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Original Article

Mechanisms of Huangqi Decoction Granules (黄芪汤颗粒剂) on Hepatitis B Cirrhosis Patients Based on RNA-Sequencing*

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ABSTRACT **Objective:** To explore the action mechanisms of Huangqi Decoction Granules (黄芪汤颗粒剂, HQDG) on hepatitis B cirrhosis. **Methods:** A total of 85 patients with hepatitis B cirrhosis were randomly divided into HQDG group (42 cases) and control group (43 cases) by a random number table and were treated with HQDG or placebo for 48 weeks (6 g per times and orally for 3 times a day), respectively. After RNA-sequencing of serum samples extracted from the patients, the differentially expressed genes (DEGs) in HQDG and control groups before and after treatment were separately screened. The DEGs were then performed pathway enrichment analysis and protein-protein interaction (PPI) network analysis. The expression levels of key genes were detected by quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** After the investigation, 4 and 3 cases were respectively excluded from HQD and control groups because of the incomplete data. Additionally, 3 and 5 cases were lost to follow up in HQD and control groups, respectively. Finally, a total of 70 cases with good compliance were included for further DEGs analysis. A total of 1,070 DEGs (including 455 up-regulated genes and 615 down-regulated genes) in HQDG group and 227 DEGs (including 164 up-regulated genes and 63 down-regulated genes) in the control group were identified after treatment. Compared with the control group, 1,043 DEGs were specific in HQDG group. Besides, 1 up-regulated transcription factor (TF, such as GLI family zinc finger 1, GLI1) and 25 down-regulated TFs (such as drosophila mothers against decapentaplegic proteinfamily member 2, SMAD2) were identified. Pathway enrichment analysis showed that down-regulated Ras homolog gene family member A (RHOA) was enriched in pathogenic *Escherichia coli* infection. In the PPI network, up-regulated epidermal growth factor receptor (EGFR), and down-regulated cell division cycle 42 (CDC42) as well as v-akt murine thymoma viral oncogene homolog 1 (AKT1) had higher degrees. Moreover, long non-coding RNAs (lncRNA) growth arrest-specific 5 (GAS5) was involved in the lncRNA-target regulatory network. Furthermore, qRT-PCR revealed that expression levels of CDC42 and GLI1 had significant differences in HQDG group before and after treatment ($P<0.05$). **Conclusions:** CDC42 and GLI1 may be the targets of HQDG in patients with hepatitis B cirrhosis. Additionally, SMAD2, EGFR, AKT1, RHOA and GAS5 might be associated with the curative effect of HQDG on hepatitis B cirrhosis patients.

KEYWORDS hepatitis B cirrhosis, Huangqi Decoction Granules, differentially expressed gene, transcription factor, long non-coding RNAs, Chinese medicine

Hepatitis B induced by hepatitis B virus (HBV) is an infectious disease of the liver, which may eventually develop into cirrhosis, liver failure, even liver cancer.[1] The transmission ways of hepatitis B include blood transfusions, sexual contact, mother-to-fetus transmission, and re-use of contaminated syringes and needles.[2-4] Cirrhosis can develop from hepatic inflammation and chronic hepatitis B.[5] Cirrhosis patients may have serious complications, such as bleeding from dilated stomach veins or veins in the esophagus, hepatic encephalopathy, even liver cancer.[6] Cirrhosis has been reported to cause 1.2 million deaths in 2013.[7] Thus, it is of great importance to explore a highly effective therapy strategy for hepatitis B cirrhosis.

As a classical Chinese medicine recipe, Huangqi Decoction (黄芪汤, HQD) can improve the quality of

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life and liver function on patients with liver disease.[8-11] HQD is composed of *Radix et Rhizoma Glycyrrhizae* and *Radix Astragali*, mixed in a ratio of 6:1 (w/w). Previous study has reported that HQD exerted significant therapeutic effects in the treatment of liver cirrhosis induced by dimethylnitrosamine (DMN) in rats.[12] Using quantitative real-time polymerase chain reaction (qRT-PCR), Liu, et al[10] found that HQD could decrease the expression of collagen type Ⅰ alpha 1 chain (COL1A1), COL1A2, COL5A2, platelet-derived growth factor receptor alpha (PDGFRA), PDGFRB, PDGFB, PDGFD, and thrombospondin 1 (THBS1), as well as PDGF and transforming growth factor β (TGF-β) signaling pathways in the DMN-induced rat liver fibrosis. During the development of liver fibrosis to cirrhosis, HQD plays a positive protective effect through inhibiting hepatocyte apoptosis and facilitating CD68 expression.[10] HQD has a higher effect than its components in relieving DMN-induced liver fibrosis, and may function in inhibiting proliferation and activating chenodeoxycholic acid (CDCA)-induced hepatic stellate cells (HSCs).[13] Through a double-blind randomized clinical study, we found that HQD contributed to improve liver function in patients with HBV-induced cirrhosis, improve the degree of esophageal gastric varices and reduce portal vein diameter.[14] However, the action mechanisms of HQD on hepatitis B cirrhosis remain unclear. Therefore we designed this experiment to explore the action mechanisms of HQD on hepatitis B cirrhosis.

METHODS

Diagnostic, Inclusion and Exclusion Criteria

The diagnostic criteria are according to "The guideline of prevention and treatment for chronic hepatitis B (2010 version)":[15] medical history of chronic hepatitis B; auxiliary examinations including abdominal ultrasound and liver biopsy are in accordance with liver cirrhosis standards. Abdominal ultrasound criteria: the ultrasound imaging diagnosis of liver cirrhosis shows obvious uneven (serrated or wavy) surface of liver; hepatic parenchymal echogenicity is enhanced, uneven, and nodular; the inner diameters of portal vein and splenic portal vein are widened, and hepatic vein is thinned and distorted; gastroscopic examination reveals esophageal varices. Esophageal varices is graded using Palmer classification.[16]

The inclusion criteria are as follows: according with diagnostic criteria of chronic hepatitis B (CHB); age ranging from 18 to 65 year-old; patients voluntarily participated in this study and signed informed consent. The patients met the following criteria were excluded: cirrhosis patients with alanine transaminase (ALT)> 2 × upper limits of normal (ULN), or total bilirubin (TBIL) > 2 × ULN, complicated with hepatic encephalopathy, hepatorenal syndrome, or primary hepatic carcinoma; patients combined with serious heart, lung, kidney, endocrine, hematopoietic system and mental nerve diseases; the disabled in legal sense; pregnancy or lactation women, and fertile women without taking effective contraceptives; people with allergic constitution, or were allergic to the ingredients of HQD; patients who had participated in other clinical trials.

Participants

From July 2011 to December 2014, uighur inpatients or outpatients aged 18–65 years and diagnosed with decompensated hepatitis B cirrhosis and mild to moderate esophageal varices in Xinjiang Kashgar Second People's Hospital were included in this study. Totally 85 enrolled patients were divided into HQD Granules (HQDG) group (42 cases, male 28 cases, female 14 cases; mean age 42.56 ± 9.25 year-old) and control group (43 cases, male 26 cases, female 17 cases; mean age 43.58 ± 10.15 year-old) by a random number table. There was no significant difference between 2 groups in baseline information (P>0.05). This study got approval from the Ethics Review Committee of Xinjiang Kashgar Second People's Hospital (ethical approval No. 2010CB504801).

Intervention

The patients in HQDG group or control group were treated with HQDG (batch No. 0906133, *Radix Astragalus*: *Radix Glycyrrhizae* root=6:1) or placebo (batch No. 0905254), 6 g per bag (equal to 40 g rude drug), orally for 3 times a day for 48 weeks. The placebo included 10% HQDG, flavoring agent and excipient. Weight, color, smell, taste and packaging appearance in placebo group were similar to HQDG. HQDG and placebo were both prepared by Jiangyin Tianjiang Pharmaceutical Co., Ltd., China. In this study, the enrolled patients were with mild to moderate esophageal varices. There is currently no clinical intervention for such patients to block the disease progress. Intervention, surgery, or non-selective beta receptor blockers are only available in patients with severe esophageal varices at risk of bleeding.

Therefore, placebo used in this study conformed to the requirements of the ethics.

RNA Extraction and RNA-seq Library Construction

The serum samples (1 mL) were extracted from the patients before and after treatment, and each sample had 3 repeats. Using Norgen's total RNA purification kit (37500; NorgenBiotek Corporation, Thorhold, Canada), total RNA were extracted from the serum samples according to the manufacturer's instructions. Then, 1–2 μL RNA was quantified by spectrophotometer (Nanodrop, Wilmington, USA). RNA-seq library was constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (E7770; New England Biolabs, Ipswich, USA). Briefly, mRNAs were purified and fragmented. Subsequently, the fragmented RNA was reversely transcribed into cDNA, and then double-stranded cDNA library were constructed, followed by PCR amplification. Finally, the library was sequenced on Illumina Hiseq 4000 (PE150, Illumina, San Diego, USA). The sequencing data were deposited in the Sequence Read Archive (SRA) database of National Center for Biotechnology Information (NCBI) with the accession number of SRP081348.

Quality Control and Differentially Expressed Gene Screening

The fastx_toolkit (http://hannonlab.cshl.edu/fastx_toolkit/)[17] and prinseq-lite (http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi)[18] tools were used to perform quality control for the sequencing data. The bases with continuous quality lower than 10 at both ends of sequences, the reads in which less than 80% bases having quality larger than 20, and the reads shorter than 50 nt were filtered out. The filtered data were then mapped to human genome hg19 by tophat2 software.[19] Combined with the annotation information of the known coding genes and long non-coding RNAs (lncRNAs) provided by GENCODE v24 database (http://genome.imim.es/gencode/),[20] the differentially expressed gene (DEGs) in 2 groups before and after treatment were identified by Cufflinks tool (http://cole-trapnell-lab.github.io/cufflinks/).[21] The genes with *q*-value< 0.05 and |log2 fold change (FC)| > 1 were considered as DEGs.

Based on the VennDiagram package (http://cran.r-project.org/web/packages/VennDiagram/VennDiagram.pdf)[22] in R, venn diagram was drawn for DEGs. Additionally, the transcription factors (TFs) among the DEGs specific in HQDG group were identified based on the TRANSFAC database (http://transfac.gbf-braunschweig.de/TRANSFAC/index.html).[23] The genes targeted by the TFs were identified using chiPBase database(http://deepbase.sysu.edu.cn/chipbase/).[24]

Functional and Pathway Enrichment Analyses

Gene Ontology (GO, http://www.geneontology.org) database can be used to describe gene products from the following aspects: molecular function, subcellular location, and biological process.[25] The Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.ad.jp/kegg) database include functions of genes or other molecules.[26] Using Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) online tool, the DEGs were performed with GO functional and KEGG pathway enrichment analyses. *P* value < 0.05 was used as the threshold.

Protein-Protein Interaction Network Construction

Search Tool for the Retrieval of Interacting Genes (STRING, http://string-db.org/) has integrated known and predicted associations covering more than 1,100 organisms.[27] The STRING online tool was utilized to predict the interaction relationships among the proteins encoded by the DEGs specific in HQDG group, and then protein-protein interaction (PPI) network was constructed by Cytoscape software (http://www.cytoscape.org).[28] The combined score > 4 was set as the cut-off criterion.

Regulatory Network Construction

After further screening the differentially expressed lncRNAs (DE-lncRNAs) from the DEGs, the genes targeted by the DE-lncRNAs were predicted by calculating pearson correlation coefficients.[29] The false discovery rate (FDR)< 0.05 was set as the threshold. The target genes were then performed functional and pathway enrichment analyses using the cluster Profiler package (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html)[30] in R, with *q* value < 0.05 as the threshold. The lncRNA-target gene regulatory network was constructed by Cytoscape software.[28]

qRT-PCR Analysis

The primers for qRT-PCR reaction were designed and synthesized by Primer Premier 6.0 software (Premier Software Inc., Cherry Hill, NJ, USA) and Sangon Biotech Co., Ltd. (Sangon Biotech Co., Ltd., Shanghai, China), respectively (Table 1). The

expression levels of key genes in the serum of 2 groups before and after treatment were detected by SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA). The 20 μL reaction system was the mixture of 10 μL SYBR Premix Ex Taq (2×), 8 μL cDNA template (being diluted into the same concentration by ddH_2O), 1 μL forward primer (10 μmol/L), and 1 μL reverse primer (10 μmol/L). The reaction condition was as follows: 50 ℃ for 3 min, 95 ℃ for 3 min, 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. A melting curve was created to obtain the results. All experiments were repeated for 3 times, and GAPDH was used as the reference gene. Based on the $2^{-\triangle\triangle Ct}$ method,[31] the expression levels of key genes were calculated.

Table 1. The Primers Used for Quantitative Real-Time PCR Amplification

Primer	Primer sequence (5'–3')	Primer length (bp)
GLI1	F: TCATATCCTGACCCCACCCAA	21
	R: TGGCTTGACTTGCACTTGTCC	21
CDC42	F: CAAACAGAAGCCTATCACTCC	21
	R: GCAGCCAATATTGCTTCG	18
GAPDH	F: TGACAACTTTGGTATCGTGGAAGG	24
	R: AGGCAGGGATGATGTTCTGGAGAG	24

Notes: F: forward; R: reverse

Statistical Analysis

All data were shown as mean ± standard error of mean ($\bar{x} \pm$SEM). The *t*-test in Graphpad prism (Graphpad Software, San Diego, CA, USA) was used to analyze the data. $P<0.05$ was taken as the threshold for significant difference.

RESULTS

DEGs Analysis

After the investigation, 4 cases from HQD group and 3 cases from control group were respectively excluded because of the incomplete data. Additionally, 3 and 5 cases were lost to follow up in HQD and control groups, respectively. Finally, a total of 70 cases with good compliance were included for further DEGs analysis.

In HQD group, a total of 1,070 DEGs (including 455 up-regulated genes and 615 down-regulated genes) were identified after treatment. Meanwhile, there were 227 DEGs (including 164 up-regulated genes and 63 down-regulated genes) in control group. Venn diagram showed that HQDG group had 1,043 specific DEGs (including 441 up-regulated genes and 602 down-regulated genes) compared with the control group (Figure 1). According to transcription factor database (TRANSFAC), 1 up-regulated TF (GLI family zinc finger 1, GLI1) and 25 down-regulated TFs (such as SMAD family member 2, SMAD2; Spi-1 proto-oncogene, SPI1; and TATA-box binding protein associated factor 1, TAF1) were identified from the 1,043 specific DEGs. Especially, SPI1 targeted 164 specific DEGs (including 29 up-regulated genes and 135 down-regulated genes) and TAF1 targeted 696 specific DEGs (including 463 up-regulated genes and 233 down-regulated genes) in HQDG group.

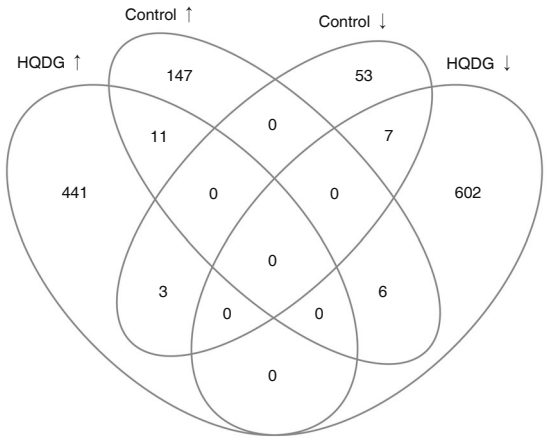

Figure 1. Result of Venn Diagram Analysis
Note: ↑: up-regulated genes; ↓: down-regulated genes

Functional and Pathway Enrichment Analyses

The top 10 GO functions and KEGG pathways enriched by the DEGs in HQDG group after treatment are listed in Appendix 1. For the up-regulated genes, the enriched terms included flagellum (GO), cilium (GO), sensory perception of chemical stimulus (GO) and nitrogen metabolism (KEGG pathway). The down-regulated genes were enriched in translational elongation, structural constituent of ribosome (GO), translation (GO) and pathogenic *Escherichia coli* infection (KEGG pathway; involving Ras homolog family member A, RHOA).

In addition, the top 10 GO functions enriched by the DEGs in control group after treatment are listed in Appendix 2. For the up-regulated genes, the enriched GO terms included protein transport, establishment of protein localization and cofactor binding. For the down-regulated genes, the enriched GO terms included reduction of cytosolic calcium ion concentration, DNA clamp loader activity and protein-DNA loading ATPase activity. There were no pathways enriched by the DEGs in control group after treatment.

The top 10 GO functions and KEGG pathways enriched by the DEGs specific in HQDG group after treatment are listed in Appendix 3. For the up-regulated genes, the enriched terms included flagellum, cilium, sensory perception of smell and nitrogen metabolism. The down-regulated genes were enriched in translational elongation, cytosolic ribosome, cytosol and ribosome.

PPI Network Analysis

The PPI network for the up-regulated genes specific in HQDG group had 175 nodes and 261 interactions (Figure 2). Meanwhile, the PPI network constructed for down-regulated genes specific in HQDG group consisted of 91 nodes and 239 interactions (Figure 3). Specially, epidermal growth factor receptor (EGFR, degree = 11) in the PPI network for the up-regulated genes, as well as cell division cycle 42 (CDC42, degree = 27) and v-akt murine thymoma viral oncogene homolog 1 (AKT1, degree = 21) in the PPI network for the down-regulated genes had higher degrees.

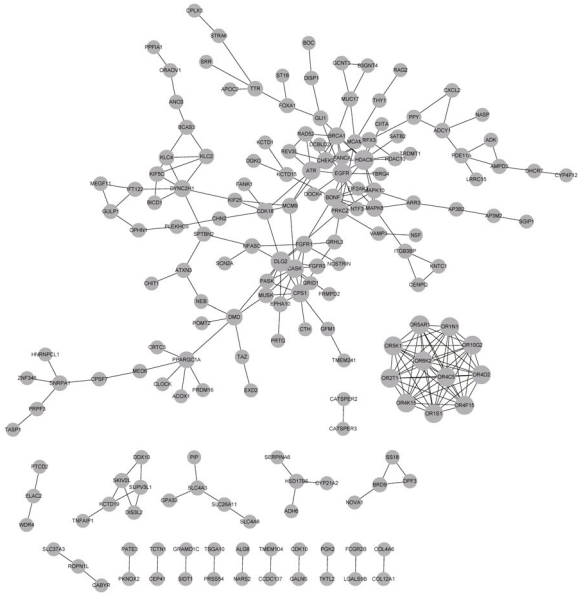

Figure 2. Protein-Protein Interaction Network for the Up-Regulated Genes Specific in Huangqi Decoction Granules Group

Regulatory Network Analysis

Based on the lncRNA annotation information in GENCODE v24 database, a total of 25 DE-lncRNAs were screened out from all of the DEGs, including 14 up-regulated genes and 8 down-regulated genes in HQDG group, as well as 2 up-regulated genes and 1 down-regulated gene in control group. A total of 1,002 genes targeted by 15 DE-lncRNAs (including growth arrest-specific 5, GAS5) were predicted. Then, the

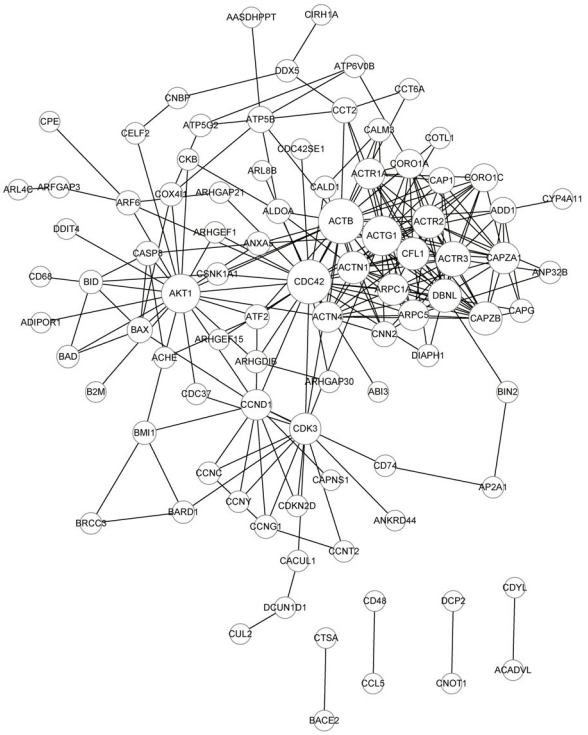

Figure 3. Protein-Protein Interaction Network for the Down-Regulated Genes Specific in Huangqi Decoction Granules Group

lncRNA-target gene regulatory network was constructed, which contained 1,017 nodes and 1,004 interaction pairs (Figure 4). Furthermore, the genes targeted by the up-regulated lncRNAs were enriched in molecular function, biological process and cellular component. The genes targeted by the down-regulated lncRNAs

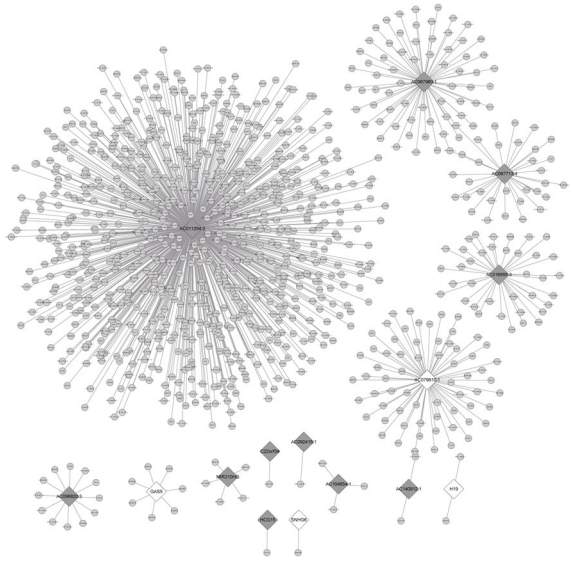

Figure 4. Long Non-Coding RNA-Target Gene Regulatory Network

Notes: The circles represent target genes. The grey and white diamonds represent up-regulated and down-regulated lncRNAs, respectively.

were enriched in molecular function, cytokine activity and endomembrane system organization (Appendix 4). There were no pathways enriched for the target genes.

qRT-PCR Analysis

As a result, CDC42 expression in HQDG group after treatment was significantly lower than pre-treatment and control group after treatment (P<0.05, Figure 5A). While the expression level of GLI1 in HQDG group after treatment was significantly higher than pre-treatment and control group after treatment (P<0.05, Figure 5B). Nevertheless, the expression levels of other genes were not detected.

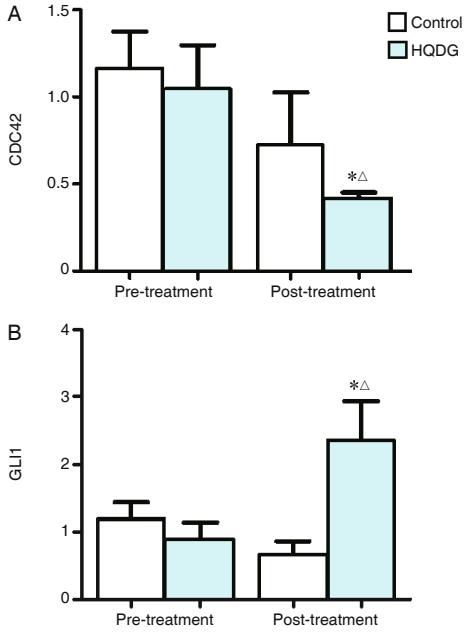

Figure 5. Expression Levels of CDC42 (A) and GLI1 (B) in the Serum of Two Groups

Notes: *P<0.05 *vs*. HQDG group before treatment; △P<0.05 *vs*. control group before treatment

DISCUSSION

In this study, a total of 1,070 DEGs and 227 DEGs were separately identified in HQDG group and control group, respectively. Venn diagram analysis further screened 1,043 DEGs (including 441 up-regulated genes and 602 down-regulated genes) specific in HQDG group compared with the control group. One up-regulated TF (GLI1) and 25 down-regulated TFs (including SMAD2) were identified from the 1,043 specific DEGs. Through promoting the protein stability and nucleus translocation of GLI proteins, hepatitis B virus X (HBx) regulates hedgehog-GLI activation in liver cancer cells.[31] Cortical activation of GLI1 suppresses the induction of hepatic encephalopathy and TGF β 1 inhibits GLI1 in neurons, thus GLI1 activation or TGF β 1 inhibition may be potential therapeutic strategies for hepatic encephalopathy.[32] A recent study found that cryptolepine derivative-6h blocked Shh signaling, resulting in the decreased protein expression of GLI1 in liver fibrosis,[33] suggesting that GLI1 may be a target in the treatment of liver fibrosis. During and after the formation of liver fibrosis, SMAD2 and SMAD4 are mainly expressed by HSCs, myofibroblasts and fibroblasts, and their expression levels increase as hepatic fibrosis becoming more serious.[34] Through shifting phosphorylated SMAD2 and SMAD3 signaling from fibro-carcinogenesis to tumor suppression, hepatitis C virus (HCV) clearance plays a critical role in controlling fibrosis and decreasing the incidence of hepatocellular carcinoma (HCC).[35] These declared that GLI1 and SMAD2 might be involved in the HQD treatment process of hepatitis B cirrhosis. As a GTPase protein belongs to Rho family, CDC42 plays a role in regulating HCV infectivity.[36] The results of qRT-PCR showed that CDC42 and GLI1 levels had significant differences in HQDG group between after and before treatment, indicating that CDC42 and GLI1 may be the targets of HQDG in the treatment of patients with hepatitis B cirrhosis. However, the expression levels of other genes could not be detected by qRT-PCR, which may be due to the low RNA content in serum.

Epidermal growth factor (EGF) is related to the progression of cirrhosis and HCC, and EGFR inhibition can reduce fibrogenesis and prevent HCC in patients with high-risk cirrhosis.[37] As a EGFR ligand induced before liver injury, amphiregulin (AR) functions in liver fibrosis by promoting the expression of fibrogenic mediators and contributing to the survival and growth of fibrogenic cells.[38] Akt and endothelial nitric oxide synthase (eNOS) phosphorylation show a significant reduction in cirrhotic liver, which can be repaired by constitutively active Akt (myr-Akt) gene therapy.[39] Clinical study indicates that Akt phosphorylation plays a critical role in the aggressiveness of HCC, thus Akt phosphorylation can serve as a risk factor for poor prognosis and early recurrence in HCC.[40] In the PPI networks, up-regulated EGFR and down-regulated AKT1 had higher degrees, indicating that EGFR and AKT1 might be associated with the curative effect of HQDG to hepatitis B cirrhosis.

Pathway enrichment analysis showed that down-regulated RHOA was enriched in pathogenic

Escherichia coli infection. In cirrhosis, reduced nitric oxide availability and elevated RhoA/Rho-kinase signaling contribute to the increasing of intrahepatic resistance and portal hypertension.[41] Through suppressing the activation of RhoA and Rho-associated coiled coil-forming protein kinase (ROCK) Ⅱ and the downstream myosin phosphatase target subunit 1 (MYPT1) phosphorylation, salvianolic acid B can reduce portal pressure and weaken endothelin-1-induced contraction of HSCs in rats with DMN-induced cirrhosis.[42] A total of 1,002 genes targeted by 15 DE-lncRNAs (including GAS5) were predicted. GAS5 can inhibit the activation of HSCs and suppress liver fibrogenesis through acting as a competing endogenous RNA for miR-222 and subsequently increasing the p27 protein level.[43] Thus, RHOA and GAS5 might also be targets of HQDG in patients with hepatitis B cirrhosis.

In conclusion, CDC42 and GLI1 may be the targets of HQD in patients with hepatitis B cirrhosis. In addition, SMAD2, EGFR, AKT1, RHOA and GAS5 might be associated with the curative effect of HQD to hepatitis B cirrhosis. However, further experimental researches are still needed to confirm these results.

Conflict of Interest

None.

Author Contributions

Cheng Y and Liu P carried out the conception and design of the research, participated in the acquisition of data and drafted the manuscript. Hou TL carried out the analysis and interpretation of data. Maimaitisidike M and Ababaikeli R participated in the statistical analysis. Abudureyimu A and Cheng Y participated in the study design and performed the statistical analysis. All authors read and approved the final manuscript.

Electronic Supplementary Material: Supplementary materials (Appendixes 1–4) are available in the online version of this article at http://dx.doi.org/10.1007/s11655-018-3013-3.

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