Identification of Key Genes for Hepatitis Delta Virus-Related Hepatocellular Carcinoma by Bioinformatics Analysis

Cheng Zhang[1], Shan Wu[1], Xiao-Dong Yang[1], Hui Xu[1], Tai Ma[2], Qi-Xing Zhu[1]

[1]*Anhui Provincial Cancer Institute, The First Affiliated Hospital of Anhui Medical University, Anhui, China*
[2]*Department of Oncology, The First Affiliated Hospital of Anhui Medical University, Anhui, China*

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ABSTRACT

Background: *It has been proposed that hepatitis delta virus (HDV) induces hepatic carcinogenesis by distinct molecular events compared with hepatocellular carcinoma (HCC) that is commonly induced by other hepatitis viruses. This study aimed to explore the underlying mechanism by identifying the key genes for HDV-HCC using bioinformatics analysis.*

Methods: *The GSE107170 dataset was downloaded and the differentially expressed genes (DEGs) were obtained by the online tool GEO2R. Gene otology (GO) functional analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using R packages. The protein-protein interaction (PPI) network was constructed by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). Hub genes were selected by Cytoscape software according to degree algorithm. The hub genes were further validated in terms of expression and survival analysis based on public databases.*

Results: *A total of 93 commonly upregulated genes and 36 commonly downregulated genes were found. The top 5 upregulated hub genes were TFRC, ACTR2, ARPC1A, ARPC3, and ARPC2. The top 5 downregulated hub genes were CTNNB1, CCND1, CDKN1B, CDK4, and CDKN1A. In the validation analysis, the expressions of ARPC1A, ARPC3, and CDK4 were promoted in general liver cancer samples. Higher expressions of ARPC2 and CDK4 and lower expressions of CDKN1A, CCND1, and CDKN1B were associated with worse prognosis in general HCC patients.*

Conclusion: *The present study identifies a series of key genes that may be involved in the carcinogenesis of HDV-HCC and used as prognostic factors.*

Keywords: *Hepatocellular carcinoma, hepatitis delta virus, computational biology*

INTRODUCTION

According to the GLOBOCAN 2018, liver cancer is the sixth most commonly diagnosed cancer (4.7%) and the fourth leading cause of cancer death (8.2%) worldwide in 2018.[1] Rates of both incidence and mortality are 2 to 3 times higher among men in most world regions.[1] The highest age-standardized rate (ASR) for male is observed in Eastern Asian (26.7 per 100 000 person-years).[1] For primary liver cancer, the majority of the cases (about 75-85%) are hepatocellular carcinoma (HCC). Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is one of the main risk factors for HCC. In the region which reports the highest ASR of liver cancer, co-infections of HBV with HCV or hepatitis δ virus (HDV) contributes to the high burden.[2]

HDV was first recognized as a new HBV antigen,[3] but now it has been confirmed as a unique RNA virus.[4] This virus is now estimated to affect 15-20 million people worldwide.[5] Chronic HDV infection is associated with a severe course of hepatitis that frequently leads to rapid fibrosis progression, hepatic decompensation, and HCC development.[6] Although the molecular events involved in HBV- or HCV-related HCC has been extensively studied and reviewed elsewhere,[7] the key genes and pathways that participated in HDV-related HCC are poorly known. It has been proposed that HDV and HBV promote carcinogenesis by distinct molecular mechanisms regardless of the dependence of HDV on HBV.[8] The high-throughput DNA microarray analysis is a helpful tool for us to better understand the underlying mechanisms and general genetic alterations in cancer initiation and metastasis. In recent years, the bioinformatics analysis on microarray data has been applied to investigate HCC carcinogenesis by exploring the most crucial genes, the otology, and the key pathways.[9-11] The present study aims to investigate the potential molecular mechanisms of HDV-related HCC pathogenesis by exploring hub genes

Corresponding authors: ***Hui Xu*** *or* ***Tai Ma*** *or* ***Qi-Xing Zhu****, e-mail:* ***xuhui52088@163.com*** *or* ***matai@ahmu.edu.cn*** *or* ***zqxing@yeah.net***
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and pathways in HDV-related HCC cases using bioinformatics approaches.

MATERIALS AND METHODS

Microarray Data Source and Analysis

To identify the differentially expressed genes (DEGs) between HDV-HCC samples and control samples, we searched for human microarray gene expression data related to "hepatitis D virus" and "hepatocellular carcinoma" in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo). Two datasets, GSE98383 and GSE107170, were retrieved. Since GSE107170 was the larger dataset that fully included the samples of GSE98383, we used GSE107170 as the only eligible dataset. This dataset was submitted by Diaz et al.[8] in November 2017 and updated in March 2019. This database included 12 liver tumor specimens from 5 HDV-HCC patients (57.2 ± 3 years) and 29 cirrhotic liver specimens from 7 HDV non-HCC cirrhosis patients (55.8 ± 1 years),[8] which were the overlapping part of GSE98383 and GSE107170. In addition, this database reused GSE55092 data and included 39 liver tumor specimens from 11 HBV-HCC patients (59.6 ± 2 years).[12] Regarding the serological HBV profile, all patients were positive for hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc), and antibody to hepatitis B e antigen (anti-HBe), and negative for hepatitis B e antigen (HBeAg) and antibody to HBsAg (anti-HBs). Regarding the serological HDV profile, all HDV patients were positive for serum HDV RNA and IgG anti-HDV, whereas IgM anti-HDV was positive in 3 of 5 patients with HCC and in all patients with HDV cirrhosis. Only 1 HBV patient was found to be positive for IgG anti-HDV but repeatedly negative for IgM anti-HDV and serum HDV RNA, whereas the remaining 10 HBV patients were negative for all markers of HDV infection. Since this study was based on the public database, ethical committee approval and informed consent were not applicable here.

Gene profiling was performed using Affymetrix Human Genome U133 Plus 2.0 Array (Platform GPL570). The explorations of (1) the DEGs between HDV-HCC and HDV-cirrhotic tissues and (2) the DEGs between HDV-HCC and HBV-HCC tissues were performed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). An adjusted *P*-value < .05 and a $|\log_2(\text{fold change})\ (FC)| > 1.0$ were used as the threshold for DEGs identification. The DEGs that were simultaneously found in both comparisons were illustrated using Venny diagram.

Statistical Analysis Process

The enrichment analyses of the DEGs were performed using clusterProfiler package[13] in RStudio 1.1.456. Briefly, the list of commonly up- and downregulated expressed genes were transformed from symbol to entrez ID. Then the Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed. The GO analysis included biological processes (BP), cellular components (CC), and molecular functions (MF). Top 10 ontologies or pathways in each category were illustrated using dot plots. The cut-off criterion was a *P*-value < .05.

To illustrate the interactions of the DEGs, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/) was used to construct protein-protein interaction (PPI) network with default parameters. The network was then imported to Cytoscape software 3.6.1[14] for the following analyses. The highly interconnected cluster in the PPI network was computed using MCODE plugin.[15] The centrality of the protein was computed using CytoNCA plugin.[16] The top 5 hub genes were found using cytoHubba plugin via the degree algorithm.

The expression information of the hub genes between HCC patients and controls were acquired and illustrated using the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/), a newly developed interactive web server for analyzing the RNA sequencing expression data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects.[17] A *P*-value < .05 and a $\log_2(FC) > 1.0$ were considered as significant.

The survival analyses of the hub genes in HCC patients were performed using the Kaplan-Meier plotter,[18] which is an online tool to draw survival plots that are based on gene chip and RNA-seq data from GEO and TCGA. The overall survival (OS) and progression-free survival (PFS) were evaluated. Patients were split into high and low expression groups according to the median gene level. A log-rank *P*-value < .05 was considered significant.

RESULTS

Identification of the Common DEGs for HDV-HCC

As shown in Figure 1A, the value data of the two comparisons were median centered, which indicated that the dataset is suitable for DEGs analysis using GEO2R. For the comparison between HDV-HCC and HDV-cirrhosis, a total of 635 upregulated and 968 downregulated DEGs

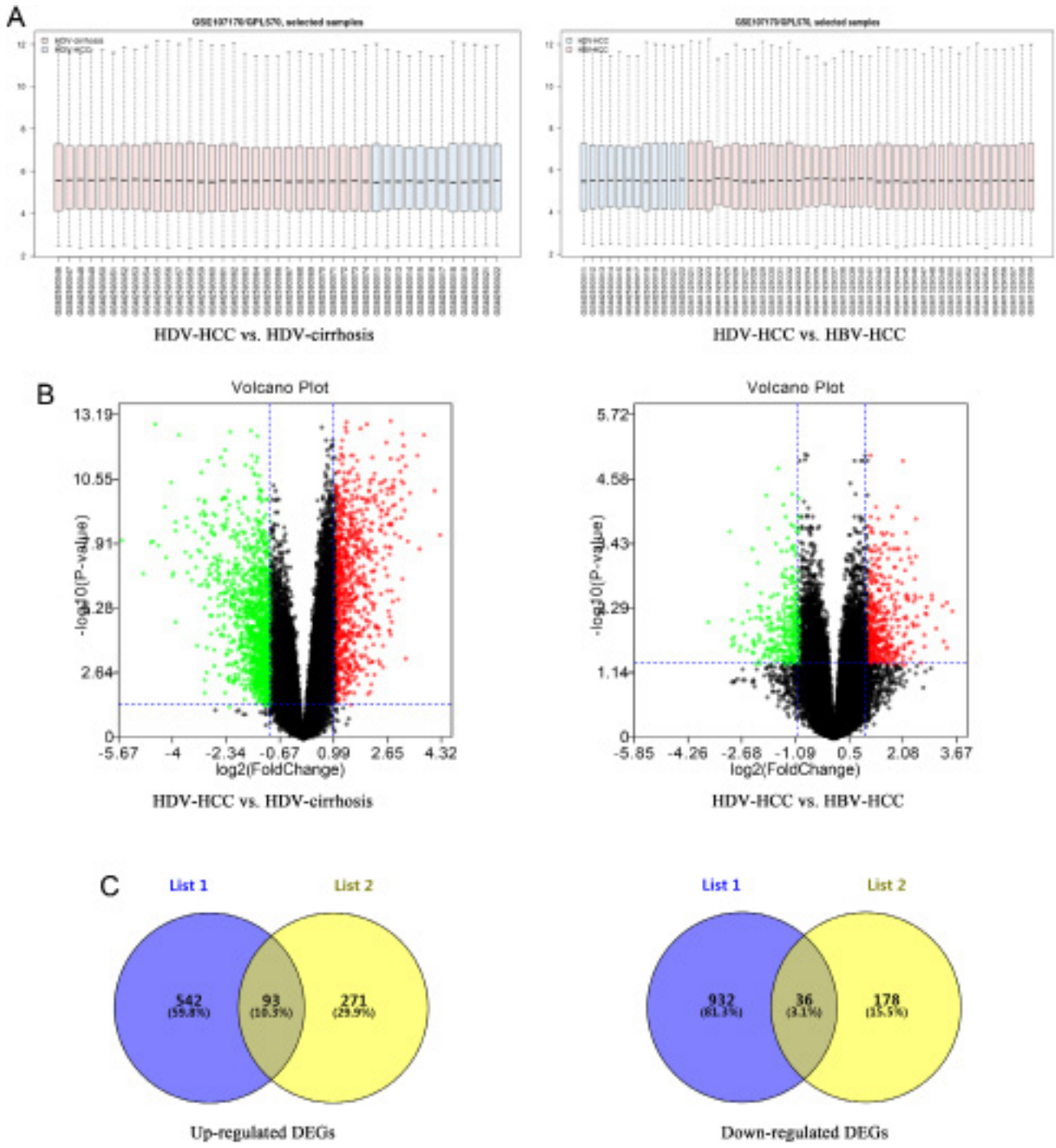

Figure 1. Identification of the DEGs for HDV-HCC. (A) The HDV-HCC versus HDV cirrhosis comparison (left) and the HDV-HCC versus HBC HCC comparison (right) were performed using the GSE107170 dataset. The distributions of value data for the two comparisons were presented as boxplots, which showed that the value data were median centered across the selected samples. (B) Volcano plot of gene data. Each dot represented a gene. The red dots represented upregulated DEGs and the green dots represented downregulated DEGs. (C) Venny diagrams showed the common DEGs across the two comparisons. Ninety-three common upregulated DEGs (left) and 36 common downregulated DEGs (right) were identified. List 1 represented the HDV-HCC versus HDV cirrhosis comparison and List 2 represented the HDV-HCC versus HBC HCC comparison. DEG, differentially expressed gene; HDV, hepatitis delta virus; HCC, hepatocellular carcinoma.

were found (Figure 1B). For the comparison between HBV-HCC and HDV-HCC, a total of 364 upregulated and 214 downregulated DEGs were found (Figure 1B). As shown in the Venny diagrams, 93 genes were commonly upregulated, while 36 genes were commonly downregulated (Figure 1C).

GO and KEGG Pathway Enrichment Analysis

To investigate the functions of the DEGs, GO analysis was performed. For upregulated genes, the BP part of GO analyses demonstrated that the DEGs were significantly enriched for muscle tissue development, organophosphate catabolic process, nucleotide catabolic process, and negative regulation of target of rapamycin (TOR) signaling. The CC part showed that the genes were mainly involved in the apical part of cell, sarcomere, contractile fiber, myofibril, and melanosome. The MF part showed that the genes were mainly enriched in protein heterodimerization activity, ubiquitin-protein ligase binding, GTPase activity, and core promoter sequence-specific DNA binding. For downregulated genes, the majority of the BP part was associated with regulation of lipid metabolic process, response to a steroid hormone, biological events about DNA transcription, and canonical Wnt signaling pathway. The CC part showed that the genes were predominantly enriched in the transcription factor complex, plasma membrane, and beta-catenin-T cell transcription factor (TCF) complex. The MF part showed that the genes were mainly associated with vitamin transmembrane transporter activity, nuclear receptor activity, steroid hormone receptor activity, and beta-catenin binding. For the top 10 enriched ontologies, see Figure 2A.

To further explore the mechanism of the DEGs, KEGG pathway analysis was also performed. The upregulated DEGs were mostly involved in endocytosis, phagosome, circadian rhythm, amino sugar and nucleotide sugar metabolism, and bile secretion. The downregulated DEGs were predominantly enriched for Cushing syndrome, focal adhesion, thyroid cancer, hedgehog signaling pathway, and endometrial cancer. For the top 10 KEGG pathways, see Figure 2B.

PPI Network Construction and Hub Genes Identification

The PPI networks of both upregulated and downregulated DEGs, as well as predicted functional partner molecules, were established. Regarding the upregulated DEGs, 93 nodes and 60 edges were displayed on the network, with an average local clustering coefficient of 0.335 and an enrichment *P*-value of .001. The average node degree was 1.29. According to the size of the nodes, transferrin receptor and actin-related proteins play a central role. In addition, three highly interconnected regions were identified, which are shown as clusters of purple, red, and yellow nodes (Figure 3 left panel). Regarding the down-regulated DEGs, the network included 46 nodes and 44 edges, with a coefficient of 0.335 and a *P*-value of .003. The average node degree was 1.91. Catenin-beta and proteins for cell cycle regulation play a central role. Only 1 cluster was illustrated in the PPI network (Figure 3, right panel).

To further select the hub genes that encoded the most interactive proteins, further calculation was performed based on degree algorithm. Based on the score, the sequence of the top 5 upregulated genes were *transferrin receptor* (*TFRC*), *actin-related protein 2* (*ACTR2*), *actin-related protein 2/3 complex subunit 1A* (*ARPC1A*), *actin-related protein 2/3 complex subunit 3* (*ARPC3*), and *actin-related protein 2/3 complex subunit 2* (*ARPC2*). The sequence of the top 5 downregulated genes were *catenin beta 1* (*CTNNB1*), *cyclin D1* (*CCND1*), *cyclin-dependent kinase inhibitor 1B* (*CDKN1B*), *cyclin-dependent kinase 4* (*CDK4*), and *cyclin-dependent kinase inhibitor 1A* (*CDKN1A*).

Validation of the Hub Genes

To valid the hub genes mentioned above, we first used GEPIA to compare the expression of these genes between HCC patients and controls. As shown in Figure 4, the expressions of *ARPC1A*, *ARPC3,* and *CDK4* were significantly upregulated in cancer samples. The expressions of the other common DEGs were not substantially regulated in overall HCC patients compared with non-tumorous controls.

To further study the prognostic value of the hub genes, survival information was also investigated. For the upregulated hub genes, only *ARPC2* was associated with a higher risk of overall death. None of the upregulated hub genes predicted PFS. For the downregulated hub genes, *CDKN1A* predicted both better OS and PFS. In addition, *CCND1* and *CDKN1B* predicted better PFS. Interestingly, *CDK4* was associated with both shorter OS and PFS. For details, see Table 1.

DISCUSSION

HDV infection is associated with a severe course of hepatitis and is the only chronic hepatitis virus infection without a US Food and Drug Administration

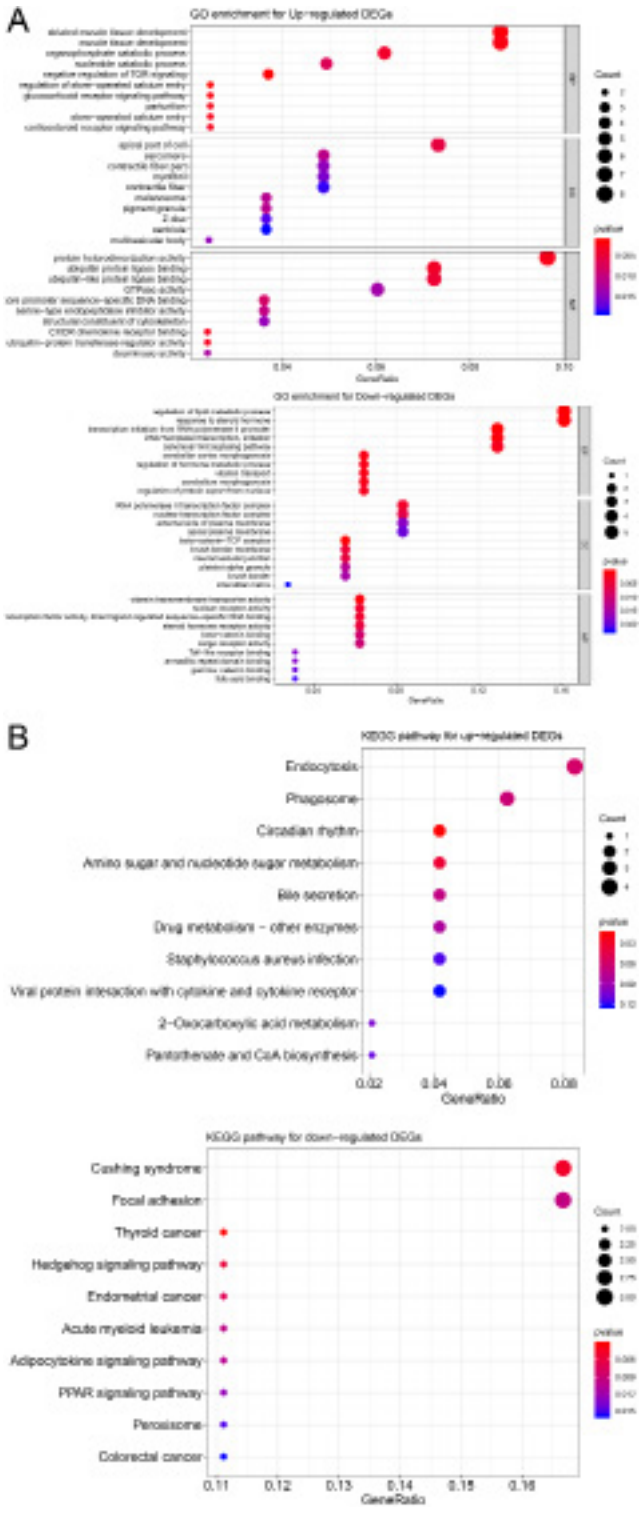

Figure 2. GO functional analysis and KEGG pathway enrichment analysis. (A) The GO enrichment analyses for the common up- and downregulated DEGs for HDV-HCC. (B) The KEGG pathway analyses for the common up- and downregulated DEGs for HDV-HCC. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; HDV, hepatitis delta virus; HCC, hepatocellular carcinoma.

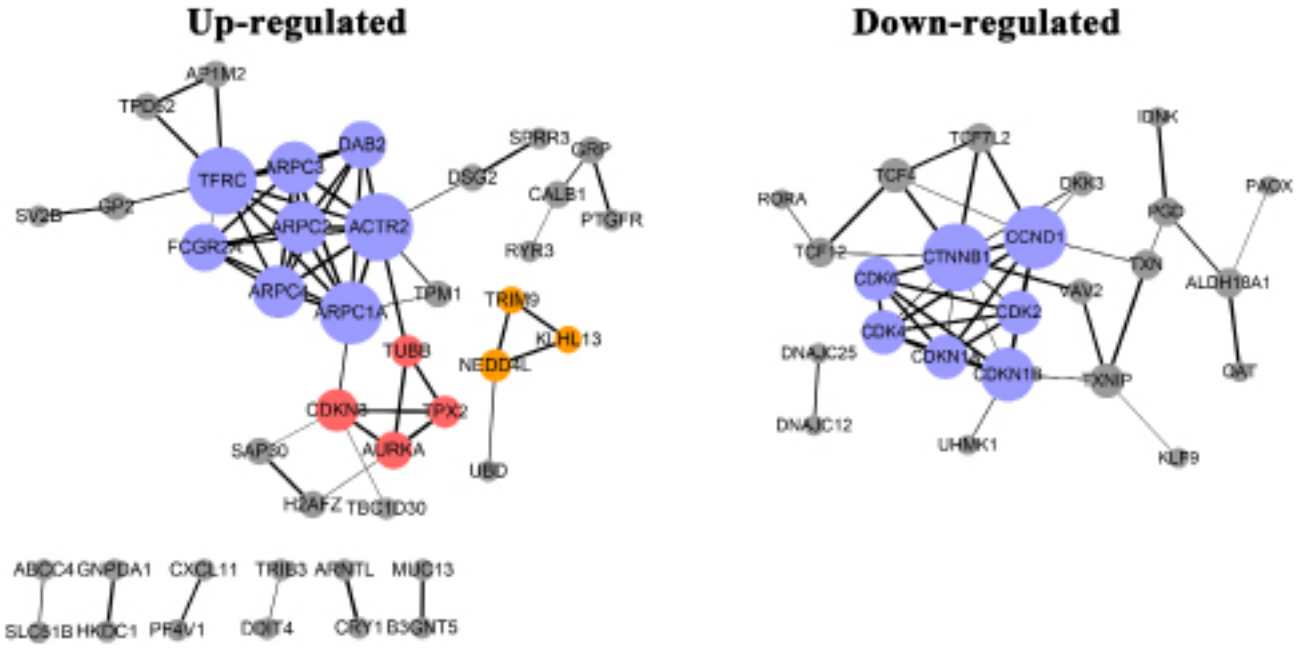

Figure 3. The PPI networks of the up- and downregulated DEGs. Each node represented a protein. The nodes with the same color (except for the grey nodes) were in the same cluster calculated by the MCODE plugin of Cytoscape software. The size of the nodes indicated the centrality of the protein calculated by CytoNCA plugin of Cytoscape software. The size of the edge indicated the combined score calculated by STRING. PPI, protein-protein interaction; DEG, differentially expressed gene; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins.

(FDA) approved therapy. Only interferon α (IFN-α) has proven antiviral activity against HDV, and treatment with pegylated-IFN-α leads to HDV clearance in about 25% of patients.[19] The risk of HCC is increased twofold with the presence of HDV.[20] Four main points are commonly proposed to contribute to hepatitis virus-related HCC formation, which are oxidative stress, accumulation of genomics alterations/instability, activation of regeneration pathways, hepatocyte turnover and subsequent clonal expansion, and formation of fibrotic/cirrhotic microenvironment.[7] However, the current evidence on HDV-HCC mechanism is limited, and the molecular events of HDV-HCC are proposed to be distinct from the carcinogenesis induced by other hepatitis virus.[8] The present study analyzed the dataset GSE107170 which enrolled HDV-HCC patients as a case group and HCC-cirrhosis patients plus HBV-HCC patients as two control groups. Ninety-three upregulated genes and 36 downregulated genes were both found in the HDV-HCC versus HCC-cirrhosis comparison and the HDV-HCC versus HBV-HCC comparison.

The PPI network illustrated that transferrin receptor, actin-related proteins, catenin-beta, and proteins for cell cycle regulations were of importance. We further identified hub genes based on degree algorithm. Five upregulated hub genes (*TFRC*, *ACTR2*, *ARPC1A*, *ARPC3*, and *ARPC2*) were obtained. The gene *TFRC* encodes transferrin receptor 1, a membrane glycoprotein that imports iron by binding transferrin. The mechanism by which transferrin receptor 1 affects cancer has been widely studied and reviewed elsewhere.[21] Briefly, this protein is commonly upregulated and induces iron overload in cancer cells, thus affecting the network of oncogenes expression.[21] Recently, it has been consistently demonstrated that *TFRC* is related to disease status and prognosis in patients with HCC.[22,23] Here we also identified *TFRC* as a hub gene for HDV-HCC; however, it was not substantially regulated in general HCC nor correlated with HCC prognosis in validation analysis. The proteins encoded by the other 4 hub genes, *ACTR2*, *ARPC1A*, *ARPC3*, and *ARPC2*, are the unique components of the human actin-related protein 2/3 complex (Arp2/3), a key nucleator for a branched actin network.[24] This protein complex may be a crucial regulator of cell migration and invasion in cancer.[24,25] In the present study, the validation analysis based on public databases demonstrated that the expressions of *ARPC1A* and *ARPC3* in general HCC were promoted significantly, and *ARPC2* promotion was associated with poor OS in general HCC.

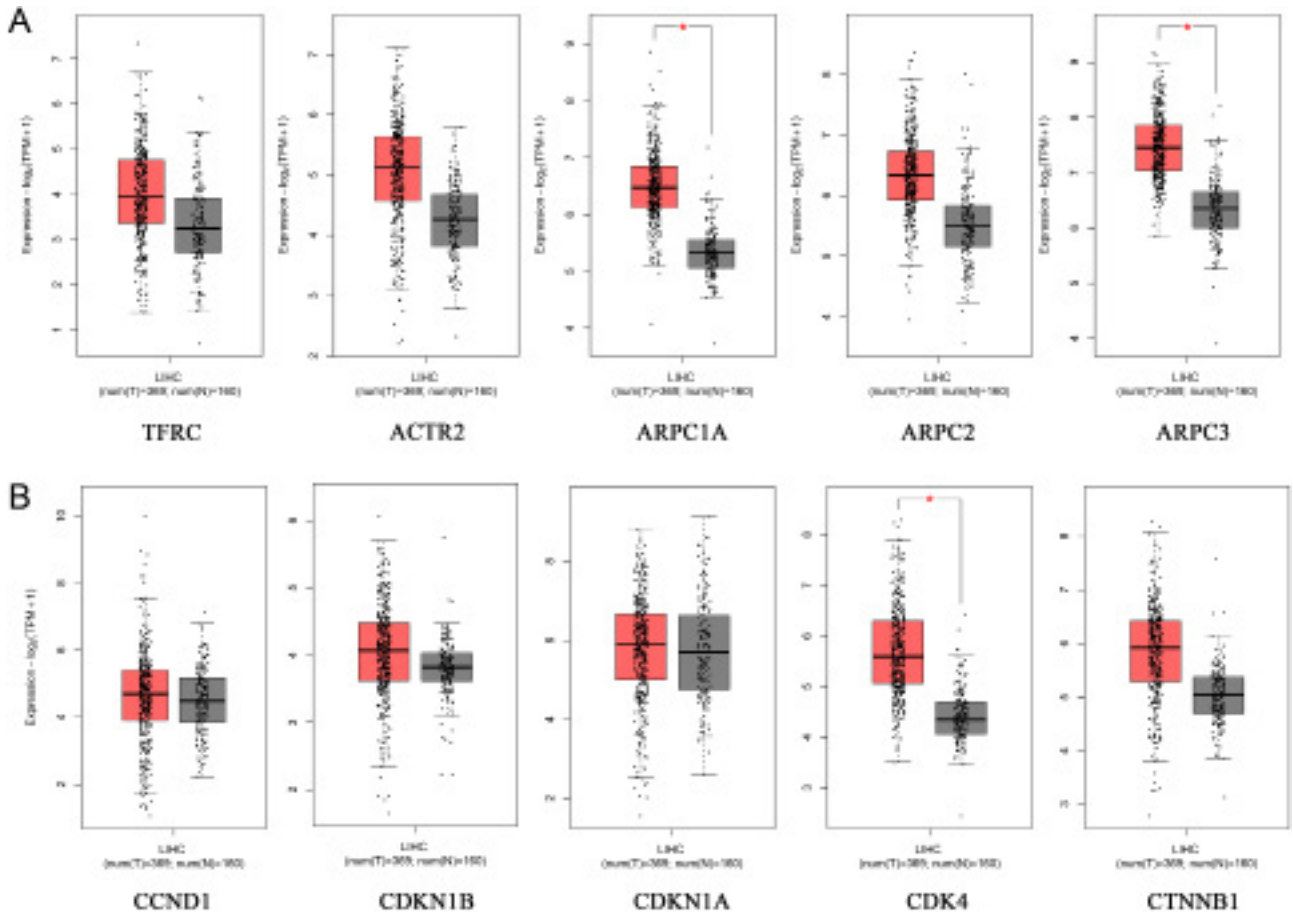

Figure 4. The expression levels of hub genes for HDV-HCC based on the TCGA/GTEx database. The pink box indicated the HCC group and the grey box indicated control group. Each dot represented a sample. The star symbol indicated statistical significance. (A) The boxplots of expressions of the top 5 upregulated genes. (B) The boxplots of expressions of the top 5 downregulated genes. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GTEx, The Genotype-Tissue Expression.

Besides, 5 downregulated genes (*CTNNB1*, *CCND1*, *CDKN1B*, *CDK4*, and *CDKN1A*) were also obtained. The gene *CTNNB1* encodes catenin beta 1, a protein that mediates the canonical Wnt signaling pathway and contributes to the malignant transformation and expansion of liver cells.[26] Of note, aberrant activation of Wnt signaling pathway exists in hepatic precancerous lesions that is commonly induced by virus infection.[26] Here we identified *CTNNB1* as a hub gene for HDV-HCC. The other 4 hub genes were crucial components of the process of cell cycle regulation. It is clear that defects in cell cycle regulation can result in cancerous growth and developmental abnormalities.[27] Cyclin D1 (encoded by *CCND1*) stimulates G0 cells to enter the cell cycle.[27] CDK4 is a cell-cycle-related member of the cyclin-dependent kinase (CDK) family, which can be negatively regulated by inhibitor p21 and p27 (encoded by *CDKN1A* and *CDKN1B*, respectively).[28] The genes from the CDK family[29-31] and CDKN family[29] were frequently identified as hub genes in recent bioinformatics studies on HCC. In this study, *CCND1*, *CDKN1B*, and *CDKN1A* predicted better survival in patients with overall HCC. Interestingly, higher expression of *CDK4* was observed in overall HCC specimens and was associated with a considerable risk of poor OS (HR = 2.15) and PFS (HR = 1.96) according to public databases. However, this gene was inhibited in the HDV-HCC patients from the present study.

A critical question is whether HDV-HCC is induced by HDV per se or is a complication of HDV-induced liver cirrhosis. If HDV-HCC is complication of hepatitis virus-induced liver cirrhosis, the molecular pattern of it would be similar to HCC induced by HBV-associated liver cirrhosis. The present study presumed that the molecular

Table 1. The Survival Analysis of HCC Based on the Hub Genes

	Overall Survival (*N* = 364)		Progression-Free Survival (*N* = 366)	
Gene	HR (95% CI)	*P*	HR (95% CI)	*P*
Upregulated hub genes				
TFRC	1.36 (0.96-1.92)	.08	1.15 (0.86-1.54)	.35
ACTR2	1.02 (0.53-1.95)	.95	1.06 (0.79-1.42)	.71
ARPC1A	1.00 (0.71-1.41)	1.00	0.95 (0.71-1.28)	.75
ARPC3	1.25 (0.84-1.86)	.27	1.22 (0.88-1.70)	.22
ARPC2	1.55 (1.09-2.20)	.01	0.92 (0.68-1.23)	.56
Downregulated hub genes				
CTNNB1	1.17 (0.83-1.65)	.37	0.72 (0.52-1.00)	.05
CCND1	0.78 (0.55-1.10)	.16	0.61 (0.45-0.82)	<.01
CDKN1B	0.71 (0.51-1.00)	.05	0.69 (0.51-0.92)	.01
CDK4	2.15 (1.49-3.10)	<.01	1.96 (1.43-2.68)	<.01
CDKN1A	0.62 (0.43-0.89)	<.01	0.67 (0.49-0.92)	.01

HCC, hepatocellular carcinoma; HR, hazard ratio; TFRC, transferrin receptor; ACTR2, actin-related protein 2; ARPC1A, actin-related protein 2/3 complex subunit 1A; ARPC3, actin-related protein 2/3 complex subunit 3; ARPC2, actin-related protein 2/3 complex subunit 2; CTNNB1, catenin beta 1; CCND1, cyclin D1; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDK4, cyclin-dependent kinase 4; CDKN1A, cyclin-dependent kinase inhibitor 1A.

events of HDV-HCC pathogenesis are, to some extent, distinct from the profile of HBV-HCC. First, the key genes were differentially expressed in HDV-HCC cases compared with HDV-cirrhosis and HBV-HCC cases (adjusted *P*-value < .05 and $|\log_2(FC)| > 1.0$). In addition, the expression and prognostic indication of the hub genes of HDV-HCC were different from the general HCC based on public databases, and these hub genes were biological oncogenes based on the aforementioned evidence. Compared with other studies that investigated the key genes of HBV-HCC,[32-37] the majority of the key DEGs of HBV-HCC is different from hub genes in the present study. The overlapping part is all about molecules for cell cycle regulation, such as the CDKN family and CDK family. Taken together, it is indicated that the molecular carcinogenesis of HCC in HDV cases is at least partly different from that in HBV cases. Therefore the hypothesis of partial contribution of HDV per se on HCC is indirectly supported, and more direct and rigorous evidence is needed.

The main limitation of the current study is lack of experimental verification. This study explored the underlying mechanism in silico. To confirm these findings, more studies in vitro and in vivo that focus on the molecular event and biological process are required. The number of patients is also limited; however, we believe this pilot study sheds new light on the molecular mechanism of HDV-HCC. Obtaining liver samples from HDV-HCC patients is difficult, and so this dataset took advantage of the collection of liver specimens from patients who underwent surgery. To ensure comparability, none of the features were statistically different between the groups. Besides, multiple biopsies from an individual were taken to enlarge the sample size. Another limitation in this study is based on a single dataset, and more public datasets are needed for an integrated bioinformatics analysis.

CONCLUSION

129 common DEGs are identified for HDV-HCC compared with HDV cirrhosis and HBV-HCC. The molecular mechanism of HDV-HCC is likely to be different from the general HCC. A set of key genes may be involved in the carcinogenesis of HDV-HCC and used as prognostic factors.

Ethics Committee Approval: N/A.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: The authors have no conflict of interest to declare.

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