MiRNA-target network analysis identifies potential biomarkers for Traditional Chinese Medicine (TCM) syndrome development evaluation in hepatitis B caused liver cirrhosis

Yamin Liu1, Mei Wang2, Yunquan Iuo3, Chenjian Chen1, Yiyu Lu4, Yulin Shi1, Chenchen Tang1, Qianmei Zhou1, Hui Zhang1, Yuanjia Hu4, Shibing Su1 & Qilong Chen1

Hepatitis B is one of most etiologies of Liver cirrhosis in China, and clinically lacks the effective strategy for Hepatitis B caused cirrhosis (HBC) therapy. As a complementary and alternative medicine, Chinese Traditional Medicine (TCM) has special therapeutic effects for HBC. Here, we focus on the evolution process of HBC TCM syndromes, which was from Excessive (Liver-Gallbladder Dampness-Heat Syndrome, LGDHS) to Deficient (Liver-Kidney Deficiency Syndrome, LKYDS) via Excessive-Deficient syndrome (Liver-Depression and Spleen-Deficiency Syndrome, LDSDS). Using R package, 16 miRNAs in LGDHS/Normal, 48 miRNAs in LDSDS/LGDHS, and 16 miRNAs in LKYDS/LDSDS were identified, respectively. The miRNA-target networks show that the LDSDS was most stability and complicated. Subsequently, 4 kernel miRNAs with LGDHS-LDSDS process, and 5 kernel miRNAs with LDSDS-LKYDS process were screened. Using RT-qPCR data, p1 (hsa-miR-17-3p, -377-3p, -410-3p and -495) and p2 miRNA panel (hsa-miR-377-3p, -410-3p, -27a-3p, 149-5p and 940) were identified by Logistic Regression Model, which clearly improve the accuracy of TCM syndrome classification. The rebuilt miRNA-target network shows that the LDSDS is a critical point and might determine the evolution directions of HBC TCM syndrome. This study suggests that the identified kernel miRNAs act as potential biomarkers and benefit to evaluate the evolution tendency of HBC TCM syndromes.

Liver Cirrhosis has been recognized by the development of acute deterioration of liver function1. Importantly, hepatitis B Virus (HBV) caused cirrhosis (HBC) is one of most etiologies of Liver cirrhosis and annually about 1.5 million people are suffering in China. As a chronic and complicated liver disease, the 5-year survival rate of patients with severe HBC only is about 50%, especially, hepatocellular carcinoma (HCC) was estimated that occurs almost exclusively in patients with HBC. During the past decades, clinically lacks the effective strategy for the therapy of HBC, fortunately, as a complementary and alternative medicine, Chinese Traditional Medicine (TCM) has special therapeutic effects for HBC treatment. In previous study, we found that PNP, AQ7P, and PSMD2 may be involved in TCM syndromes differentiation of HBC. However, the characteristics of HBC TCM syndromes are unclear, especially, the development mechanism from Excessive (Liver-Gallbladder Dampness Heat Syndrome, LGDHS) to Excessive-Deficient (Liver-Depression and Spleen-Deficiency Syndrome, LDSDS) syndrome, and/or Excessive-Deficient (Liver Depression and Spleen Deficiency Syndrome, LKYDS) develop to Deficient (Liver-Kidney Deficiency Syndrome, LKYDS) syndrome still rigorously lacks in HBC progression.

MicroRNA (miRNA) is a class of small nonconding RNA molecules (18–24 nucleotides), which was involved in many liver disease, including liver metabolism, fibrosis, regeneration, and HCC. Importantly, the exceptional
stability of miRNA in serum or plasma is valued for clinical use, such as playing biomarkers to distinguish chronic HBV hepatitis, their corresponding cirrhosis and HCC\(^9,10\). In this work, we analyzed the dynamical expression levels of miRNAs in the evolutionary process of HBC TCM syndromes based on transcriptional profiles. The miRNA-target network suggests that the LDSDS might act as an important critical point in HBC TCM syndromes development. Using the RT-qPCR data, we have established stepwise logistic regression models with promising diagnostic performances for TCM syndrome classification. Our findings provide useful information for developing novel tools to evaluate the evolution tendency of HBC TCM syndromes.

Results

Differential expressed miRNAs of TCM syndromes in HBC. Using the Random variance model of R package, 16 differential expressed (DE) miRNAs in LGDHS/Normal, 5 DE miRNAs in LDSDS/Normal, and 7 DE miRNAs in LKYDS/Normal were identified, respectively (Fig. 1A–C). It suggests that the expression statuses of miRNAs are great difference among the three HBC TCM syndromes. In this study, we mainly focus on the miRNAs levels among the LGDHS/Normal, LDSDS/LGDHS, and LKYDS/LDSDS, because they represent a consecutive evolutionary stage of HBC TCM syndromes, which was evolved from Excessive syndrome (LGDHS) to Deficient (LKYDS) via Excessive-Deficient (LDSDS) syndrome. Subsequently, 48 DE miRNAs in LDSDS/LGDHS (Fig. 1D) and 16 DE miRNAs in LKYDS/LDSDS (Fig. 1E) were obtained, respectively, and suggesting that these DE miRNAs are high correlated with the process of LGDHS developed to LDSDS and LDSDS evolved to LKYDS. Furthermore, 6 overlapped miRNAs, including hsa-miR-376a-3p, -376c-3p, -377-3p, -381-3p, -410-3p, and -654-3p, were selected from the LGDHS/Normal, LDSDS/LGDHS, and LKYDS/LDSDS and defined as co-expressed miRNAs in the evolutionary process of TCM syndromes, suggesting these miRNAs maybe play critical roles for HBC development.

To investigate the biological functions of the DE miRNAs in evolutionary process of HBC TCM syndromes, the miPath (v3.0) program were performed for pathways analysis\(^11\), and significant terms were determined when \(P\)-value < 0.001. Compare with the normal controls, Steroid hormone biosynthesis, Prion diseases, Metabolism of xenobiotics by cytochrome P450, TGF-beta signaling pathway, Signaling pathways regulating pluripotency of
stem cells, Proteoglycans in cancer, Lysine degradation, and Hippo signaling pathway are highly associated with LGDHS (Fig. 2A). In the process of LGDHS developed to LDSDS, the pathways mainly focus on ECM-receptor interaction, TGF-beta signaling pathway, Signaling pathways regulating pluripotency of stem cells, Steroid hormone biosynthesis, Proteoglycans in cancer, and Prion diseases (Fig. 2B). Furthermore, the TGF-beta signaling pathway, Prion diseases, Signaling pathways regulating pluripotency of stem cells, Lysine degradation, and Huntington’s disease are highly correlated with the process of LDSDS develop to LKYDS (Fig. 2C). Interestingly, three pathways thread throughout the whole evolutionary process of LGDHS-LDSDS-LKYDS, such as Prion diseases, Signaling pathways regulating pluripotency of stem cells, and TGF-beta signaling pathway. It suggests that these pathways are indispensable for the evolutionary process of HBC TCM syndromes.

**miRNA target genes prediction and enrichment analysis.** Using the prediction programs, the miRNA target genes were predicted respectively. After redundancy analysis, the final database of LGDHS/Normal (16 DE miRNAs with 2917 target genes), LDSDS/LGDHS (48 DE miRNAs with 6531 target genes) and LKYDS/ LDSDS (16 DE miRNAs with 2594 target genes) were built, respectively. The functional enrichment was conducted by DAVID program, GO terms (10% top terms), KEGG pathways and Disease terms were shown in Fig. 3. In addition to the same GO, KEGG and Disease terms, the LGDHS related biological functions mainly associated with regulation of transcription, cell division, G1/S transition of mitotic cell cycle, cell cycle arrest, Focal adhesion, Pancreatic cancer, Chronic myeloid leukemia, Glioma, Hippo signaling pathway, TGF-beta signaling pathway, Hepatitis B, and ErbB signaling pathway. The LDSDS mainly associated with metal ion binding, transcription factor activity, ubiquitin conjugating enzyme activity, ubiquitin-dependent protein catabolic process, Viral carcinogenesis, Progesterone-mediated oocyte maturation, Estrogen signaling pathway, leucine and isoleucine degradation, Melanogenesis, Fatty acid degradation, Dopaminergic synapse, and Alzheimer’s disease. The LKYDS mainly associated with poly(A) RNA binding, cellular response to DNA damage stimulus, cell-cell adherens junction, protein kinase binding, Hepatitis B, Dorso-ventral axis formation, Neurotrophin signaling pathway, MAPK signaling pathway, ErbB signaling pathway, and many tumors. These results suggested that the biological functions are great difference among the three HBC TCM syndromes.

Here, we notice that the co-expressed miRNAs thread throughout the whole evolutionary process of HBC TCM syndrome, which might play important roles for TCM syndrome evolution. GO terms ($P < 10^{-5}$) show
that these co-expressed miRNAs target genes are mainly associated with nucleus, protein binding, nucleic acid binding, nucleoplasm, transcription factor activity (Fig. 3A). KEGG pathways show an impressive functional association with various signal-related and cancer-related pathways, such as Signaling pathways regulating pluripotency of stem cells ($P = 6.78 \times 10^{-5}$), p53 signaling pathway ($P = 1.07 \times 10^{-4}$), and PI3K-Akt signaling pathway ($P = 1.02 \times 10^{-5}$), Pathways in cancer ($P = 6.62 \times 10^{-5}$), Non-small cell lung cancer ($P = 8.70 \times 10^{-5}$) (Fig. 3B). Furthermore, Disease terms show that the co-expressed miRNAs target genes mainly associated with Bone Mineral Density, Brain Neoplasms, Colonic Neoplasms, plasma HDL cholesterol levels, Apolipoproteins B, Type 2 diabetes, Mouth Neoplasms, Breast Cancer, esophageal adenocarcinoma, stomach cancer, Osteoporosis, head and neck cancer (Fig. 3C). Although none of the Disease terms directly correlated with hepatitis B or fibrosis, in fact, many terms belong to the complicated disease and may be relevant to the genesis of liver cirrhosis.

**miRNA-target networks building and kernel miRNAs screening.** In the evolution process of HBC TCM syndromes, the miRNA-target networks of LGDHS, LDSDS and LKYDS (Fig. 4A–C) were constructed, respectively. The topological profiles show that three networks are more likely similar to ‘Medusa’ model, which consists of regulatory core framework by hub nodes and represent most determinants in the network. This model suggests that the hub nodes have prominently functions in the realized network profiles, and the periphery nodes should be regulated. Such being the case, the network parameters were calculated, including Betweenness Centrality (BC), Closeness Centrality (CC) and Degree (De), and the hub nodes of networks were defined as $BC \geq \text{Avg (BC)}$, $CC \geq \text{Avg (CC)}$ and $De \geq \text{Avg (De)}$. The determinate nodes were considered as kernel miRNAs and play important roles for TCM syndrome evolution in HBC progression. Finally, 7 kernel miRNAs in LGDHS network, 17 kernel miRNAs in LDSDS network, and 8 kernel miRNAs in LKYDS network were screened, respectively (Supplementary Table 1). Furthermore, we noticed 4 kernel miRNAs shared by LGDHS and LDSDS, 5 kernel miRNAs overlapped between LDSDS and LKYDS, and 2 kernel miRNAs (hsa-miR-377-3p, -410-3p) mutually shared among the LGDHS, LDSDS and LKYDS. The results indicated that these kernel miRNAs might

---

**Figure 3.** The biological functions analysis of differential expressed miRNAs target genes. (A) Represent the GO terms distributions in the HBC TCM syndrome development, (B) represent the KEGG pathways distributions in the HBC TCM syndrome development, and (C) represent the Disease terms distributions in the HBC TCM syndrome development.
directly regulate the Excessive (LGDHS) develop to Excessive-deficient syndrome (LDSDS) or Excessive-deficient (LDSDS) develop to Deficient syndrome (LKYDS).

The co-network was knockout from the miRNA-target network. Furthermore, the co-network of three TCM syndromes was isolated from above networks (Fig. 4D). To investigate the functions of co-expressed miRNAs, the co-network was knockout form the three miRNA-target networks, respectively, (E) represents the LGDHS knockout network, (F) represents the LDSDS knockout network, and (G) represents the LKYDS knockout network. (H) Represents the change rate of network parameters between before and after knockout, and (I) represents the Robustness levels in three networks.

**Validating the co-expressed miRNAs and kernel miRNAs.** The RT-qPCR was performed to measure the expression levels of 6 co-expressed miRNAs in LGDHS-LDSDS-LKYDS process. At the transcriptional levels, most co-expressed miRNAs expression levels have statistically significance in whole evolutionary process of TCM syndromes, expect hsa-miR-376c-3p, -377-3p and -381-3p in Normal/LGDHS, and hsa-miR-376c-3p and -654-3p in LKYDS/LDSDS (P > 0.05)(Fig. 5A). Based on the RT-qPCR and microarray data, the expression tendency of co-expressed miRNAs are coherent in the evolutionary process of HBC TCM syndromes (Fig. 5B,C). This result suggests that the dynamical expression levels of co-expressed miRNAs might benefit to evaluate the development tendency of TCM syndromes in HBC progression.
To explore whether the kernel miRNAs play important roles in each evolution stage of HBC TCM syndromes, the selected kernel miRNAs also were validated (Fig. 5D,E). The statistical significance was noted for 4 miRNAs in LGDHS develop to LDSDS process (hsa-miR-17-3p, -377-3p, -410-3p, and -495), and 5 miRNAs in LDSDS develop to LKYDS process (hsa-miR-377-3p, -410-3p, -149-5p, 27a-3p, and -940). The results indicate that the biological functions of these miRNAs might highly correlate to the HBC TCM syndrome development.

Identifying the excellent miRNA panel. Using RT-qPCR data, a Stepwise Logistic Regression Model was designed to calculate the optimal combination from these kernel miRNAs. Consequently, an excellent miRNA panel with hsa-miR-17-3p, -377-3p, -410-3p, and -495 were identified to evaluate the process of Excessive (LGDHS) develop to Excessive-deficient syndrome (LDSDS), and hsa-miR-377-3p, -410-3p, -149-5p, 27a-3p, and -940 were identified evaluate the Excessive-deficient (LDSDS) evolved to Deficient syndrome (LKYDS) process. The logit model were (p1 = miRNAs) = 2.485 - 0.035 * hsa-miR-17-3p - 0.076 * hsa-miR-495-3p - 0.070 * hsa-miR-377-3p - 0.062 * hsa-miR-410-3p, and (p2 = miRNAs) = 1.117 + 0.072 * hsa-miR-377-3p + 0.059 * hsa-miR-410-3p - 0.051 * hsa-miR-149-5p + 0.051 * hsa-miR-27a-3p - 0.062 * hsa-miR-940, which were used to construct the ROC-curve. The AUC for the test were p1 = 0.796 (95% CI: 0.687 to 0.872, Fig. 5F) and p2 = 0.860 (95% CI: 0.708 to 0.884, Fig. 5G). This result suggested that multiple miRNAs can combined an excellent miRNA panel.
expression34. Importantly, hsa-miR-495 could be a potential therapeutics in early tumorigenic progression, which was from Excessive (LGDHS) to Deficient (LKYDS) via Excessive-Deficient syndrome (LDSDS). The miPath (v3.0) analysis shows Prion diseases, Signaling pathways regulating pluripotency of stem cells, and TGF-beta signaling pathway are highly correlated with the LGDHS, LDSDS, and LKYDS of HBC progression. Importantly, act as a central regulator, TGF-beta be involved in many disease progression in chronic liver disease, which from initial liver injury through inflammation/fibrosis to cirrhosis or hepatocellular carcinoma15. TGF-beta overexpression also was correlated with tumor progression, metastasis, angiogenesis and poor prognostic outcome16–17. It suggests that these pathways are important for understanding the mechanisms of HBC TCM syndromes development.

In the evolution process, six co-expressed miRNAs were identified among the LGDHS, LDSDS and LKYDS, which might essential links for HBC TCM syndrome development. The RT-qPCR demonstrates the expression tendencies of these co-expressed miRNAs are coherent with the microarray levels in the process of LGDHS develop to LKYDS. GO terms and KEGG pathways show that the target genes mainly associated with Binding, Transcription, Activity, Nucleoplasm, Prostate cancer, Non-small cell lung cancer, Glioma, Chronic myeloid leukemia, p53 signaling pathway and PI3K-Akt signaling pathway. The p53 signaling pathway was considered as highly correlated with the pathogenesis of numerous cancer20. In hepatitis B caused hepatocellular carcinoma (HCC), the transcriptional activity of p53 was impaired by interacting with NUMB and consequently HCC development21. Importantly, the serum p53 was increased in HBV-related cirrhosis patients22, and the serum p53 protein expression were more pronounced in patients with liver cirrhosis more than liver fibrosis23. Furthermore, p53 also can act as potential biomarker for liver cirrhosis and HCC diagnosis24. This phenomenon suggests that the co-expressed miRNAs might regulate p53 signaling pathway related genes, and then to regulate the TCM syndromes development in whole HBC progression.

Furthermore, we found that 4 kernel miRNAs were associated with Excessive (LGDHS) develop to Excessive-Deficient (LDSDS), and 5 kernel miRNAs were correlated with Excessive-Deficient (LDSDS) develop to Deficient syndrome (LKYDS) based on RT-qPCR data. Using a Stepwise Logistic Regression Model, p1 miRNA panel (hsa-miR-17-3p, -377-3p, -410-3p and -495) and p2 miRNA panel (hsa-miR-377-3p, -410-3p, -27a-3p, 149-5p and 940) was identified, respectively, which was clearly improve the diagnostic accuracy of TCM syndrome in HBC progression. It suggests that these kernel miRNAs might act as useful tools to evaluate the evolution levels of HBC TCM syndromes. Interestingly, hsa-miR-377-3p and -410-3p not only overlapped between p1 and p2 miRNA panels, but also co-expressed in the whole evolutionary process of HBC TCM syndromes.

In p1 miRNA panel, all miRNAs are down expressed when LGDHS develop to LDSDS. Hsa-miR-377 was involved in the regulation of endogenous cell growth and associated with the problem pregnancies25. In nucleus pulposus cells, has-miR-377-3p was correlated with PKCε activation and might lead to ADAMTS5 long-term down-regulation26. Has-miR-377-3p also was demonstrated have tumor suppressive role and inhibited tumormetastasis by targeting EZF3 in NSCLC cell27. Has-miR-410 is a key regulatory factor and regulating the expression of IL-10 by targeting STAT3 in pathogenesis of Systemic lupus erythematosus (SLE)28. In liver and colorectal tumors, has-miR-410 have oncogenic properties and up-regulated by regulating FHL129. Has-miR-410 also acts as a tumor suppressor gene in some malignancies, such as gastric cancer30, gliomas31, and pancreatic cancer32. Has-miR-17-3p was involved in cell cycle regulation and overexpressed in various types of human tumors33. In synovial sarcoma, has-miR-17-3p act as an oncogene and promote the tumor growth by directly inhibiting p21 expression34. Importantly, has-miR-499 could be a potential therapeutics in early tumorigenic progression, which was recover RUNX3 expression under hypoxic conditions35. In breast cancer, has-miR-495 can facilitate tumor progression through the repression of JAM-A36, especially, it can inhibit the G1-S phase transition and suppress proliferation and tumorigenicity37. Furthermore, has-miR-495 also was considered as a tumor suppressor and associated with many tumors regulation38–42.

In p2 miRNA panel, hsa-miR-377-3p, -410-3p and -27a-3p are up expressed, whereas, 149-5p and 940 are down expressed when LDSDS develop to LKYDS. Hsa-miR-27a-3p was reported as a candidate biomarker for Alzheimer disease (AD)39, and combines miR-23a to target SMAD5 and regulate cell apoptosis by the FasL-Fas pathway in human granulocyte41. In human livers, has-miR-27a can regulate the CYP3A4 gene expression42, and act as pharmacologically relevant modulators in liver Dihydropyrimidinid dehydrogenase (DPD) function43. Has-miR149-5p was associated with cellular migration, proliferation and apoptosis in renal cell carcinoma44, act as potential prognosis of glioma45, mediate the crosstalk between tumor cells and cancer-associated fibroblasts in gastric cancer46, and increase hepatocellular carcinoma (HCC) risk47–48. The clinical evidence indicates that miR-149 is a potential prognostic biomarker of HCC, which was act as a tumor suppressive miRNA to inhibit tumorigenesis and repress metastasis of HCC39, 44. In HCC tissues and cell lines, has-miR-940 was remarkably decreased, and the lower expression level can promote cellular proliferation by targeting Estrogen-related receptor gamma (ESRRG)49. Importantly, the decreased has-miR-940 was contributed to poor overall survival of HCC, because it
can decrease HCC invasion and migration by down-regulated CXCR2 expression\(^{56}\). Furthermore, has-miR-940 was consistently down-regulated in breast cancer tissues\(^{57}\), it can play oncogenic role in gastric cancer\(^{58}\), activates the Wnt/beta-catenin signaling activation by targeting GSK3\(\beta\) and sFRP1 in pancreatic carcinoma\(^{59}\), and act as a diagnostic and prognostic tool for prostate cancer\(^{60}\).

Compare the biological functions of these miRNAs, mainly of them were focus on tumor suppressive or acted as prognostic biomarkers for human tumors. Importantly, hsa-miR-149-5p and 940 of p2 miRNA panel were highly correlated with tumorigenesis and metastasis of HCC. The p2 miRNA panel was associated with the evolution process of Excessive-Deficient (LDSDS) to Deficient syndrome (LKYDS), and it suggested that the stage of LDSDS evolved to LKYDS was more dangerous and increased hepatocellular carcinoma (HCC) risk in HBC progression.

The miRNA-target networks show that the structure of LDSDS was most complicated in the evolutionary process of HBC TCM syndromes. Subsequently, the co-network was knockout from LGDHS, LDSDS and LKYDS related miRNA-target networks, respectively. The robustness levels show the topological profile of LDSDS network is stronger than others, and suggests the LDSDS is an important stage for HBC TCM syndrome development. Furthermore, a new miRNA-target network was rebuilt using p1 and p2 miRNA panel, and interestingly, a Tai-Chi Diagram appeared on the topological profile of network. In the Diagram, Yang map represents Excessive syndrome (LGDHS), Yin map represents Deficient syndrome (LKYDS), and the Yin-Yang map (whole diagram) represents Excessive-Deficient syndrome (LDSDS) of HBC progression. At previous, Yin-Yang background was used to describe the Yin-Yang theory of TCM\(^{61}\) and possible relationship between Cold-Hot ZHENG networks in Neuro-Endocrine-Immune (NEI) system\(^{62}\). Here, we infer that the Excessive-deficient syndrome (LDSDS) is an important critical functional point in the evolutionary routes of HBC TCM syndromes. A large number of biological events may be heavily concentrated in LDSDS stage, such as cell cycle, cell apoptosis and proliferation, tumorigenesis, tumor invasion, migration and metastasis, moreover, many regulation events like as p53 signaling pathway and TGF-beta signaling pathway also were occurred in this stage (Fig. 6). Obviously, these biological events might playing important roles in the LDSDS stage, and may determine the “right” or “left” evolved routes of TCM syndromes in HBC progression, especially, a mutual transformation between Excessive (LGDHS) and Deficient syndrome (LKYDS) may occur in given changes. Generally, the prognosis of Excessive (LGDHS) is better than that of Excessive-deficient syndrome (LDSDS) and Deficient syndrome (LKYDS) in HBC progression.

In conclusion, we have identified a panel miRNAs to evaluate the development process of HBC TCM syndromes. Further analysis of the expression profiles of kernel miRNAs in different stages revealed that these
miRNAs were distinctly expressed in certain stages of HBC TCM syndromes, which were significantly correlated with indicators for HBC TCM syndromes evolution. The topological profile of networks shows that the Excessive-Deficient syndrome (LDSDS) was more complicated than Excessive (LGDHS) and Deficient syndrome (LKYDS). All these findings would help to understand the expression profiles of miRNAs in different stages that might act as novel diagnostic tools for TCM syndromes classification, and furthermore, the Excessive-Deficient syndrome (LDSDS) is a critical point of TCM syndromes when Excessive (LGDHS) develop to Deficient syndrome (LKYDS), which acts as a mediated process and determined the evolution direction of HBC TCM syndrome.

Methods

Clinical specimens. In this study, 162 clinical serum samples were collected from Shanghai Shuguang Hospital, which was including LGDHS (n = 36), LDSDS (n = 62), LKYDS (n = 34), and Normal controls (n = 30). The diagnostic criteria of western medicine for HBC followed the guidelines that defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005. The TCM syndrome system for HBC applied by the 3 senior TCM doctors of each diagnosis was accepted according to the standards of TCM differential syndrome. The diagnosis criteria of western medicine for HBC followed the guidelines that defined by the Chinese Society of Infectious Diseases in 2005. The TCM syndrome system for HBC applied by the 3 senior TCM doctors of each diagnosis was accepted according to the standards of TCM differential syndrome. The diagnostic criteria of western medicine for HBC followed the guidelines that defined by the Chinese Society of Infectious Diseases in 2005. The TCM syndrome system for HBC applied by the 3 senior TCM doctors of each diagnosis was accepted according to the standards of TCM differential syndrome.

miRNA profiles detection and analysis. The miRNA profiles were generated using Agilent Human miRNA microarray V3 (Agilent Technologies Inc, Santa Clara). All raw data were transformed to log2 and normalized each expression by zero mean and unit sample variance.

Using R package, a random variance model was designed to indentify the differential expressed (DE) miRNAs among the three TCM syndromes of HBC, such as LGDHS/Normal, LDSDS/Normal, LKYDS/Normal, LGDHS/LKYDS/LDSDS, where the fold-change > 1.5 and P < 0.05 were considered to be significant. The differential expressed (DE) miRNAs and predicted targets were combined and to construct miRNA-target network. Being built network, miRNAs were weighted by the fold change (|log2|), target genes were weighted based on the distribution of degree. Subsequently, all nodes were ranked according to their weights and tested the similarity, then, the obtained nodes were used to remap the network. In the network, the node represents miRNA or target, the edge represents the connection strength.

miRNA target genes prediction. The miRNA target genes were predicted using three database involve TarBase (v7.0), miRecords and miRTarBase, which contained the largest collection of manually curate experimentally data. Furthermore, miRanda, miRDB, miRWALK, and RNAhybrid programs were used to predict the non-experimental targets, where the P < 0.001 were considered to be significant. The predicted target genes were analyzed using DAVID online, significance analysis was defined as P value adjusted by False Discovery Rate (FDR), and gene sets containing less than 5 genes overlapping were removed. In this study, Gene ontology (GO), pathway and disease term with an FDR-adjusted P-value of less than 0.05 were retained.

miRNA-target network construction. The differential expressed (DE) miRNAs and predicted targets were combined and to construct miRNA-target network. Being built network, miRNAs were weighted by the fold change (|log2|), target genes were weighted based on the distribution of degree. Subsequently, all nodes were ranked according to their weights and tested the similarity, then, the obtained nodes were used to remap the network. In the network, the node represents miRNA or target, the edge represents the connection strength.
In this study, the co-network was knockout from raw network, and the robustness (R) level was used to evaluate the stability of network. The formula as follows,
\[ R = \frac{C}{(N - N_T)} \]
where the numerator C is the maximum connected component after network knockout, N is the raw network data, and N_T is the number of knockout nodes. Furthermore, the consecutive parameters of network were generated, such as Betweenness Centrality (BC), Closeness Centrality (CC) and Degree (De). In this work, the kernel node of network was defined as BC ≥ Avg (BC), CC ≥ Avg (CC) and De ≥ Avg (De). The determinate miRNAs were considered as kernel miRNAs and play important roles in differential HBC TCM syndromes.

**Quantification of co-expressed miRNAs and kernel miRNAs.** The RT-qPCR was used to identify the co-expressed miRNAs and kernel miRNAs in 162 serum samples (LGDS n = 36, LDSDS n = 62 and LKYDS n = 34, Normal controls n = 30). The quantification of miRNA was performed with SYBR Green PCR Master Mixture (TOYOBO, LTD, Japan) according to the manufacturer’s instructions using a Rotor-Gene 6000 Real-time PCR machine (Corbett Life Science, Sydney, Australia). The specificity of each PCR product was validated by melting curve at the end of PCR cycles. All miRNAs were validated in triplicate, the Ct was considered as the number of cycle requirement and for the fluorescent signal to reach the threshold. The levels of miRNAs were calculated using 2^-ΔΔCt where ΔCt = Ct of internal reference – Ct of target miRNA. The differences in miRNAs expression levels between groups were compared using the Student’s t-test and P-value < 0.05 was considered have statistically significant difference.

**Experimental data analysis.** Using RT-qPCR data, a stepwise logistic regression model was performed to screen diagnostic miRNA panel, which was considered as potential markers in the evolutionary process of HBC TCM syndromes. The predicted probability of being diagnosed with TCM syndromes evolution was used as surrogate marker to construct a receiver operating characteristic (ROC) curve. The area under the ROC-curve (AUC) represents an accuracy index for evaluating the diagnostic performance of the selected miRNA panel. The two-tailed test was used and \( P < 0.05 \) was considered have statistically significant.

**References**
1. Arroyo, V., Moreau, R., Jalan, R. & Gines, P. Acute-on-chronic liver failure: A new syndrome that will re-classify cirrhosis. *Journal of hepatology* 62, S131–143 (2015).
2. Hsu, A., Lai, C.-L. & Yuen, M. F. Update on the Risk of Hepatocellular Carcinoma in Chronic Hepatitis B Virus Infection. *Curr Hepat Rep* 10, 106–111 (2011).
3. de Jongh, F. E. et al. Survival and prognostic indicators in hepatitis B surface antigen-positive cirrhosis of the liver. *Gastroenterology* 103, 1630–1635 (1992).
4. Tan, Y. J. Hepatitis B virus infection and the risk of hepatocellular carcinoma. *World J Gastroenterol* 17, 4853–4857 (2011).
5. Chen, Q. et al. Transcriptional Profiling and miRNA-Target Network Analysis Identify Potential Biomarkers for Efficacy Evaluation of Fuzheng-Huayu Formula-Treated Hepatitis B Caused Liver Cirrhosis. *International journal of molecular sciences* 17 (2016).
6. Lu, Y. Y. et al. Study of ZHENG differentiation in hepatitis B-caused cirrhosis: a transcriptional profiling analysis. *BMC complementary and alternative medicine* 14, 371 (2014).
7. Budhu, A. et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology (Baltimore, Md.)* 47, 897–907 (2008).
8. Bala, S. et al. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology (Baltimore, Md.)* 56, 1946–1957 (2012).
9. Jin, B. X. et al. MicroRNA panels as disease biomarkers distinguishing hepatitis B virus infection caused hepatitis and liver cirrhosis. *Scientific reports* 5, 15026 (2015).
10. Qi, P. et al. Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. *PloS one* 6, e28486 (2011).
11. Vlachos, I. S. et al. DIANA-miPath v3.0: deciphering microRNA function with experimental support. *Nucleic acids research* 43, W460–466 (2015).
12. Guo, Y., Feng, Y., Trivedi, N. S. & Huang, S. Medusa structure of the gene regulatory network: dominance of transcription factors in cancer subtype classification. *Exp Biol Med (Maywood)* 236, 628–636 (2011).
13. Chen, Q.-L. et al. Characteristic Analysis from Excessive to Deficient Syndromes in Hepatocarcinoma Underlying miRNA Array Data. *Evid Based Complement Alternat Med* 2013, 324636 (2013).
14. Chen, Q.-L. et al. Progression from Excessive to Deficient Syndromes in Chronic Hepatitis B: A Dynamical Network Analysis of miRNA Array Data. *Evidence-Based Complementary and Alternative Medicine* 2013, 945245 (2013).
15. Dooley, S. & ten Dijke, P. TGF-beta in progression of liver disease. *Cell and tissue research* 347, 245–256 (2012).
16. Zhang, L. et al. TRAF4 Promotes TGF-beta Receptor Signaling and Drives Breast Cancer Metastasis. *Molecular cell* (2013).
17. Saito, A. et al. An integrated expression profiling reveals target genes of TGF-beta and TGF-alpha possibly mediated by microRNAs in lung cancer cells. *PloS one* 8, e65873 (2013).
18. Ly, S. et al. Crkl Efficiently Mediates Cell Proliferation, Migration, and Invasion Induced by TGF-beta Pathway in Glioblastoma. *Journal of molecular neuroscience: MN* (2013).
19. Mu, X. et al. TGF-beta signaling is often attenuated during Hepatotumorigenesis, but is retained for the malignancy of hepatocellular carcinoma cells. *PloS one* 8, e63436 (2013).
20. Stegh, A. H. Targeting the p53 signaling pathway in cancer therapy - the promises, challenges and perils. *Expert Opin Ther Targets* 16, 67–83 (2012).
21. Liu, D. et al. Hepatitis B e antigen and its precursors promote the progress of hepatocellular carcinoma by interacting with NUMB and decreasing p53 activity. *Hepatology (Baltimore, Md.)* 64, 390–404 (2016).
22. Shahzad, P. et al. The Increased Level of Serum p53 in Hepatitis B-Associated Liver Cirrhosis. *Iranian journal of medical sciences* 39, 446–451 (2014).
23. Attallah, A. M. et al. Expression of p53 protein in liver and sera of patients with liver fibrosis, liver cirrhosis or hepatocellular carcinoma associated with chronic HCV infection. *Clinical biochemistry* 42, 455–461 (2009).
Yuan, B., Liang, Y., Wang, D. & Luo, F. MiR-940 inhibits hepatocellular carcinoma growth and correlates with prognosis of human nucleus pulposus cells. PloS one 8, e60205 (2013).

Sun, C. et al. Long non-coding RNA NEAT1 promotes non-small cell lung cancer progression through regulation of miR-377-3p-E2F3 pathway. Oncotarget 7, 51784–51814 (2016).

Liu, D. et al. MiR-410 Down-Regulates the Expression of Interleukin-10 by Targeting STAT3 in the Pathogenesis of Systemic Lupus Erythematosus. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 39, 303–315 (2016).

Wang, Y. et al. MiR-410 is overexpressed in liver and colorectal tumors and enhances tumor cell growth by silencing FHL1 via a direct/indirect mechanism. PloS one 9, e80780 (2014).

Shen, J. et al. MicroRNA-410 suppresses migration and invasion by targeting MDM2 in gastric cancer. PloS one 9, e104510 (2014).

Chen, L. et al. MiR-410 regulates MET to influence the proliferation and invasion of gloma. The international journal of biochemistry & cell biology 44, 1711–1717 (2012).

Guo, R., Gu, J., Zhang, Z., Wang, Y. & Gu, C. MicroRNA-410 functions as a tumor suppressor by targeting angiotsentin II type 1 receptor in pancreatic cancer. IJUJB life 67, 42–53 (2015).

Volina, S. et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proceedings of the National Academy of Sciences of the United States of America 103, 2257–2261 (2006).

Minami, Y. et al. SS18-SSX-regulated miR-17 promotes tumor growth of synovial sarcoma by inhibiting p21WAF1/CIP1. Cancer science 105, 1152–1159 (2014).

Lee, S. H., Jung, Y. D., Choi, Y. S. & Lee, Y. M. Targeting of RUNX3 by miR-130a and miR-495 cooperatively increases cell proliferation and tumor angiogenesis in gastric cancer cells. Oncotarget 6, 33269–33278 (2015).

Cao, M. et al. MicroRNA-495 induces breast cancer cell migration by targeting JUN-A. Protein & cell 5, 862–872 (2014).

Wang, L. et al. Downregulated miR-495 [Corrected] Inhibits the G1-S Phase Transition by Targeting Bmi-1 in Breast Cancer. Medicine 94, e718 (2015).

Li, Z. et al. Methylation-associated silencing of miR-495 inhibits the migration and invasion of human gastric cancer cells by directly targeting PRL-3. Biochemical and biophysical research communications 456, 344–350 (2015).

Jiang, X. et al. MiR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia. Proceedings of the National Academy of Sciences of the United States of America 109, 19397–19402 (2012).

Zhang, B. et al. Hsa-miR-495 acts as a tumor suppressor gene in glioma via the negative regulation of MYB. Molecular medicine reports 14, 977–982 (2016).

Li, J. Z. et al. MicroRNA-495 Regulates Migration and Invasion in Prostate Cancer Cells Via Targeting Akt and mTOR Signaling. Cancer investigation 34, 181–188 (2016).

Xu, Y. Y., Tian, J., Hao, Q. & Yin, L. R. MicroRNA-495 downregulates FOXC1 expression to suppress cell growth and migration in endometrial cancer. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine 37, 239–251 (2016).

Sala Frigerio, C. et al. Reduced expression of hsa-miR-27a-3p in CSF of patients with Alzheimer disease. Neurology 81, 2103–2106 (2013).

Nue, M. et al. miR-23a and miR-27a promote human granulosa cell apoptosis by targeting SMAD5. Biology of reproduction 93, 98 (2015).

Shi, Y. et al. Hsa-miR-27a is involved in the regulation of CYP3A4 expression in human livers from Chinese Han population. Pharmacogenomics 16, 1379–1386 (2015).

Offer, S. M. et al. microRNAs miR-27a and miR-27b directly regulate liver dihydroxypridine dehydrogenase expression through two conserved binding sites. Molecular cancer therapeutics 13, 742–751 (2014).

Jin, L. et al. Tumor suppressor miR-1-19-5p is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma. Molecular medicine reports 13, 5386–5392 (2016).

Xue, L., Wang, Y., Yue, S. & Zhang, J. Low miR-149 expression is associated with unfavorable prognosis and enhanced Akt/mTOR signaling in glioma. International journal of clinical and experimental pathology 8, 11178–11184 (2015).

Li, P. et al. Epigenetic silencing of microRNA-149 in cancer-associated fibroblasts mediates prostaglandin E2/interleukin-6 signaling in the tumor microenvironment. Cell research 25, 588–603 (2015).

Wang, X. H., Wang, F. R., Tang, Y. F., Zou, H. Z. & Zhao, Y. Q. Association of miR-194C>T and miR-499A>G polymorphisms with the risk of hepatocellular carcinoma in the Chinese population. Genetics and molecular research: GMR 13, 5048–5054 (2014).

Wang, R. et al. Association study of miR149 rs2292832 and miR608 rs4919510 and the risk of hepatocellular carcinoma in a largescale population. Molecular medicine reports 10, 2736–2744 (2014).

Liu, M. F., Chen, W. Q., He, Y. Z. & Gu, Y. L. Role of miR-194C>T polymorphisms on the risk of hepatocellular carcinoma in the Chinese population. Genetics and molecular research: GMR 13, 7184–7189 (2014).

Zhang, Y. et al. Comprehensive analysis of microRNA-regulated protein interaction network reveals the tumor suppressive role of microRNA-149 in human hepatocellular carcinoma via targeting AKT/mTOR pathway. Molecular cancer 13, 253 (2014).

Luo, G. et al. miR-149 represses metastasis of hepatocellular carcinoma by targeting actin-regulatory proteins PPM1E. Oncotarget 6, 2257–2261 (2015).

Yuan, B., Liang, Y., Wang, D. & Luo, F. MiR-940 inhibits hepatocellular carcinoma growth and correlates with prognosis of hepatocellular carcinoma patients. Cancer science 106, 819–824 (2015).

Ding, D. et al. miR-940 Suppresses Tumor Cell Invasion and Migration via Regulation of CXCR2 in Hepatocellular Carcinoma. Scientific reports 5, 8336 (2015).

Liu, X. et al. MicroRNA-940 promotes tumor cell invasion and metastasis by downregulating ZNF724 in gastric cancer. BioMed research international 6, 25418–25428 (2015).

Wang, H. W. et al. Over-expression of microRNA-940 promotes cell proliferation by targeting GSK3beta and sFRP1 in human pancreatic carcinoma. Biomedicine & pharmacotherapy = Biomedicine & pharmacotheraphy 83, 593–601 (2016).

Rajendiran, S. et al. MicroRNA-940 suppresses prostate cancer migration and invasion by regulating MIEN1. Molecular cancer 13, 250 (2014).

Shao, L. Mapping ancient remedies: applying a network approach to traditional Chinese medicine. Science 350, 572–574 (2015).

Li, S. et al. Understanding ZHENG in traditional Chinese medicine in the context of neuro-endocrine-immune network. IET systems biology 1, 51–60 (2007).

Hepatology, C. So, Association, C. M., Diseases, C. So. I. & Association, C. M. Guideline on prevention and treatment of chronic hepatitis B in China (2005). Chin Med J 120, 2159–2173 (2007).
64. The standards of TCM differential syndromes of viral hepatitis. Defined in December 1991 by Internal Medicine Hepatopathy Committee of Chinese Traditional Medicine Association.

65. Vlachos, I. S. et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic acids research* **43**, D153–159 (2015).

66. Xiao, F. et al. miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Res* **37**, D105–110 (2009).

67. Hsu, S. D. et al. miRTarBase: a database curates experimentally validated microRNA-target interactions. *Nucleic Acids Res* **39**, D163–169 (2011).

68. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44–57 (2009).

69. Huang da, W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**, 1–13 (2009).

Acknowledgements

This work was supported by Program of National Natural Science Foundation of China (81473443, 81503478), Natural Science Foundation of Shanghai Science and Technology Commission (14ZR1438800), Key Program of National Natural Science Foundation of China (81330084), and the “Yang Fan” Program of Shanghai Committee of science and Technology Fund annotation (14YF1411400).

Author Contributions

S.S.B. and C.Q.L. designed the research project. L.Y.M., W.M., L.Y.Q., C.J. and Z.H. performed all the experiments. L.Y.Y., S.Y.L., T.C.C and Z.Q.M. conducted the bioinformatics analysis. H.Y.J. helped with the results analysis. L.Y.M. and C.Q.L. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-11351-5

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017