-performance liquid chromatography/mass spectrometry coupled with pattern recognition approach. J Pharm Biomed Anal 2013; 74: 22–30
47. Sun H, Wu F, Zhang A, Wei W, Han Y, Wang X. Profiling and identification of the absorbed constituents and metabolites of schisandra lignans by ultra-performance liquid chromatography coupled to mass spectrometry. Biomed Chromatogr 2013; 27(11): 1511–1519
48. Zhang A, Sun H, Qiu S, Wang X. Advancing drug discovery and development from active constituents of yinchenhao tang, a famous traditional Chinese medicine formula. Evid Based Complement Alternat Med 2013; 2013: 257909
49. Sun H, Zhang A, Yan G, Han Y, Sun W, Ye Y, Wang X. Proteomics study on the hepatoprotective effects of traditional Chinese medicine formulae Yin-Chen-Hao-Tang by a combination of two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. J Pharm Biomed Anal 2013; 75: 173–179
50. Sun H, Zhang HL, Zhang AH, Zhou XH, Wang XQ, Han Y, Wang XJ. Network pharmacology combined with functional metabolomics discover bile acid metabolism as a promising target for mirabilite against colorectal cancer. RSC Adv 2018; 8(53): 30061–30070
51. Sun H, Wang H, Zhang A, Yan G, Zhang Y, An N, Wang X. Berberine ameliorates nonbacterial prostatitis via multi-target metabolic network regulation. OMICS 2015; 19(3): 186–195
52. Du H, Zhao X, Zhang A. Identifying potential therapeutic targets of a natural product Jujuboside B for insomnia through network pharmacology. Plant Sci Today 2014; 1(2): 69–79