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Retrospective Study

Baseline metabolites could predict the responders with HBV-related liver fibrosis for entecavir or combined with FuzhengHuayu tablet

Metabolites predict HBV-related liver fibrosis responders

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Abstract

BACKGROUND

After receiving entecavir or combined with FuzhengHuayu tablet (FZHY) treatment, some sufferers with HBV-related liver fibrosis could achieve a histological improvement while the others may fail to improve even worsen. Serum metabolomics at baseline in these patients who were effective in treatment remain unclear.

AIM

We are about to explore baseline serum metabolites characteristics in responders.

METHODS

A total of 132 patients with HBV-related liver fibrosis and 18 volunteers as healthy controls were recruited. First, all subjects were divided into training set and validation set. Second, the included patients were subdivided into entecavir responders (E-R), entecavir no-responders (E-N), FZHY+entecavir responders (F-R), and FZHY+entecavir no-responders (F-N) following the pathological histological changes after 48 wk' treatments. Then, Serum samples of all subjects before treatment were tested by

HPLC-MS. Data processing was conducted using multivariate PCA and OPLS-DA. Diagnostic tests of selected differential metabolites were used for Boruta analyses and logistic regression.

RESULTS

As for the intersection about differential metabolic pathways between the groups E-R *vs* E-N and F-R *vs* F-N, results showed that 4 pathways including Linoleic acid metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism were screened out. As for the differential metabolites, these 7 intersected metabolites including Hydroxypropionic acid, Tyrosine, Citric acid, TCDCA, Benzoic acid, 2-Furoic acid, and Propionic acid were selected.

CONCLUSION

Our findings showed that 4 metabolic pathways and 7 differential metabolites have potential usefulness in clinical prediction of the response of entecavir or combined with FZHY on HBV fibrotic liver.

Key Words: Serum metabolomics; Differential metabolites; Therapeutic responders; entecavir; FuzhengHuayu tablet; HBV-related liver fibrosis

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Core Tip: This study will use HPLC-MS and multivariate statistical modelings to predict serum metabolites of the treatment (entecavir or entecavir+FZHY) that effectively reversed HBV-related liver fibrosis. It is of great theoretical and practical

significance to prevent the transformation of liver fibrosis to cirrhosis or even hepatocellular carcinoma and reduce the social burden.

INTRODUCTION

Fiver fibrosis, with the characteristics of progressive and reversible accumulation of fibrillar extracellular matrix (ECM) components in the liver, is harmful to the physiological architecture of liver and leads to almost half of all-cause mortality for the majority of liver diseases around the world [1-2]. Hepatitis B virus (HBV) is a common cause of many acute and chronic liver diseases and its infection is the main cause of liver fibrosis [3]. According to the findings of epidemiological study [4], over 240 million patients suffered from HBV infection. As a progressive disease, if chronic hepatitis B (CHB) was not controlled in time, it would progress to fibrosis or cirrhosis or even hepatocellular carcinoma (HCC) [5]. Therefore, antiviral agents should be taken into consideration in treating HBV and entecavir is a representative one.

In recent years, the study of liver fibrosis has always been a hot topic in medical research [6]. As a reversible lesion, liver fibrosis is the intermediate link from chronic liver diseases further development to cirrhosis [7]. Currently, there is still a lack of effective treatment for cirrhosis. Therefore, anti-liver fibrosis is an important treatment strategy. FuzhengHuayu tablet (FZHY) is a new anti-fibrosis traditional Chinese medicine (TCM) drug, which is widely used in the clinical treatment of liver fibrosis and cirrhosis [8]. Moreover, our previous multi-center clinical study also confirmed that entecavir+FZHY treatment could significantly improve the histological reversal rate of CHB fibrosis [9], but about one-third of patients still lacked a significant histological response [10]. Therefore, clarifying the biological characteristics of these sufferers responding to the entecavir or entecavir+FZHY will help improve the curative effect of precision therapy.

So far, there is no single biomarker or scoring system with the ideal sensitivity and specificity to detect and identify liver fibrosis [11]. Although liver biopsy is still the gold standard for staging diagnosis of liver fibrosis, it has some limitations such as

invasiveness, sample error, and a risk of complications [12]. And this method is not convenient to dynamically observe the pathological evolution and therapeutic effects of liver fibrosis. Naturally, the non-invasive diagnosis methods of liver fibrosis including transient elastography (Fibroscan), elastography and diffusion-weighted magnetic resonance imaging (MRI) have made rapid progress and been widely used in clinical practice. However, these techniques are often interfered with by factors such as a patient's body mass index (BMI), liver inflammation or hepatocyte degeneration [13].

Metabolomics, a promising discipline following genomics, transcriptomics and proteomics, is a new kind of systematic study on the changes of small molecule metabolites produced by body metabolism [14]. Meanwhile, it is named as the "terminal" of the genome and proteome for the systematic analysis of various metabolites and their metabolic pathways in a population, high throughput and model. Moreover, metabolomics can reveal the downstream products of gene and protein expression in an organism, reflecting all physiological activities of the body. Because it is closer to disease phenotype, metabolomics is more suitable for disease typing and biomarker discovery. In this study, we are about to use the high performance liquid chromatography-mass spectrometry (HPLC-MS) and multivariate statistical modelings to predict serum metabolites of the treatment (entecavir or entecavir+FZHY) that effectively reversed HBV-related liver fibrosis. It is of great theoretical and practical significance to prevent the transformation of liver fibrosis to cirrhosis or even HCC and reduce the social burden.

MATERIALS AND METHODS

Patient selection

This is a cross-sectional study with multi-center randomized controlled clinical trials. A total of 132 patients with HBV-related liver fibrosis and 18 volunteers as healthy controls were recruited from September 9, 2014 to October 25, 2018. There were two sets including training set and validation set in this study. All participants were from 20 hospitals around the China and they all volunteered to sign informed consent. And this

research protocol was Ethics Committee of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Ethical approval number: 2014-331-27-01). Diagnostic criteria for HBV-related liver fibrosis was based on the guidelines for the prevention and treatment of chronic hepatitis B (2019) [15]. In this study, the primary focus of research was on the progression of liver fibrosis. Liver histopathology was employed as the indicator for therapeutic assessment, with fibrosis severity evaluated according to the Ishak scoring system. Meanwhile, the primary outcome was the proportion of patients with a 1-point improvement of liver fibrosis stage using the Ishak score from baseline to week 48. Liver biopsies were performed before and 48 wk after initiation of combination TCM and graded independently by 3 pathologists. A decrease in Ishak score of 1 or greater, was considered fibrosis regression [16]. The final fibrotic scores were obtained by consensus from 2 or more pathologists. If there was disagreement, liver biopsy samples were evaluated and decided by central pathologist. However, a detailed distinction of inflammation levels was not conducted. On the noninvasive diagnosis and staging of liver fibrosis, APRI and FIB-4 were predominantly used as adjunct diagnostic tools for assessing liver fibrosis severity.. Accordingly, the two treatment groups were ultimately subdivided into four subgroups including entecavir responders (E-R), entecavir no-responders (E-N), FZHY+entecavir responders (F-R), and FZHY+entecavir no-responders (F-N). Meanwhile, in this study, inclusion criteria included both age at least 18 years and meeting the above diagnostic criteria. However, exclusion criteria were as follows: liver fibrosis without HBV infection; Cardia-cerebrovascular or infectious diseases or other digestive system diseases; Pregnant women or lactating women; Patients with poor compliance.

Sample collection

All subjects were asked to have normal regular diets and schedules on the day before blood collection, and venous blood was collected on an empty stomach the next morning. 500μL serum was centrifuged at 4℃at 4000r/min and stored in a -80℃for later use.

Sample processing

The cryopreserved serum was thawed on ice-bath in case of degradation. 25μL of serum was added to a 96-well plate for the transferring to the Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, California, USA). 120μL of methanol was automatically added to each serum and vortexed for 5 minutes. The plate was centrifuged at 4000g for half an hour and it was returned back to the workstation. 30μL of supernatant fluid was transferred to a clean 96-well plate, where each well was filled with 20μL of freshly prepared derivative reagents. Then the plate was sealed for derivatization at 30°Cfor an hour and the sample was diluted by 330μL of ice-cold 50% methanol solution. Next, the plate was left at -20°Cfor 20 minutes and centrifuged at 4°Cfor half an hour. Finally, 135μL of supernatant fluid was taken to a new 96-well plate, which was sealed for LC-MS analysis.

Quality control analysis

All samples were mixed into one quality control sample for quality control. The quality control samples were analyzed 6 times and randomly respectively tested 2 times before, during and after analysis. The total ion flow chromatograms of the quality control samples were overlapped and the total principal component analysis (PCA) was performed. It would show good repeatability if the results of the quality control samples were close to each other.

Materials and reagents

Formic acid (Optima Grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (Optima LC-MS) and acetonitrile (Optima LC-MS) were purchased from Thermo-Fisher Scientific (FairLawn, NJ, USA). The experimental water was distilled water.

Instrument analysis platform

We used a ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) in order to quantitate all targeted metabolites in this study. A briefly description of the optimized instrument settings can be shown in Supplementary

material Table S1. Meanwhile, the instrument performance optimization and routine maintenance were conducted every week.

LC-MS analysis

Extraction of ion flow chromatograms based on HPLC-MS. (i) Chromatographic elution gradient: the initial gradients were 5% solution B (acetonitrile + 0.1% formic acid) and 95% solution A (distilled water + 0.1% formic acid), whose elution time lasted 2-10 minutes. Meanwhile, solution B increased linearly to 95% for 5 minutes and then dropped back to 5%. The injection volume was 4 ul and the automatic sampler temperature was 4°C (ii) Mass spectrometry scanning mode: positive and negative ions were used for detection by mass spectrometry. The ion scanning time was 0.03 s, the time interval was 0.02s, and the data collection range was 50-100m/z.

Screening and identification of potential metabolites

The data of group A and group B were analyzed by total PCA, then partial least squares discriminant analysis (PLS-DA) was used, and finally the supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was used for modeling analysis. Variable importance in the projection (VIP) values (threshold > 1) based on the OPLS-DA model, combined with p-values ($P < 0.05$) of T test, were used to find metabolites which were differentially expressed. Potential metabolites were identified by searching online database (http://metlin.scripps.edu/) to compare the mass charge ratio or molecular mass of mass spectrometry.

Potential metabolite enrichment analysis and metabolic pathway analysis

Metabo-Analyst online analysis software (https://www.metaboanalyst.ca) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (https://www.kegg.jp/) were used for metabolic pathway analysis and enrichment analysis of the identified potential metabolites so as to determine the metabolic pathways involved in the potential metabolites, and to evaluate the diagnostic performance of the potential metabolites enriched in pathways.

Diagnostic tests

In order to validate the applicability and stability of the selected differential metabolites, Random Forest (RF), Support Vector Machine (SVM) and Boruta analyses were conducted for each selected metabolite in sequence. Boruta analysis, the maximum number of runs with 1000, was an RF-based feature selection method that it selects key features with more significant distinguishing ability than random lag features. When provisional features were included, a secondary selection was made to determine whether certain metabolites with large fluctuations should be included in the selected features.

These differential metabolites used for subsequent model construction were modeled and predicted using logistic regression. After modeling, sensitivity and specificity values were calculated to evaluate the model effects through drawing the receiver operating characteristic curve (ROC). Meanwhile, the closer the area under the curve (AUC) value is to 1, the better the sensitivity, specificity and diagnostic abilities. The conventional AUC of metabolites with the value ≥ 0.75 indicated relatively good sensitivity and specificity.

Statistical analysis

Statistical analysis software packages in R studio (http://cran.r-project.org/) were performed for the statistical algorithms. All the included data were calculate with mean±standard deviation (SD) or median-interquartile range (M, IQR). The Mann-Whitney U test or T test was used for the statistical differences in pairwise comparison. Multivariate statistical modelings including PCA, PLS-DA, and OPLS-DA were used for the multi-class classification and identification of differently altered metabolites. Among these modelings, each spatial dot in the K-dimensional space represented an individual sample with the samples color-coded based on grouping information. R^2X and R^2Y respectively represented the fraction of the variance of X matrix and Y matrix, while Q^2Y represented the predictive accuracy of the model. Cumulative values of R^2X and R^2Y approaching 1.0, along with Q^2Y greater than 0.2 (permutation test), indicated a model with a satisfactory predictive ability. Those variables with VIP greater than 1.0 are considered significantly different between classes. If

multidimensional statistics cannot establish a robust discriminant model (such as uneven distribution of inter-group sample categories or large intra-group deviation), differential metabolites between the two groups would be acquired with the aid of univariate analysis.

RESULTS

Baseline clinical characteristics of participants

In the training set, there were 23 sufferers in each subgroup and 13 normal volunteers as control. In the validation set, there were 10 patients in each subgroup and 5 volunteers as control. Details of the baseline clinical characteristics of the two datasets can be found in **Table 1**. Specifically, there were no significant differences in the gender, age, BMI, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin (TBIL), creatinine (Cr), prothrombin time (PT), platelet count (PLT), alpha fetoprotein (AFP), fibrosis index based on the 4 factor (FIB-4), aspartate aminotransferase-to-platelet ratio index (APRI), Ishak score in the training set ($P > 0.05$). However, in the validation set, the serum ALB and TBIL levels significantly differed between the F-R and F-N patients ($P < 0.05$), but the other indexes were not statistically significant ($P > 0.05$).

The pathological histological results of the liver biopsy

The obtained tissues *via* liver biopsy were fixed in 10% formalin and embedded in paraffin. Sections of each liver tissue were cut and stained using hematoxylin-eosin (HE) staining for histopathological analysis. Based on the HE staining results and Ishak score, staging of liver fibrosis was determined as F1 to F6 [17]. Briefly, F1: some portal areas have fibrosis but no fibrous septum; F2: many portal areas have fibrosis along with one fibrous septum; F3: many portal areas have fibrosis along with two or three fibrous septa; F4: portal areas have obvious portal-junction bridge fibrosis along with more than four fibrous septa; F5: portal areas have obvious portal-junction bridge fibrosis or portal-central bridge fibrosis along with one to three pseudolobuli; F6: more

than three pseudolobuli. Details of relevant figures can be found in Supplementary material Figure S1.

Overall metabolomics analysis of serum samples

Representative NMR spectra with targeted metabolites are exhibited in Supplementary material Figure S2. The serum spectra included high-intensity signals from Maleic acid, Glycine (G1 VS G2), Dihomo-gamma-linolenic acid, Arachidonic acid, Hydroxypropionic acid, (G3 VS G4), 2-Furoic acid, 2-Phenylpropionate, Arachidonic acid, Benzoic acid, Butyric acid, Aconitic acid, Citric acid, Dimethylglycine, GCDCA, Homovanillic acid, Hydrocinnamic acid, Hydroxyphenyllactic acid, Isocitric acid, Tyrosine, Phenyllactic acid, Propionic acid, TCDCA, TCA (G9 VS G1-G4). Because all patients were suffered from HBV-related liver fibrosis in this study, statistical assessment by PCA indicated not clear separation in each group (E-R *vs* E-N; F-R *vs* F-N; patients vs volunteers) (Supplementary material Figure S3). Besides, in order to exclude the possible confounding factors irrelevant to the group differences and to assess the statistical meaning of those signals, OPLS-DA was conducted and the result showed that the discrimination model could differentiate the two groups despite within a small overlap in one orthogonal component (**Figure 1A-1C**). Moreover, as shown in **Figure 1D-1F**, the models with $R^2(Y)$ of 0.512 (E-R *vs* E-N), 0.572 (F-R *vs* F-N), 0.401 (patients vs volunteers) suggested relatively good predictability and no potential over-fit. However, the models with $Q^2(Y)$ of -0.612 (E-R *vs* E-N), 0.0819 (F-R *vs* F-N), and 0.208 (patients vs volunteers) indicated the potential risk of over-fit.

Serum metabolites relevant to responders and HBV-related liver fibrosis

Due to the possibility of potential risk of the over-fit in these models, differential metabolites between the two groups were acquired with the aid of univariate analysis instead of analysis together with the VIP values from the above OPLS-DA model. Furthermore, in order to explore the applicability and stability of the distinctive models, serum samples from all the included patients and volunteers were collected and analyzed using the training set and validation set for the subsequent analyses.

In order to find out potential metabolites involving in responders and HBV-related liver fibrosis among the thousands of variables, a pairwise comparison in each group was conducted. According to the threshold value ($P < 0.05$ and $|\log2FC| \geqq 0$, FC: fold change), a total of 2 (E-R *vs* E-N), 16 (F-R *vs* F-N) and 35 (patients vs volunteers) potential metabolites in the training set (**Figure 2A-2C**) were obtained while a total of 8 (E-R *vs* E-N), 7 (F-R *vs* F-N) and 23 (patients vs volunteers) potential metabolites in the validation set (**Figure 2D-2F**) were acquired.

Selection of potential metabolites in different sets

By taking intersection and union set in terms of the aforementioned obtained unidimensional and multidimensional potential metabolites, these metabolites that may have biological significance can be selected on the basis of OPLS-DA (VIP > 1) and univariate ($P < 0.05$ and $|\log2FC| \geqq 0$) analyses. A total of 53 potential metabolites in the training set and 38 potential metabolites in the validation set were obtained. Detailed information of these selected potential metabolites were shown in **Table 2**. The distribution of data for all the metabolites in each group can be found in Supplementary material Figure S4. Furthermore, a heat map, together with Z-score, was used for analysis of these selected metabolites and the results suggested that the pairwise comparisons between the two groups could be separated no matter which data set was **(Figure 3)**.

Metabolic pathways related to the selected metabolites in different sets

Both topological centrality (impact value > 0) and enrichment significance ($-\ln(p) > 2.99$, namely $P < 0.05$) were used to evaluate the analyses of enrichment and metabolic pathways for the selected potential metabolites. As shown in **Figure 4**, there were 2 pathways (Butanoate metabolism, Nicotinate and nicotinamide metabolism) (E-R *vs* E-N), 1 pathway (Fatty acid biosynthesis) (F-R *vs* F-N), and 11 pathways (Primary bile acid biosynthesis, Nitrogen metabolism, Butanoate metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Phenylalanine metabolism, Glycine, serine and threonine metabolism, Glyoxylate and dicarboxylate metabolism, Citrate cycle (TCA cycle), Thiamine metabolism, Alanine, aspartate and glutamate metabolism)

(patients vs volunteers) in the training set (**Figure 4A-4C**); and there were 6 pathways (Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Nitrogen metabolism, Linoleic acid metabolism, Thiamine metabolism, Alanine, aspartate and glutamate metabolism) (E-R *vs* E-N), 5 pathways (Nitrogen metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism, beta-Alanine metabolism) (F-R *vs* F-N), and 6 pathways (Phenylalanine metabolism, Primary bile acid biosynthesis, Citrate cycle (TCA cycle), Tyrosine metabolism, Ubiquinone and other terpenoid-quinone biosynthesis, Nitrogen metabolism) (patients vs volunteers) in the validation set (**Figure 4D-4F**).

Selection of differential metabolites in different sets

In order to find differential metabolites from these selected potential metabolites, RF, SVM and Boruta analyses were conducted for each selected metabolite in sequence. And intersection of these potential metabolites in the three analyses can be found in Supplementary material Figure S5. Specifically, there were Maleic acid and Adipic acid (E-R *vs* E-N), Hydroxypropionic acid, 10Z-Heptadecenoic acid, and Linoleylcarnitine (F-R *vs* F-N), Tyrosine, Benzoic acid, 2-Furoic acid, Aconitic acid, and Butyrylcarnitine (patients vs volunteers) in the training set while there were Linoelaidic acid, gamma-Linolenic acid, Glycylproline, Proline, Asparagine, and Carnitine (E-R *vs* E-N), Hydroxypropionic acid, Aspartic acid, Dihomo-gamma-linolenic acid, and Tyrosine (F-R *vs* F-N), Dimethylglycine, Citric acid, GCDCA, and 2-Phenylpropionate (patients vs volunteers) in the validation set.

In the results of Boruta analysis (**Figure 5)**, the metabolites marked as confirmed are the differential metabolites obtained by the final screening for subsequent model construction. As shown in **Figure 5A-5C**, in addition to the above intersection metabolites, there were Arachidonic acid, Oleylcarnitine, and DHA (F-R *vs* F-N), Butyric acid, TCDCA, Arachidonic acid, Citric acid, and Propionic acid (patients vs volunteers) confirmed in the training set. As shown in **Figure 5D-5F**, in addition to the above intersection metabolites, there were TCDCA, Benzoic acid, Tyrosine, 2-Furoic

acid, Butyric acid, TCA, Isocitric acid, Hydrocinnamic acid, and Propionic acid (patients vs volunteers) confirmed in the validation set.

Evaluation of model effects in different sets

In the training set, there were good sensitivity and specificity with the AUC value of 0.851 (F-R *vs* F-N) and 0.985 (patients vs volunteers) except for 0.733 (E-R *vs* E-N) (**Figure 6A-6C**). In the validation set, there were good sensitivity and specificity with the AUC value of 1 (E-R *vs* E-N, patients vs volunteers) and 0.94 (F-R *vs* F-N) (**Figure 6D-6F**). On the whole, the above AUC values of the two sets indicated good diagnostic capability in this study.

DISCUSSION

As the prevalence of HBV-related liver fibrosis rises around the world, accurately targeting the effect population responding to entecavir or entecavir+FZHY are very important for precise treatment and improving clinical efficacy. Metabolomics can be used for biomarker discovery [18]. In this study, the HPLC-MS and multivariate statistical modelings were applied to predict the serum differential metabolites of the interventions that effectively reversed HBV-related liver fibrosis. Our findings showed that 7 metabolic pathways (E-R *vs* E-N) including Linoleic acid metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism, Nitrogen metabolism, Butanoate metabolism, Nicotinate and nicotinamide metabolism; 7 metabolic pathways (F-R *vs* F-N) including Linoleic acid metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism, Nitrogen metabolism, beta-Alanine metabolism, Fatty acid biosynthesis; 3 metabolic pathways (patients vs volunteers) including Nitrogen metabolism, Primary bile acid biosynthesis, Citrate cycle (TCA cycle) were selected. As for the intersection about differential metabolic pathways between the groups E-R *vs* E-N and F-R *vs* F-N, the results of this study showed that 4 pathways including Linoleic acid metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism were screened out.

As for the linoleic acid metabolism, a study suggested that dietary linoleic acid and the ratio of unsaturated to saturated fatty acids were inversely associated with significant liver fibrosis risk [19]. Another clinical study showed that specific changes in linoleic acid metabolites in individuals who were heavy drinkers could distinguish individuals with moderate alcohol-associated hepatitis from those with mild alcohol-associated liver disease, but no matter what kind of alcohol-associated liver diseases was, its common characteristics were ranging from steatosis to steatohepatitis, fibrosis, and cirrhosis [20]. As for the aminoacyl-tRNA biosynthesis, an animal experiment showed that Ganfule capsule could alleviate liver injury and liver fibrosis caused by bile duct ligation in mice, which were associated with regulating and controlling these metabolic pathways including glutamine metabolism; valine, leucine, and isoleucine biosynthesis; aminoacyl-tRNA biosynthesis [21]. Besides, results of a nonalcoholic fatty liver disease rat model suggested that the occurrence of metabolic disturbance was mainly associated with aminoacyl-tRNA biosynthesis, nitrogen metabolism, lipid metabolism, glyoxylate and dicarboxylate metabolism, and amino metabolism [22]. As for the alanine, aspartate and glutamate metabolism, a study aimed to investigate the role of Wnt/β-catenin signaling pathway and glutamine aminohydrolase enzyme (l-glutaminase) in the pathogenesis of liver fibrosis and the potential benefits of niclosamide in treating liver fibrosis indicated that the group of niclosamide-and-CCl_4-treated rats showed a significant decrease in total bilirubin, alanine transaminase, aspartate transaminase, β-catenin, l-hydroxyproline, l-glutaminase activity, which concluded that Niclosamide protected rats against liver fibrosis by inhibiting the Wnt/β-catenin pathway and glutaminolysis [23]. In a word, these metabolism pathways screened out in this study were closely related to the occurrence and development of liver fibrosis.

The study of baseline differential metabolites to predict the response of entecavir or entecavir+FZHY on HBV fibrotic liver has revealed important insights that could lead to better-tailored treatments for individuals. In particular, the findings suggested that the certain differential metabolites mentioned above were associated with the response

to entecavir and entecavir+FZHY in HBV fibrotic liver. Moreover, this study suggested that these baseline differential metabolites could be used in conjunction with clinical parameters to improve the accuracy of individualized treatment for patients suffering from HBV-related liver fibrosis. This was key to reducing the number of failures that can result from inappropriate treatments. In addition, the findings from this research were valuable for the development of biomarker-guided precision medicine, in which differential metabolites could be used to predict disease progression, select appropriate treatments, and monitor treatment outcomes in HBV patients. Furthermore, this study gave us a starting point for finding novel metabolites or biomarkers that were better predictors of the response to entecavir or entecavir+FZHY in HBV fibrotic liver. Finally, these findings will help to further our knowledge about the molecular mechanisms of HBV-related liver fibrosis and may present opportunities to evaluate the efficacy of individual treatments more accurately. By understanding how these differential metabolites were associated with the response to entecavir and entecavir+FZHY, physicians could optimize their treatment options for each individual patient and maximize the effects of their HBV-related liver fibrosis therapy. The results of this study may also serve as a resource for the development of future pharmacological treatments that were more effective and target different pathways to combat HBV-related liver fibrosis.

There were several limitations which need to be noticed in our study. First, all of the sample sources were from China. This geographically limited distribution could also fail to popularize the universality of the therapeutic schedule. Second, there was no single FZHY group. As all participants included in this study were CHB patients, and the occurrence of liver fibrosis in these patients was directly or indirectly caused by HBV, antiviral therapy was the fundamental treatment. Treating HBV-related liver fibrosis patients solely with FZHY would be inconsistent with clinical ethical standards; therefore, the observation of the therapeutic effect of FZHY as a standalone treatment was lacking. As for the FZHY monotherapy group, in order to further validate the identified differential metabolites and metabolic pathways, it may be considered for

future research to select other etiologies of liver fibrosis for validation, or to explore the differences between monotherapy and combination therapy in animal experiments. Third, our study only focused on patients with hepatitis B, but whether the conclusions can be generalized to the treatment of liver fibrosis caused by other causes needs to be further studied. Finally, due to the cross-sectional nature of this study, external reproducibility needs to be further predicted by prospective studies.

CONCLUSION

In summary, using metabolomis analysis, we identified 4 metabolic pathways and 7 differential metabolites from serum that accurately differentiated responders from no-responders in the treatment of HBV-related liver fibrosis. If validated in future studies, these metabolic pathways and differential metabolites will be useful in improving the curative effect of entecavir+FZHY and promoting the development of precision medicine.

ARTICLE HIGHLIGHTS

Research background

After receiving entecavir or combined with FuzhengHuayu tablet (FZHY) treatment, some sufferers with HBV-related liver fibrosis could achieve a histological improvement while the others may fail to improve even worsen. Serum metabolomics at baseline in these patients who were effective in treatment remain unclear.

Research motivation

The key significance of this cross-sectional study is to predict the serum metabolites of the treatment (entecavir or entecavir+FZHY) that effectively reversed HBV-related liver fibrosis.

Research objectives

We are about to explore serum differential metabolites and metabolic pathways at baseline in HBV-related liver fibrosis patients who are response to the treatments.

Research methods

A total of 132 patients with HBV-related liver fibrosis and 18 volunteers as healthy controls were recruited. First, all subjects were divided into training set and validation set. Second, the included patients were subdivided into entecavir responders (E-R), entecavir no-responders (E-N), FZHY+entecavir responders (F-R), and FZHY+entecavir no-responders (F-N) following the pathological histological changes after 48 wk' treatments. Then, Serum samples of all subjects before treatment were tested by HPLC-MS. Data processing was conducted using multivariate PCA and OPLS-DA. Diagnostic tests of selected differential metabolites were used for Boruta analyses and logistic regression.

Research results

As for the intersection about differential metabolic pathways between the groups E-R *vs* E-N and F-R *vs* F-N, results showed that 4 pathways including Linoleic acid metabolism, Aminoacyl-tRNA biosynthesis, Cyanoamino acid metabolism, Alanine, aspartate and glutamate metabolism were screened out. As for the differential metabolites, these 7 intersected metabolites including Hydroxypropionic acid, Tyrosine, Citric acid, TCDCA, Benzoic acid, 2-Furoic acid, and Propionic acid were selected.

Research conclusions

Our findings showed that 4 metabolic pathways and 7 differential metabolites have potential usefulness in clinical prediction of the response of entecavir or combined with FZHY on HBV fibrotic liver.

Research perspectives

It is of great theoretical and practical significance to prevent the transformation of liver fibrosis to cirrhosis or even hepatocellular carcinoma and reduce the social burden.

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