Abstract. Pien Tze Huang (PZH), a common hepatoprotective Traditional Chinese Medicine that has been found to be an effective treatment for carbon tetrachloride-induced hepatic damage, including liver fibrosis. Circular RNAs (circRNAs) serve a crucial role in regulating gene expression levels via circRNA/micro (mi)RNA/mRNA networks in several human diseases and biological processes. However, whether circRNAs are involved in the underlying mechanism of the therapeutic effects of PZH on liver fibrosis remains unclear. Therefore, the aim of the present study was to investigate these effects using circRNA expression profiles from PZH-treated fibrotic livers in model mice. A case-control study on >59,476 circRNAs from CCl₄-induced (control group, n=6) and PZH-treated (case group, n=6) mice was performed using circRNA sequencing in liver tissues. PZH treatment resulted in the differential expression of 91 circRNAs, including 58 upregulated and 33 downregulated circRNAs. Furthermore, the construction of competing endogenous networks also indicated that differentially expressed circRNAs acted as miRNA sponges. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of miRNA targets demonstrated that PZH-affected circRNAs were mainly involved in biological processes such as ‘positive regulation of fibroblast proliferation’, ‘cellular response to interleukin-1’ and ‘regulation of DNA-templated transcription in response to stress’ and in a number of important pathways, such as ‘TNF signaling pathway’, ‘PI3K-Akt signaling pathway’, ‘IL-17 signaling pathway’ and ‘MAPK signaling pathway’. To further validate the bioinformatics data, reverse transcription-quantitative PCR was performed on seven miRNA targets in a human hepatic stellate LX-2 cell model. The results suggested that seven of the miRNAs exhibited regulatory patterns that were consistent with those of the transcriptome sequencing results. Kaplan-Meier survival analysis demonstrated that the expression levels of dihydrodiol dehydrogenase and solute carrier family 7, member 11 gene were significantly associated with patient survival, 269 patients with liver hepatocellular carcinoma from The Cancer Genome Atlas database. To the best of our knowledge, this was the first study to provide evidence that PZH affects circRNA expression levels, which may serve important roles in PZH-treated fibrotic liver through the regulation of functional gene expression. In conclusion, the
present study provided new insights into the mechanism underlying the pathogenesis of liver fibrosis and identified potential novel, efficient, therapeutic targets against liver injury.

Introduction

Liver fibrosis may be caused by acute or chronic liver injury and has been associated with severe morbidity and mortality (1). The progression from hepatitis to liver fibrosis, cirrhosis and even hepatic carcinoma has been reported to be accelerated by the occurrence of metabolic liver disease, alcoholism and viral hepatitis (2,3). The development of liver fibrosis is a result of the interaction among various types of resident hepatocytes, inflammatory cells, mediators and extracellular matrix (ECM) components, which are usually characterized by increased matrix protein production and decreased matrix remodeling (4,5). Liver fibrosis accelerates the progression of acute liver disease to its chronic form by disrupting the normal liver parenchyma (6). Furthermore, animal models that simulate liver fibrosis have improved our understanding of liver injury, and carbon tetrachloride (CCl4)-induced liver fibrosis is widely used in mouse models to study hepatotoxic mechanisms (3,7). CCl4-induced acute liver injury leads to the complex regulation of cellular responses. It has previously been established that continuous exposure of mice to a low dose of CCl4 (0.2 ml/kg), beginning at 8 weeks of age and lasting up to 6 weeks, leads to the development of liver fibrosis and compensatory cell proliferation (8). A CCl4-induced liver fibrosis mouse model has been demonstrated to be able to effectively simulate the formation of liver injury in vivo, which has been widely used in the study of the mechanism of hepatotoxicity (3,8-11).

Currently, treatments for liver fibrosis mainly include eliminating the primary causes and suppressing inflammation. However, there is still a lack of effective preventative methods or therapeutic drugs for liver fibrosis (12,13). The drug discovery process has paid great attention to the investigation of the efficacy drugs used in traditional medicine, as they are cheaper and have fewer side effects. Pien Tze Huang (PZH), a complex combination of Panax notoginseng (85%); Calculus bovis (5%); snake gallbladder (7%), obtained from the dry gall bladder of snake; and musk (3%), obtained from the preputial gland located in the abdomen of male musk deer, has been used as a traditional medicine with anti-inflammatory and soothing effects on skin boils and abscesses (14). A previous study has demonstrated that PZH exerts an important protective effect on CCl4-induced liver injury in mice (15). Additional studies have also suggested that PZH is effective in inhibiting liver damage caused by excessive drinking (16), improving histopathological damage in the liver and positively affecting physiological and biochemical indexes, such as serum alanine aminotransferase and aspartate aminotransferase, to a certain extent (15,17). Cell necrosis and swelling, microvesicular steatosis and lymphocyte infiltration in the injured liver have been reported to be significantly reduced following PZH treatment for liver disease (18).

Circular RNAs (circRNAs) are a recently discovered type of non-coding RNAs (ncRNAs). Most circRNAs are endogenous ncRNAs, and their formation does not have a covalent ring structure at the 3' and 5' end (19). Owing to their special structure, circRNAs cannot be easily degraded by nucleic acid exoenzyme ribonuclease R (RNase R) and are more stable than linear RNA (20). Furthermore, circRNAs regulate alternative splicing and transcription in a variety of diseases by acting as efficient microRNA (miRNA/miR) sponges, which can efficiently prevent the suppression of their mRNA targets (21,22). A previous study confirmed the regulatory role of circRNAs in circRNA/miRNA-gene regulatory networks by building a database of circRNAs derived from transcriptome sequencing data (23). Therefore, the interactions among circRNA, miRNA and mRNA have been considered important in transcriptional and post-transcriptional regulation.

An increasing number of studies have demonstrated that circRNAs can act as promising and technically suitable biomarkers for the occurrence and development of various diseases (24-28). Recently, Xu et al (29) analyzed the regulatory role of the circRNA/miRNA/mRNA network and revealed that circRNA_0001178 and circRNA_0000826 may be potential diagnostic markers for colorectal cancer metastasis. Furthermore, a recent study revealed that Mus musculus (mmu)_circRNA_002381 may influence the regulatory process of the transcription of certain genes affected by cocaine-induced neuroplasticity via the circRNA/miRNA interaction network (30). However, it remains unclear as to how the circRNA/miRNA/mRNA network modulates improvements in the fibrotic liver tissue of PZH-treated subjects.

The present study investigated the hepatoprotective activity of PZH against CCl4-induced liver fibrosis, and aimed to determine the circRNA-based molecular mechanisms by which it may confer its protection on the mice liver against CCl4-induced liver damage. Differentially expressed circRNAs were successfully identified using high-throughput RNA-sequencing (RNA-seq) in liver tissues from CCl4-induced and PZH-treated mice. The sponging action of differentially expressed circRNAs and related miRNA targets was further examined using multiple bioinformatics tools. Moreover, functional circRNA/miRNA/mRNA networks were established to provide novel insights into the treatment of liver fibrosis with PZH. The functional characteristics of the representative circRNA candidates were further analyzed by performing reverse transcription-quantitative PCR (RT-qPCR) on the mRNAs associated with the differentially expressed circRNAs in hepatic stellate cells (HSCs).

Materials and methods

Animal experiment. A total of 45 C57BL/6 mice of each group (age, 6 weeks; weight, 20-30 g) were purchased from Shanghai Model Organisms Center, Inc. The mice were housed at 24±2°C, relative humidity of 55±15%, kept in clean cages under a 14/10 h light-dark cycle and fed with standard rodent diet throughout the entire experimental period. Mice were randomly divided into two groups named the ‘PZH-treated’ and ‘CCl4-induced’ (PZH-treated, hepatic fibrosis model using PZH plus CCl4; CCl4-induced, hepatic fibrosis model using CCl4 only; n=15 mice/group). Meanwhile, a no-induction group (n=15 mice/group) was added to confirm the success of the liver fibrosis model. Mice in the CCl4-induced liver fibrosis model group were intraperitoneally injected with a solvent mixture of 10 µl/g body weight CCl4 (Wuhan Yafa Biological Technology Co., Ltd.) and 10% corn oil twice a week and...
intragastrically administered with double-distilled (dd)H2O once a day. Mice in the PZH-treatment group were intraperitoneally injected with a solvent mixture of 10 µl/g body weight CCl₄ and 10% corn oil twice a week (31) and intragastrically administered with 0.25 mg/g PZH-sonicated reagent (Zhangzhou Pientzehuang Pharmaceutical Co., Ltd.; Chinese Food and Drug Administration approval no. Z35020242; the PZH drug powder was dissolved in double distilled water, and its concentration was diluted to 25 mg/ml) once a day. During the 8-week experimental period, three mice in each group were anesthetized intraperitoneally with 40 mg/kg pentobarbital (Sigma-Aldrich; Merck KGaA) and sacrificed by cervical dislocation every 2 weeks in the first 6 weeks, and their liver tissue and blood were harvested for detecting the hepatic fibrosis level and hepatic biochemical index. The remaining 12 mice were also anesthetized and sacrificed by cervical dislocation in the 8th week. Mice were subsequently subjected to a laparotomy and liver tissue was harvested for the following experiments. Throughout the animal experiment mental state, hair and the body weight of the mice were closely monitored for toxic effects caused by CCl₄. No significant differences were observed in the body weight of the mice between CCl₄-induced and PZH-treated groups (P=0.7772; Fig. S1). The experimental procedures in the present study were approved by The Institutional Review Board of Shanghai Jiao Tong University (Shanghai, China; approval no. IACUC. NO:2017-0033).

RNA isolation for high-throughput sequencing. Total RNA was extracted from mouse liver tissue samples using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Samples were then processed with RNase-free DNase to remove traces of genomic DNA. The RNA concentration and quality were determined using a NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and the Bioanalyzer 2100 System (Agilent Technologies, Inc.). The A260/A280 ratio and RNA integrity number (RIN; Agilent Technologies Deutschland GmbH) were used to assess RNA purity and integrity, respectively (32). All samples met the following criteria: A260/A280 ratio >1.8 and RNA integrity >8, which means that the RNA integrity was high quality for sequencing.

Transcriptome data analysis. RNA libraries were constructed using ribosomal RNA-depleted RNAs with TrueSeq Stranded Total RNA Library Prep Kit (cat. no. RS-122-2201; Illumina, Inc.) according to the manufacturer's protocol. Briefly, 5 µg RNA was treated using the Ribo-Zero™ kit (cat. no. MRZ211124; Illumina, Inc.) to remove all ribosomal RNAs and linear RNAs were digested using RNase R (cat. no. NRNN07250; Lucigen Corporation). Subsequently, the enriched circRNAs were fragmented and a double-stranded cDNA library was synthesized using random primers and adapters. Finally, the cDNAs were purified and amplified with a thermocycler. All samples were mixed and underwent bridge amplification to generate clusters using HiSeq 4000 Paired-End Cluster Kit (cat. no. PE-410-1001-1; Illumina, Inc.). Quality control was performed using Q30. The library was subjected to 150-bp paired-end sequencing using HiSeq 4000 SBS Kit (cat. no. FC-410-1001-1; Illumina, Inc.). FastQC (version v0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc) (33) was used to evaluate the quality of the sequencing data. Trim-galore (version 0.6.0) (34) with default parameters was used to remove double-ended adapters and perform quality control on raw sequencing data.

Identification of differentially expressed circRNAs between the two groups. The filtered data were first mapped to the reference genome/transcriptome (version mm10, downloaded from UCSC, https://genome.ucsc.edu/) using STAR software version 2.5.3 (35). Only the common circRNAs predicted by CIRI (version 2.0.6) (36) and CIRCexplorer (37) software were termed ‘identified circRNAs’ in the subsequent analysis. CircBase (http://www.circbase.org/) (38) and Circ2Traits (39) (http://gyanxet-beta.com/circdb) databases were used to annotate the identified circRNAs. circRNAs were normalized to the number of back-splice junctions spanning spliced reads per billion mapping (SRPBM). The differentially expressed circRNAs were screened according to a log₂(FC)>1 and P<0.05. RNA-seq data were deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO; accession no. GSE150883).

Target prediction and bioinformatics analysis. For the specific competing endogenous RNA (ceRNA) network of significantly dysregulated circRNAs and mRNAs, the miRNA/mRNA and miRNA/circRNA interactions were predicted using the RegRNA 2.0 (40) (http://regrna2.mbc.nctu.edu.tw/), miRWalk 2.0 (41) (http://mirwalk.umm.uni-heidelberg.de) and TargetScan 7.2 (42) (http://www.targetscan.org) databases. miRNA-binding sites on circRNAs were determined using RegRNA 2.0 with a cutoff score of ≥170 and free energy of ≤-25. The putative target genes of these miRNAs were identified using the miRWalk algorithm with a binding energy of ≤-30 (43). The intersection of predicted target genes identified by both of these algorithms was selected as the differentially expressed circRNA-related predicted target genes in the present study for further analysis. The shared mRNAs between the differentially expressed circRNA-related predicted target genes in the present study and the differentially expressed mRNAs from mRNA-seq data (GEO accession no. GSE133481) were extracted. The overlapping mRNA-related miRNAs and miRNA binding sites on these circRNAs were selected to construct the ceRNA regulatory network. Cytoscape (version 3.6.1) was used to visualize the circRNA/miRNA/mRNA network interactions (44). The R package ClusterProfiler (45) was used for Gene Ontology (GO) analysis (http://geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/) was used for pathway analysis of the differentially expressed circRNA-related target genes based on the adjusted P<0.05.

Validation of differentially expressed circRNA-related target gene candidates by RT-qPCR in LX-2 cells. The expression levels of candidate differentially expressed circRNA-related target genes in the ceRNA network were further validated by RT-qPCR in the human hepatic stellate LX-2 cell line (American Type Culture Collection). LX-2 cells were grown in an incubator with 5% CO₂ at 37°C in DMEM (Thermo Fisher
Scientific, Inc.) supplemented with 10% FBS (MilliporeSigma), 2 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium; Gibco; Thermo Fisher Scientific, Inc.). Cells were seeded into 6-well plates (1x10^6/well) and divided into the PZH-induced (0.75 mg/ml) and the untreated control groups. The expression levels of differentially expressed circrna-related target gene candidates were determined at 24, 48 and 72 h. Total RNA was extracted from LX-2 cells using TRIzol reagent, aforementioned. Total RNA was reversed transcribed into cDNA using 1 µg isolated RNA with the PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed using an aBi7500 real-time PCR System (Thermo Fisher Scientific, Inc.) and the FastStart Universal SYBR Green Master (Roche Diagnostics). Each qPCR reaction contained 25 µl FastStart Universal SYBR Green Master, 0.3 µM forward primer, 0.3 µM reverse primer, 50 ng cDNA template and ddH₂O to a final volume of 50 µl. The qPCR conditions were as follows: initial denaturation at 95˚C for 10 min; followed by 40 cycles at 95˚C for 15 and 60 sec at 60˚C. All primers are shown in Table SΙ. Relative mRNA expression levels were quantified using the 2^−ΔΔCq method and normalized to the internal reference gene GAPDH (46). At 2^−ΔΔCq >1, the mRNA expression levels were considered to be upregulated and at 2^−ΔΔCq <1 downregulated.

Statistical analysis. Data from three independent experiments were used for analysis. Data are presented as the mean ± SEM. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for all statistical analyses. Unpaired Student's t-test and one-way ANOVA with Duncan's post hoc test were used to determine the statistical differences between the control and PZH-treated groups. P<0.05 was considered to indicate a statistically significant difference.

Survival analysis. Survival analysis was performed using the survival package in R (version 3.6.3) (47) to explore the relationship between the differentially expressed circRNA-related targets and the status data of 269 clinical patients with liver hepatocellular carcinoma from The Cancer Genome Atlas (TCGA) database (48). Patients with expression levels higher than the median values were assigned to the high-expression group, and patients with expression levels lower than the median values were assigned to the low-expression group. Kaplan-Meier survival analysis was performed to plot survival curves and the log-rank test was used to assess the statistical difference in survival rates between the high- and low-expression groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Prediction and expression analysis of circRNAs. To identify differentially expressed circRNAs in PZH-treated mice exhibiting liver damage improvement compared with the CCL4-induced group, RNA-seq was used to profile circRNA expression levels in the tissue samples from six PZH-treated mice and six CCL4-induced mice with liver fibrosis. The quality control results from the sequencing data demonstrated that the Q30 of all samples reached 95% (Table I). The filtered data was subsequently mapped to the reference genome/transcriptome (GRCm38/mm10; Table II). CIRI and CIRCexplorer software were used to identify the circRNAs in all samples. A total of 59,476 intersection circRNAs were detected by circRNA-seq. Among all the types of circRNA, the proportion of exon circRNA was as high as 98.35% (Table II). CIRI and CIRCexplorer software were used to identify the circRNAs in all samples. Detailed information regarding the circRNAs identified is displayed in Table SII. The relationship between the differentially expressed circRNA-related targets and the status data of 269 clinical patients with liver hepatocellular carcinoma from The Cancer Genome Atlas (TCGA) database (48).
Identification of differentially expressed circRNAs. The overall expression profiles of the differentially expressed circRNAs between the CCl₄-induced and PZH-treated groups are displayed in the volcano plot in Fig. 2C. In total, 91 circRNAs, including 58 (63.73%) upregulated and 33 (36.27%) downregulated circRNAs, were demonstrated to be significantly differentially expressed in liver tissue samples from the PZH-treated compared with the CCl₄-induced group (Fig. 2A). An analysis of the source of these circRNAs revealed that, of the 91 differentially expressed circRNAs, 88 (96.7%)...
were derived from exons (data not shown). The results also demonstrated that the most significant differentially expressed circRNAs were transcribed from the exons of protein-coding regions (Fig. 2B). The distribution of the identified circRNAs on different chromosomes was also analyzed. The statistical analysis results demonstrated that circRNAs were distributed on all chromosomes. Moreover, all differentially expressed circRNAs were found to be markedly expressed, except for those on chromosome 16 (Fig. 2D). Hierarchical clustering was also performed to investigate these differentially expressed circRNAs across 12 samples between PZH-treated and CCL₄-induced groups (Fig. 3). Detailed information on the top 20 differentially expressed circRNAs is included in Table SIV. The analysis of the circRNA-seq data suggested that the expression levels of circRNAs differed between PZH treatment and CCL₄-induced fibrotic livers.

**Prediction of differentially expressed circRNA-related target genes and construction of circRNA/miRNA/mRNA networks.** The identification of circRNA target genes is crucial for characterizing circRNA function. One of the molecular functions of circRNAs is their role as a ceRNA, which regulate miRNAs and consequently modulate mRNA expression (24,49). The interactions among circRNA, miRNA and mRNA were assessed based on the differentially expressed circRNAs between PZH-treated and the CCL₄-induced groups; the results showed that the upregulated circRNAs had interactions with 139 miRNA binding sites and 927 target genes. A total of 103 potential miRNAs binding sites and 887 target genes of these miRNAs were also identified on downregulated circRNAs (Table SV). Multiple circRNA-related biomarkers associated with liver fibrosis were also identified using circRNA-seq. However, the number of putative biomarkers was too large to accurately identify the biomarkers that may be involved in PZH treatment of liver fibrosis. Therefore, only the predicted target genes of the differentially expressed circRNAs were selected for further analysis.

The predicted set of functional genes for up- or downregulated circRNAs was compared with a set of differentially expressed mRNA genes enriched in the mRNA-seq data (these mRNAs were enriched in the same treatment conditions as those in the present study) (GEO accession no. GSE133481). The shared genes between these two datasets were recorded. In the upregulated circRNAs, only 10 overlapping genes from the 927 predicted mRNAs and 294 upregulated mRNAs from the mRNA-seq data were identified. Subsequently, the circRNA/miRNA/mRNA regulatory network of these related miRNAs and the corresponding significantly upregulated
circRNA-binding sites were constructed (Fig. 4A). The results demonstrated that 10 upregulated mRNAs were found to interact with 15 miRNAs, which were involved in the regulation of 17 circRNAs. Among the 15 miRNAs in the circRNA/miRNA/mRNA regulatory network, mmu- miR-466i-5p was targeted by seven identified upregulated circRNAs and exhibited the largest interaction network. mir-345-3p was predicted to be negatively modulated by mmu_circ:chr:chromosome (chr)9:69999094|70004341 and mmu_circ:chr2:119076936|119086650. The following top five most upregulated circRNAs appeared to be associated with the largest binding miRNA network: mmu_circ:chr:chromosome (chr)9:69999094|70004341 and mmu_circ:chr2:119076936|119086650, mmu_circ:chr13:112363415|112368348, mmu_circ:chr17:27786071|27794063 and mmu_circ:chr9:69999094|70004341. In the downregulated circRNAs, 21 overlapping genes from the 887 predicted mRNAs and 558 downregulated mRNAs from the mRNA-seq data were identified. Similarly, the circRNA/miRNA/mRNA regulatory network of these related miRNAs and the corresponding significantly downregulated circRNA-binding sites were constructed (Fig. 4B). The results demonstrated that 21 downregulated mRNAs were found to interact with 27 miRNAs, which were involved in the regulation of 12 downregulated circRNAs. The following top five most downregulated circRNAs were found to be associated with the largest binding miRNA network: mmu_circ:chr12:54911310|54917067, mmu_circ:chr15:27611512|27619623, mmu_circ:chr4:1492025071|149206773, mmu_circ:chr5:23482401|23486400 and mmu_circ:chr5:89081301|89084718. The expression information for the dysregulated circRNAs and their related target genes involved in circRNA/miRNA/mRNA interaction networks are listed in Tables III and IV, respectively. A flowchart summarizing the circRNA/miRNA/mRNA interaction network associated with the dysregulated circRNAs, as well as the results of the subsequent target gene analysis, is displayed in Fig. 5.

Host gene enrichment analysis of differentially expressed circRNA-related target genes. The function of all differentially expressed circRNA-related target genes was further explored. The GO analysis results demonstrated that the target genes mainly participated in the biological processes of ‘positive regulation of fibroblast proliferation’, ‘response to endogenous stimulus’, ‘drug catabolic process’ and ‘regulation of DNA-templated transcription in response to stress’ (Fig. 6A). KEGG pathways were also identified for differentially expressed circRNA-related target genes. KEGG analysis identified the following enriched pathways: ‘Metabolic pathways’, ‘TNF signaling pathway’, ‘PI3K-Akt signaling pathway’, ‘IL-17 signaling pathway’, ‘MAPK signaling pathway’ and ‘apoptosis’ (P<0.05; Fig. 6B). Meanwhile, the results of GO pathway analyses showed that the differentially expressed circRNA-related target genes in this network were associated with cellular components, such as ‘fibrinogen complex’, ‘transcription factor AP-1 complex’ and ‘platelet alpha granule’; and molecular functions, such as ‘ketosteroid monooxygenase activity’ and ‘MAP kinase tyrosine/threonine and phosphatase activity’ (Fig. S2).

Validation of candidate circRNA-related target genes by RT-qPCR. To verify the results of the co-expression network analysis, seven overlapping circRNA-related target gene candidates were selected for RT-qPCR in LX-2 cells in the CCL4-induced and PZH-treated group (Table V). mRNA expression levels of the seven gene candidates were subsequently detected 24, 48 and 72 h following PZH treatment (0.75 mg/ml). The seven selected transcripts included: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ (YWHA G); solute carrier family 39, member 14 (SLC39A14); FOS-like 2, AP-1 transcription factor subunit (FOSL2); solute carrier family 7, member 11 (SLC7A11); serpin family E, member 1 (SERPINE1); ATPase sarcoplasmic/endoplasmic reticulum Ca2+ (ATP2A2); and NRAS.
Among these seven, seven (SLC39A14, FOSL2, SLC7A11, SERPINE1, ATP2A2 and NRAS) were significantly differentially expressed compared with the control (Fig. 7). SLC39A14, FOSL2, SLC7A11, SERPINE1, ATP2A2 and NRAS mRNA expression levels were significantly downregulated in the PZH-treated group compared with the control group, while YWHAG was significantly upregulated. Overall, the results of the RT-qPCR validation of overlapping candidate circRNA-related target genes were consistent with the mRNA-seq results.

Survival analysis between patient mortality and differentially expressed circRNA-related target gene expression levels in liver hepatocellular carcinoma (LIHC). To explore the relationship between the expression levels of overlapping functional genes associated with differentially expressed circRNAs and patient survival, 269 patients with LIHC from TCGA database were divided into low- and high-expression groups based on the median values. The mRNA expression of 2 out of the 31 overlapping functional genes was significantly associated with patient survival in LIHC (Fig. 8). These were dihydrodiol dehydrogenase (DHDH) and SLC7A11. Kaplan-Meier survival analysis demonstrated that patients with low DHDH expression levels exhibited a greater survival rate compared with those with high DHDH expression levels (log-rank test, \( P=0.0057 \); Fig. 8A). The results for DHDH were also consistent with the survival rate of patients with LIHC based on SLC7A11 mRNA expression analysis (log-rank test,
P = 0.017; and Fig. 8B). However, Kaplan-Meier survival analysis of the remaining genes, including ral BP1-associated eps domain-containing 2 (rePS2), SerPine1, solute carrier family 3, member 2 (Slc3a2) and solute carrier family 10, member 5 (Slc10a5), indicated that although the difference in LIPIc survival rates between high-expression and low-expression groups was not statistically significant (log-rank test, \( P = 0.07 \), \( P = 0.21 \), \( P = 0.22 \) and \( P = 0.33 \), respectively), the mRNA expression levels of these genes were still closely associated with the survival of the patients (Fig. 8C-F).

Discussion

Traditional Chinese Medicine PZH has attracted considerable attention due to its marked therapeutic effect on liver injury (15,16). PZH exposure has been reported to significantly reduce cell necrosis and swelling, microvesicular steatosis and lymphocyte infiltration in the injured liver (50). Previous studies have shown that PZH may affect the immune system by interfering with the expression of functional genes in immune-related pathways in CCl4-induced mice (15,50). CCl4 is the mostly used reagent for the induction of liver injury animal models. Our previous study demonstrated that the ratio of positive Sirius red staining against the total area was significantly decreased in the PZH treatment group compared with the non-treatment group using Sirius red staining (39). These results prompted us to explore potential molecular mechanisms underlying these observed effects in a PZH-treated ccl4-induced liver injury model. circRNAs have certain advantages in the development and application of new clinical diagnostic markers, for example, Ye et al found that circRNAs participate in the pathogenesis of hepatic injury and providing efficient targets in the therapy against liver injury (28), and increasing number of studies have reported that circRNAs may act as miRNA sponges to regulate disease occurrence and development (21,24,49). However, to the best of our knowledge, there are currently no studies on the regulatory mechanisms of differentially expressed circRNAs in PZH-treated liver damage using RNA-seq technology. Therefore, mining circRNA transcript expression profiles using RNA-seq is
important for exploring the pathological mechanism of PZH in the treatment of liver diseases, especially liver fibrosis. In the present study, circRNA-seq and bioinformatic analyses were used to explore the function of circRNAs in PZH-treated mice.
with liver fibrosis. The circRNA expression profile identified 91 differentially expressed circRNAs between the PZH-treated and CCl_4-induced groups, of which 58 were upregulated and 33 were downregulated. To the best of our knowledge this was the first study to provide evidence that circRNAs are differentially expressed in PZH-treated fibrotic liver tissue compared with the controls, which used CCl_4 only.

CircRNAs act as miRNA sponges that regulate target gene expression by binding to miRNA(s) (24). The present study predicted that there were 242 miRNA-binding sites on circRNAs and 994 differentially expressed circRNA-related target genes in the present study and the differentially expressed mRNAs from mRNA-seq data (GEO accession no. GSE133481) were extracted for further analysis. Abcd3, ATP-binding cassette subfamily D, member 3; Abhd15, abhydrolase domain-containing 15; Akap2, A-kinase-anchoring protein 2; Ces1g, carboxylic ester hydrolase; circRNA, circular RNA; Dhdh, dihydrodiol dehydrogenase; Elovl6, ELOVL fatty acid elongase 6; Fosl2, FOS-like 2, AP-1 transcription factor subunit; Kmat5b, lysine methyltransferase 5B; LIHC, liver hepatocellular carcinoma; miR, microRNA; Nras, NRAS proto-oncogene GTPase; Ppm1k, protein phosphatase Mg^2+/Mn^2+ dependent 1K; PZH, Pien Tze Huang; Reps2, RALBP1-associated Eps domain-containing 2; RT-qPCR, reverse transcription-quantitative PCR; Serpine1, serpin family E, member 1; qseq, sequencing; Slc3a2, solute carrier family 3, member 2; Slc7a11, solute carrier family 7, member 11; Slc10a5, solute carrier family, 10 member 5; Slc17a8, solute carrier family 17, member 8; Slc39a14, solute carrier family 39, member 14; TCGA, The Cancer Genome Atlas; Tprkb, TP53RK-binding protein; Ywhag, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ.

The shared mRNAs between the differentially expressed circRNA-related predicted target genes in the present study and the differentially expressed mRNAs from mRNA-seq data (GEO accession no. GSE133481) were extracted for further analysis. Abcd3, ATP-binding cassette subfamily D, member 3; Abhd15, abhydrolase domain-containing 15; Akap2, A-kinase-anchoring protein 2; Ces1g, carboxylic ester hydrolase; circRNA, circular RNA; Dhdh, dihydrodiol dehydrogenase; Elovl6, ELOVL fatty acid elongase 6; Fosl2, FOS-like 2, AP-1 transcription factor subunit; Kmat5b, lysine methyltransferase 5B; LIHC, liver hepatocellular carcinoma; miR, microRNA; Nras, NRAS proto-oncogene GTPase; Ppm1k, protein phosphatase Mg^2+/Mn^2+ dependent 1K; PZH, Pien Tze Huang; Reps2, RALBP1-associated Eps domain-containing 2; RT-qPCR, reverse transcription-quantitative PCR; Serpine1, serpin family E, member 1; qseq, sequencing; Slc3a2, solute carrier family 3, member 2; Slc7a11, solute carrier family 7, member 11; Slc10a5, solute carrier family, 10 member 5; Slc17a8, solute carrier family 17, member 8; Slc39a14, solute carrier family 39, member 14; TCGA, The Cancer Genome Atlas; Tprkb, TP53RK-binding protein; Ywhag, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ.
Figure 6. GO term annotation and KEGG pathway enrichment analyses of differentially expressed circRNA-related target genes. (A) Biological process GO terms corresponding to the differentially expressed circRNAs. (B) KEGG enriched pathways for the target genes of the differentially expressed circRNAs. The x-axes indicate fold enrichment and the ratio of enriched differentially expressed genes in each pathway. The y-axes denote the name of the enriched KEGG pathway. The area of each node represents the number of enriched differentially expressed genes. The -log_{10}(P-value) was visualized using a green to red color variation bar. P<0.05 was considered to indicate a statistically significant difference. circRNAs, circular RNAs; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
Figure 7. Validation of target genes displayed in competing endogenous RNA networks in LX-2 cells by reverse transcription-quantitative PCR. Data are presented as the median ± SEM. *P<0.05, **P<0.01 and ***P<0.001. ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca²⁺; FOSL2, FOS-like 2, AP-1 transcription factor subunit; NRAS, NRAS proto-oncogene GTPase; PZH, Pien Tze Huang; SERPINE1, serpin family E, member 1; SLC7A11, solute carrier family 7, member 11; SLC39A14, solute carrier family 39, member 14.

Figure 8. Kaplan-Meier survival analysis between the patient clinical outcomes and circular RNA-related target genes. (A) DHDH, (B) SLC7A11, (C) REPS2, (D) SERPINE1, (E) SLC3A2 and (F) SLC10A5 gene expression levels in 269 patients with liver hepatocellular carcinoma from The Cancer Genome Atlas cohort were used to plot survival curves produced using Kaplan-Meier survival analysis. Patients exhibiting gene expression levels higher than the median values were assigned to the high-expression group, whereas those exhibiting mRNA expression levels lower than the median values were assigned to the low-expression group. The log-rank test was used to compare the difference in survival rates between the high- and low-mRNA expression groups. P<0.05 was considered to indicate a statistically significant difference. DHDH, dihydrodiol dehydrogenase; REPS2, RALBP1-associated Eps domain-containing 2; Slc3a2, solute carrier family 3, member 2; SLC7A11, solute carrier family 7, member 11; SLC10A5, solute carrier family 10, member 5; SERPINE1, serpin family E, member 1.
seven of the identified upregulated circRNAs may serve an important role in the therapeutic effect of PZH in the fibrotic liver by decreasing the expression levels of miR-466i-5p and further increasing those of ELOV16. Previous studies have also reported that miR-345-3p upregulation is involved in the pathogenesis of liver fibrosis and hepatocellular carcinoma (HCC) by negatively regulating cyclin-dependent kinase inhibitor 1 in cancer cells (57,60). This result indicated that the overexpression of these two circRNAs may induce pulmonary fibrosis by decreasing miR-345-3p expression levels and consequently increasing that of ATP-binding cassette subfamily D, member 3. In the downregulated circRNAs, 21 overlapping genes were identified and the circRNA/miRNA/mRNA regulatory network of 27 related miRNAs and the corresponding 12 significantly downregulated circRNA-binding sites on these miRNAs was constructed. A previous study reported that miRNA-105 expression is markedly downregulated in both HCC cell lines and clinical HCC tissues, compared with normal human hepatocyte and adjacent non-cancerous tissues (61). The results of the present study found 12 significantly downregulated circRNA-binding sites in PZH-treated fibrotic livers in model mice. An et al (62) demonstrated that miR-467f is downregulated in acute liver failure compared with mock-treated livers. Furthermore, the increased expression of SMAD2 has been demonstrated to serve a vital role in the development of liver fibrosis (63). The results of the present study indicated that downregulated mmu_circchr:149202507:149206773 may participate in the therapeutic effects of PZH on the fibrotic liver by decreasing the expression of SMAD2. Overall, these results demonstrated that differentially expressed circRNAs may alter the expression of certain functional genes via the circRNA/miRNA/mRNA regulatory network, therefore mediating PZH-treated liver fibrosis. It was therefore hypothesized that differentially expressed circRNAs may act as miRNA sponges and serve an important role in PZH-treated liver fibrosis by preventing miRNAs from regulating their target mRNAs.

The RT-qPCR data for the six differentially expressed circRNA-related target genes in the LX-2 cell model were consistent with the trends observed in the miRNA-seq data. Among the verified genes, SLC7A11 was the one most widely investigated in liver damage. It has previously been reported that inhibiting SLC7A11 induces ferroptosis in myofibroblastic HSCs and protects against liver fibrosis (64). Zhang et al (65) reported that the upregulation of SLC7A11 is an indicator of unfavorable prognosis in liver carcinoma. Furthermore, SERPINE1 has been demonstrated to serve an important role in the development of liver fibrosis. Lodder et al (66) found that increased expression of gene SERPINE1 aggravating the degree of liver fibrosis, liver cell damage and inflammation in myeloid cells. A previous study also demonstrated that homolog Fos-related antigen 2, encoded by FOSL2, is a contributing pathogenic factor of pulmonary fibrosis in humans (67). Furthermore, NRAS is a proto-oncogene, whose activating mutation has been linked to several types of human cancer, including HCC (68,69). Previous studies have demonstrated that sustained NRAS activation resulting from the overexpression of constitutively active NRAS, induces HCC in genetically compromised mice (70,71). In the present study, the results of the RT-qPCR validation of the differentially expressed circRNA-related target genes (SLC7A11, SERPINE1, FOSL2, NRAS, SLC39A14 and ATP2A2) corresponded to the mRNA-seq results. These results further suggested that the differentially expressed circRNAs acted as miRNA sponges in the regulation of target gene expression to influence the effects of PZH-treatment on liver fibrosis.

To explore the association between the differentially expressed circRNA-related targets and the survival time of patients with LIHC, Kaplan-Meier survival analysis was performed using data from the TCGA (n=269). The upregulated genes in PZH-treated liver fibrosis (SLC7A11 and DHDH) were found to be significantly positively associated with patient survival. Yue et al (72) reported that SLC7A11 served an important role in HCC as a potential prognostic indicator and its overexpression promoted HCC development. Furthermore, SLC7A11 may be a prognostic factor for liver carcinoma, as indicated by the survival analysis (73). Elevated levels of plasminogen activator inhibitor-1 (the protein product of SERPINE1) have been reported in patients with viral infection-related HCC (74). A number of studies have also indicated that SERPINE1 may contribute to cancer dissemination mechanisms, including the prevention of excessive ECM degradation, modulation of cell adhesion and stimulation of angiogenesis and cell proliferation (75,76). Wu et al (77) reported that SLC3A2 was highly expressed in the human HCC cell membrane and may serve an important role in promoting tumor metastasis and HCC progression. Overall, these results indicated that a specific set of differentially expressed circRNA-related functional genes may act as therapeutic targets for liver injury.

In conclusion, the present study identified 91 differentially expressed circRNAs in a PZH-treated CCl4-induced liver fibrosis model and the potential functions of 6 circRNA-related target genes were validated in LX-2 cells. Kaplan-Meier survival analysis further confirmed that two target mRNAs had potential clinical prognostic value for LIHC. Meanwhile, a functional circRNA/miRNA/mRNA network was systematically established to further investigate the underlying mechanisms of action of differentially expressed circRNAs. Overall, the present study provided new insights into the mechanisms underlying the pathogenesis of liver fibrosis and may provide novel and potentially efficient therapeutic targets against liver injury. However, a limitation of the present study was that it did not include any further molecular biology experiments to validate each of the differentially expressed circRNAs, and thus this will be investigated future studies.

Acknowledgements

Not applicable.

Funding

This work was supported by grants from The 863 Program (grant nos. 2012AA02A515 and 2012AA021802), The National Nature Science Foundation of China (grant nos. 81773818, 81273596, 30900799 and 81671326), The National Key Research and Development Program (grant nos. 2017YFC0909303, 2016YFC0905000, 2016YFC0905002, 2016YFC1200200 and 2016YFC0906400), The 4th Three-year Action Plan for Public
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