

Screening of potential biomarkers in hepatitis C virus-induced hepatocellular carcinoma using bioinformatic analysis

JUN LIU¹, ZHANZHONG MA¹, YANMING LIU¹, LIANGYIN WU¹, ZHIWEI HOU² and WENLI LI²

¹Department of Laboratory Medicine and ²Reproductive Medicine Center, Yue Bei People's Hospital, Shaoguan, Guangdong 512026, P.R. China

Received December 6, 2018; Accepted June 6, 2019

DOI: 10.3892/ol.2019.10578

Abstract. Evidence suggests that hepatitis C virus (HCV) infection is among the main causes of hepatocellular carcinoma (HCC). In addition, HCV-induced HCC (HCV-HCC) exhibits adverse clinical outcomes and limited therapeutic treatments are available for this condition. To investigate key biomarkers in the occurrence and development of HCV-HCC, microarray datasets GSE62232, GSE69715 and GSE107170 were downloaded from the Gene Expression Omnibus database for analysis. The differentially expressed genes between HCV-HCC and normal tissue were identified using the GEO2R online tool. The function enrichment analyses including Gene Ontology and Kyoto Encyclopedia of Genes and Genomes were performed using the Database for Annotation, Visualization and Integrated Discovery online tool. A protein-protein interaction network was constructed using the Search Tool for the Retrieval of Interacting Genes database and visualized using Cytoscape. A total of 368 DEGs were identified, and the top 10 hub genes with a high degree of connectivity were selected for further analysis. Subsequently, overall survival and disease-free survival analysis revealed that there was a significant association between altered expression of HMMR, CCNB1 and KIF20A, and poor clinical outcome. In summary, these results indicate that HMMR, CCNB1 and KIF20A are potential targets for diagnosis and therapy of HCV-HCC.

Introduction

Hepatocellular carcinoma (HCC) is a high-incidence malignant tumor worldwide; its incidence rate is ~5.7% of all new cancer cases, and it has become the third leading cause of cancer-associated deaths (1). The chronic persistent infection

of hepatitis C virus (HCV) is a major etiological element of HCC, accounting for 25% of HCC cases (2). Numerous studies have focused on the aberrant expression and alteration of genes, a number of which are involved in the development of HCC. It has been found that DNA topoisomerase 2 α (TOP2A) may act as a biomarker and is a drug target of nitidine chloride in HCC (3). The increase of vascular endothelial growth factor plays a key role in the recurrence of HCC (4). Testicular nuclear receptor 4 may affect the capability of a patient to suppress HCC metastasis (5). In addition, the mRNA overexpression of the cognate receptors c-Met and epidermal growth factor receptor were found to be involved in tumor recurrence (6). However, the high mortality rate is due in part to the lack of early detection of HCC markers (7). Thus, it is vital to identify effective early diagnostic methods and to understand the molecular mechanism of HCC development, in order to intervene in the progression of the disease at the early stages.

Currently, large datasets, which include genomic sequencing, DNA copy number arrays and protein arrays, are uploaded to public databases, for example Gene Expression Omnibus (GEO) and Oncomine. Furthermore, these databases are available for screening differentially expressed genes (DEGs) and analyzing the molecular mechanism of carcinogenesis in HCC. Nevertheless, it is difficult to trust the results from separate microarray analysis. Therefore, in the present study, three mRNA microarray datasets of HCV-induced HCC (HCV-HCC) and non-tumor tissues were analyzed to identify DEGs, using the GEO2R web tool. Subsequently, the DEGs were analyzed using the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to identify the molecular mechanisms, biological processes and pathways involved, and a protein-protein interaction (PPI) network was constructed. Finally, in total, 368 DEGs and 10 hub genes were identified, providing several possibilities to investigate as potential biomarkers for the diagnosis of HCV-HCC and for novel drug development.

Materials and methods

Microarray data. GEO (<http://www.ncbi.nlm.nih.gov/geo>) is a high-throughput gene expression database. In the present study, three gene expression datasets (GSE62232 (8) from France, GSE69715 (9) from Italy and GSE107170 (10) from Italy) were downloaded from GEO. GSE62232 comprised nine

Correspondence to: Ms Wenli Li, Reproductive Medicine Center, Yue Bei People's Hospital, 133 Huimin South Road, Shaoguan, Guangdong 512026, P.R. China
E-mail: wangyiliwenli@163.com

Key words: hepatocellular carcinoma, hepatitis C virus, bioinformatics analysis, differentially expressed gene, survival analysis

HCV-HCC samples and 10 adjacent non-tumor tissue samples, GSE69715 comprised 37 HCV-HCC and 66 normal samples, and GSE107170 included 44 HCV-HCC tissue and normal tissue (Table I). All of these datasets were obtained from analyses performed using the GPL570 Affymetrix Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific, Inc.).

Identification of DEGs. GEO2R (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) is an online analysis tool that compares two or more groups of samples to identify DEGs. The DEGs of the three gene expression datasets including HCV-HCC and non-cancer samples were analyzed, and the \log_2 fold-change (FC) and adjusted P-values were calculated. Genes that fulfilled the criteria of $\log_2FC \geq 1.0$ and adjusted $P < 0.05$ were considered statistically significant and termed DEGs. The Venn diagram web tool (bioinformatics.psb.ugent.be/webtools/Venn/) was used to represent the overlapping DEGs from each dataset.

GO and KEGG pathway analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (version 6.7; <https://david.ncifcrf.gov/>) is an online biological information database that integrates biological data and analysis tools. GO was used to perform bioinformatic analysis and annotate function enrichment of genes. KEGG is a database containing large-scale molecular datasets generated from genome sequencing, used to explore the high-level functions and utilities of the biological system. DAVID was applied to perform the function enrichment and biological analyses of these DEGs. $P < 0.01$ and gene counts ≥ 10 were considered statistically significant.

PPI network of DEGs. The Search Tool for the Retrieval of Interacting Genes (STRING) database (version 10.0; <http://string-db.org/>) was used to construct the PPI network. Assessing the potential protein interactions enables the identification of possible mechanisms in the development of diseases. The PPI pairs with a combined score > 0.4 were considered statistically significant. Cytoscape software (version 3.4.0; www.cytoscape.org/) was used to visualize the PPI network. CytoHubba is an application of Cytoscape, and was used for calculating the degree of each protein node.

Hub gene identification and analysis. In the present study, the top 10 genes with a high degree of connectivity from the PPI network were identified as hub genes. Hierarchical clustering of hub genes was constructed using University of California Santa Cruz Cancer genomics browser (version hg19; <http://genome-cancer.ucsc.edu>). The overall survival and disease-free survival time analyses with the hub genes were performed by constructing Kaplan-Meier curves in cBioPortal (version 2.2.1; <http://www.cbioportal.org>). The datasets of Wurmbach Liver (11) and Mas Liver (12) were obtained from the online database OncoPrint (<http://www.oncoprint.org>) and used to analyze the relationship between expression patterns and HCV status. Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/detail.php>) is an online tool for analyzing gene expression data from cancer and normal tissues from The Cancer Genome Atlas and genotypic-tissue expression projects. GEPIA and The Human

Table I. Numbers of samples included in three Gene Expression Omnibus microarray datasets analyzed in the present study.

Dataset ID	HCV-HCC, n	Normal, n	Total, n
GSE62232	9	10	19
GSE69715	37	66	103
GSE107170	44	31	75

HCV-HCC, hepatitis C virus-induced hepatocellular carcinoma.

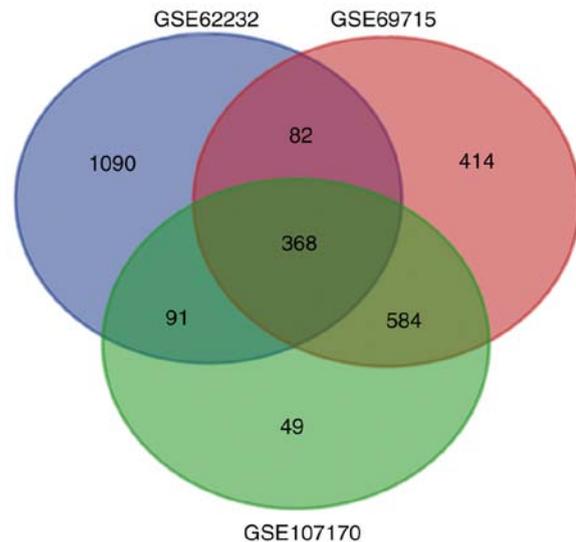


Figure 1. Venn diagram of the differentially expressed genes identified from the Gene Expression Omnibus mRNA expression datasets GSE62232, GSE69715 and GSE107170.

Protein Atlas (<https://www.proteinatlas.org>), which originated in Sweden and is designed to map all the human proteins in tissues, cells and organs, were used to verify the expression of hyaluronan mediated motility receptor (HMMR), cyclin B1 (CCNB1) and kinesin family member 20A (KIF20A) in HCV-HCC (13,14).

Results

Identification of DEGs in HCV-HCC. In the present study, three mRNA microarray datasets (GSE62232, GSE69715 and GSE107170) were selected, and DEGs (1,631 in GSE62232, 1,448 in GSE69715 and 1,092 in GSE107170) were identified. The overlap among the three gene expression profiles included 368 DEGs, displayed using a Venn diagram (Fig. 1), including 264 downregulated genes and 104 upregulated genes.

GO and KEGG analyses of DEGs. Functional enrichment analyses of DEGs, including GO and KEGG pathways, were performed using DAVID (Table II). The resulting GO terms were enriched in biological processes, including 'organic acid catabolic process', 'carboxylic acid catabolic process', 'secondary metabolic process', 'cellular amino acid catabolic process', 'amine catabolic process', 'cellular amino acid derivative metabolic process', and 'response to steroid hormone

Table II. GO and KEGG pathway functional enrichment analysis of the differentially expressed genes.

Category	Term	Description	Count	FDR
GOTERM_BP_FAT	GO:0016054	Organic acid catabolic process	16	4.38x10 ⁻⁰⁶
GOTERM_BP_FAT	GO:0046395	Carboxylic acid catabolic process	16	4.38x10 ⁻⁰⁶
GOTERM_BP_FAT	GO:0019748	Secondary metabolic process	12	4.47x10 ⁻⁰⁴
GOTERM_BP_FAT	GO:0009063	Cellular amino acid catabolic process	11	9.39x10 ⁻⁰⁴
GOTERM_BP_FAT	GO:0009310	Amine catabolic process	11	3.44x10 ⁻⁰³
GOTERM_BP_FAT	GO:0006575	Cellular amino acid derivative metabolic process	15	5.54x10 ⁻⁰³
GOTERM_BP_FAT	GO:0048545	Response to steroid hormone stimulus	16	6.39x10 ⁻⁰³
GOTERM_CC_FAT	GO:0005576	Extracellular region	73	1.08x10 ⁻⁰⁵
GOTERM_CC_FAT	GO:0044421	Extracellular region part	43	1.74x10 ⁻⁰⁴
GOTERM_CC_FAT	GO:0005615	Extracellular space	33	1.65x10 ⁻⁰³
GOTERM_MF_FAT	GO:0030246	Carbohydrate binding	25	6.44x10 ⁻⁰⁵
KEGG_PATHWAY	hsa01100	Metabolic pathways	53	3.08x10 ⁻⁰³

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

Table III. Top 10 hub genes based on degree of connectivity.

Gene symbol	Gene name	Degree of connectivity
TOP2A	DNA topoisomerase 2 α	46
CDK1	Cyclin dependent kinase 1	34
CCNB1	Cyclin B1	31
CDKN3	Cyclin-dependent kinase inhibitor 3	28
NDC80	Kinetochores complex component	27
CCNB2	Cyclin B2	27
NEK2	NIMA-related kinase 2	25
PBK	PDZ-binding kinase	25
KIF20A	Kinesin family member 20A	24
HMMR	Hyaluronan-mediated motility receptor	24

stimulus'. Molecular function analysis results indicated that the DEGs were enriched in 'carbohydrate binding'. For the results of cell component analysis, the DEGs were mainly enriched in 'extracellular region', 'extracellular region part', and 'extracellular space'. Moreover, KEGG pathway analysis revealed that the DEGs were primarily enriched in 'metabolic pathways'.

PPI network construction and hub gene selection. The PPI network of the DEGs was constructed using STRING (Fig. 2A) and the top ten genes based on connectivity degree were identified using Cytoscape (Fig. 2B). The 10 genes with the highest degree of connectivity were identified as hub genes in the PPI network (Table III) 4.

Analysis of hub genes. The hierarchical clustering of hub genes inferred that there is a difference in gene expression between

HCV-HCC samples and normal samples (Fig. 3). Subsequently, in order to identify and understand the prognostic values of the hub genes, overall and disease-free survival time analyses were performed using the Kaplan-Meier method. The results of the overall survival analysis are displayed in Fig. 4. A significant association was observed between poor prognosis and overexpression of genes CDK1, HMMR, NDC80, CCNB1, KIF20A, NEK2 and PBK, in patients with HCV-HCC. Furthermore, as shown in Fig. 5, patients with HCC and overexpression of HMMR, CCNB1 and KIF20A presented with lower disease-free survival rates. Analysis of the Wurmbach Liver and Mas Liver datasets from the Oncomine database showed that higher mRNA levels of CCNB1, KIF20A and HMMR were significantly associated with HCV-HCC (Fig. 6). GEPIA analysis of tumor vs. normal tissue demonstrated that CCNB1, KIF20A and HMMR were significantly overexpressed in HCC (Fig. 7A-C). In addition, the analysis from The Human Protein Atlas indicated that the expression of CCNB1, KIF20A and HMMR is enhanced in HCC tissue (Fig. 7D-I) (14).

Discussion

Persistent HCV infection has been considered to significantly increase the risk of developing HCC (15). However, following a review of the literature, few studies have attempted to describe the molecular mechanism of HCV-HCC (16-18). Due to an absence of appropriate diagnostic markers and targeted treatments, early diagnosis is challenging, and high mortality rates are observed amongst patients with this condition (7). Consequently, it is vital to explore definitive targets for HCV-HCC.

In the present study, 10 hub genes were identified among 368 DEGs in HCV-HCC. Most notably, overexpression of CCNB1, KIF20A and HMMR were associated with poorer prognosis. CCNB1 is an important modulator of cell cycle progression (19), and it has been reported that its overexpression contributes to the regulation of invasive

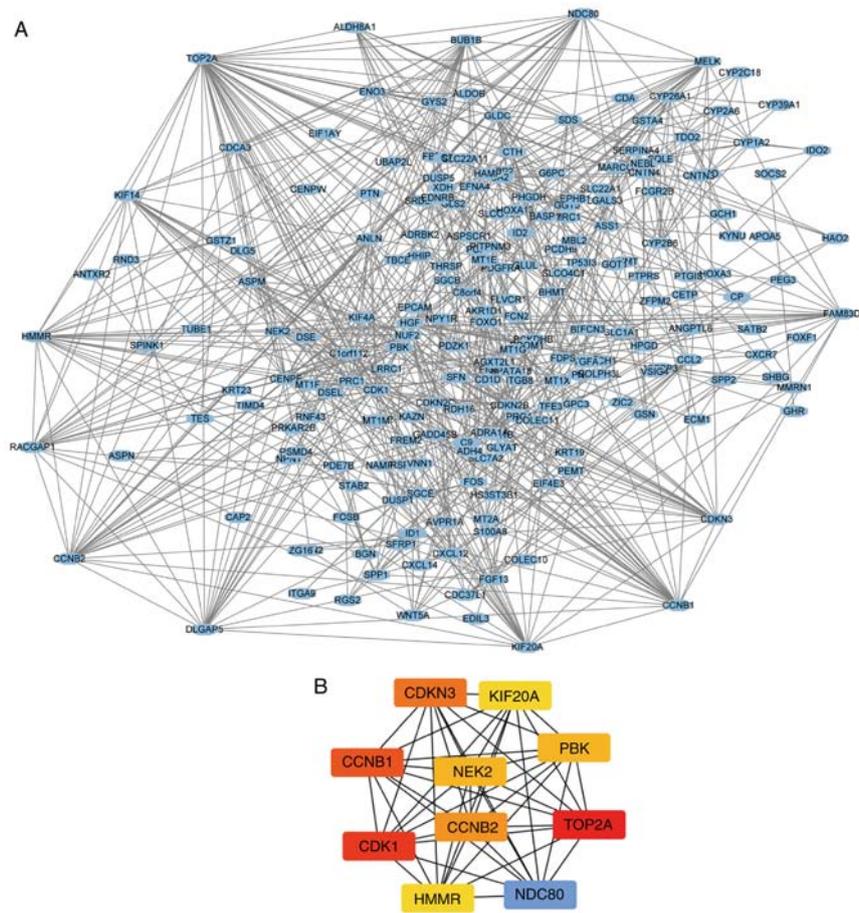


Figure 2. PPI network and the most significant modules from the differentially expressed genes. (A) A PPI network was constructed using the STRING database. (B) The top 10 hub genes from the PPI network with the highest degree of connectivity. PPI, protein-protein interaction.

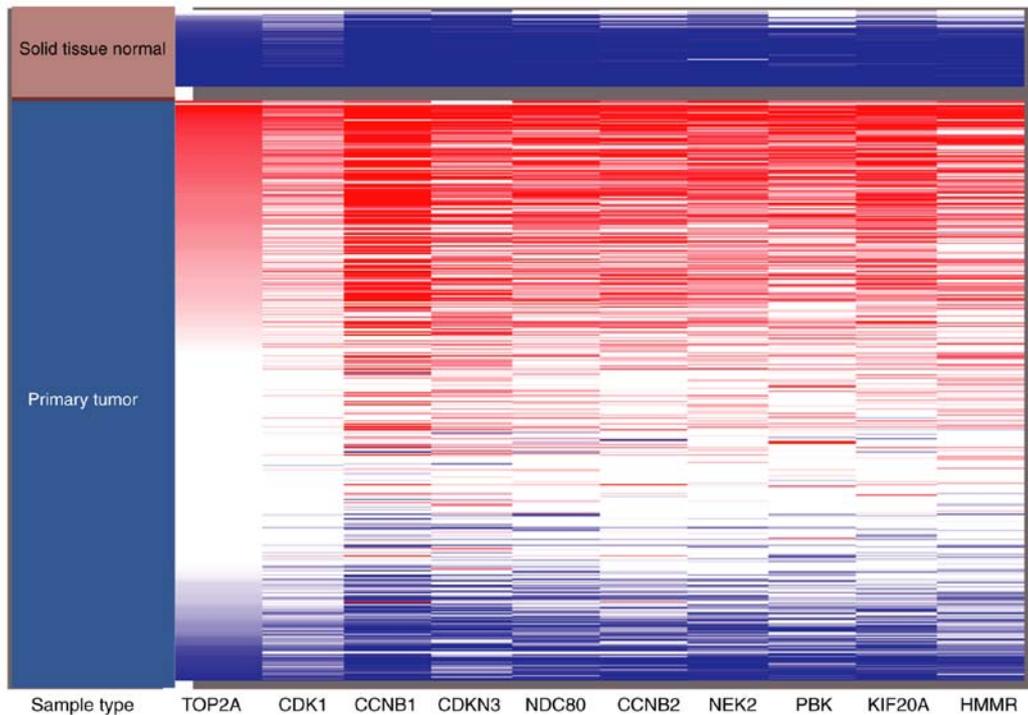


Figure 3. Hierarchical clustering of the hub genes was constructed using University of California Santa Cruz genomics browser. Upregulation of genes is marked in red, whereas downregulation of genes is marked in blue. TOP2A, DNA topoisomerase 2 α ; CDK1, cyclin dependent kinase 1; CCNB1, cyclin B1; CDKN3, cyclin dependent kinase inhibitor 3; NDC80, kinetochore complex component; CCNB2, cyclin B2; NEK2, NIMA related kinase 2; PBK, PDZ binding kinase; KIF20A, kinesin family member 20A; HMMR, hyaluronan mediated motility receptor.

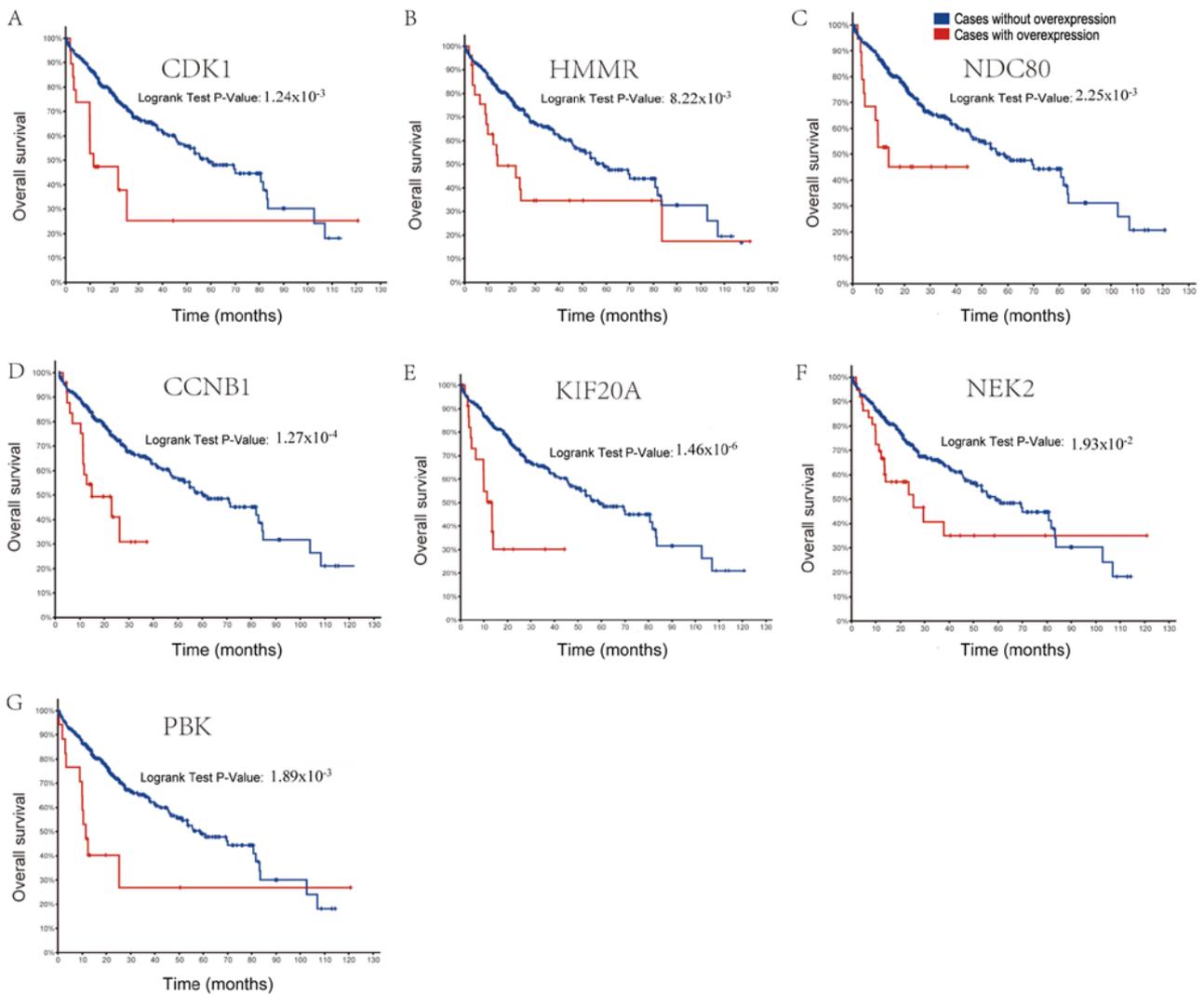


Figure 4. Overall survival time analyses of the hub genes identified in this study. Overall survival time analyses of the hub genes were carried out using cBioPortal, and are presented in Kaplan-Meier plots. (A) CDK1. (B) HMMR. (C) NDC80. (D) CCNB1. (E) KIF20A. (F) NEK2. (G) PBK. CDK1, cyclin dependent kinase 1; HMMR, hyaluronan mediated motility receptor; NDC80, kinetochore complex component; CCNB1, cyclin B1; KIF20A, kinesin family member 20A; NEK2, NIMA related kinase 2; PBK, PDZ binding kinase.

growth, metastasis and apoptosis of tumor cells (20,21). Overexpression of CCNB1 has also been demonstrated in a number of types of tumors, for example breast (22), colorectal (23) and laryngeal cancer (24). Regarding HCC, numerous studies have indicated that overexpressed CCNB1, an independent prognostic factor, is associated with shorter recurrence-free survival time and is a potential therapeutic target (25,26). In the present study, CCNB1 was found to be significantly overexpressed in HCV-HCC, and this was associated with a lower disease-free survival rate. Analogous results were revealed for KIF20A, a member of the kinesin family (27). Previous studies have characterized KIF20A as a prognostic indicator for a number of cancer types, including ovarian carcinoma, pancreatic cancer and nasopharyngeal cancer (28-30). Some of these studies suggested that overexpression of KIF20A could promote tumor cell proliferation and invasion. Shi *et al* (31) showed that KIF20A is important for proliferation of HCC cells. In the present study, overexpression of KIF20A was significantly associated with poor prognosis, and it was identified as a hub

gene in HCV-HCC. HMMR is a multifunctional oncogenic protein (32) and many studies have reported that it is overexpressed in human leukemia types and breast cancer (33-35). Tilghman *et al* (36) considered that HMMR is a candidate therapeutic target in glioblastoma, and Wang *et al* (37) suggested that overexpression of HMMR led to a poor clinical outcome. However, few studies have reported on the role of HMMR in HCC (38), whereas the present study suggests that the gene is overexpressed in patients with HCV-HCC, and that this overexpression is significantly associated with the overall and disease-free survival. Therefore, HMMR is a potential novel candidate biomarker for HCV-HCC.

According to epidemiological studies, nearly 170 million people worldwide are carriers of HCV, and have a 17-fold increased risk of developing HCC (39). HCV infection is the main cause of HCC in Latin America, Europe and Japan (40). Therefore, performing survival analyses and exploring the prognostic value of the 10 hub genes identified from the present study in cohorts with various ethnic backgrounds is important in future studies. The results of the

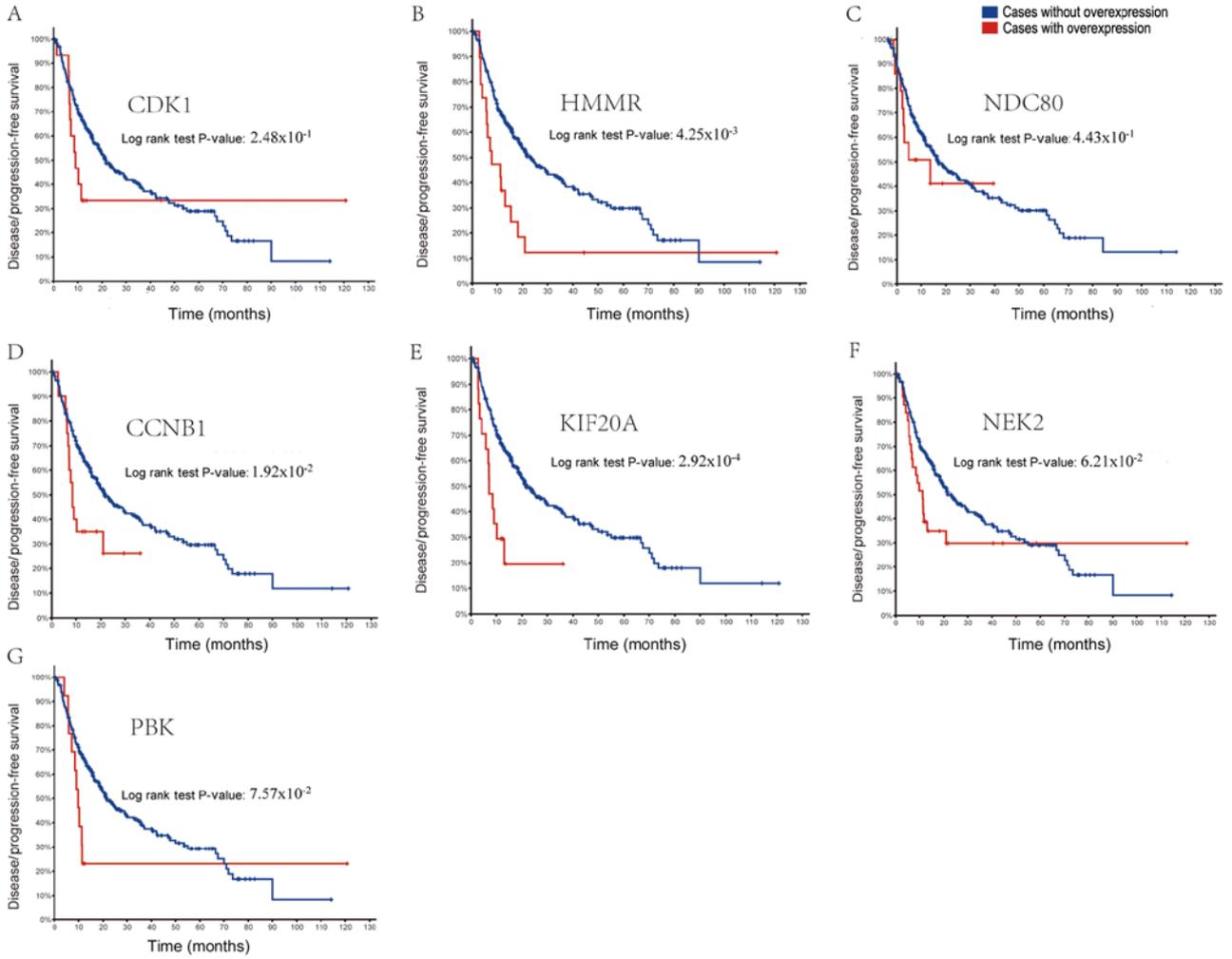


Figure 5. Disease-free survival time analyses of the hub genes identified in this study. Disease-free survival time analyses of the hub genes were carried out using cBioPortal, and are presented in Kaplan-Meier plots (A) CDK1. (B) HMMR. (C) NDC80. (D) CCNB1. (E) KIF20A. (F) NEK2. (G) PBK. CDK1, cyclin dependent kinase 1; HMMR, hyaluronan mediated motility receptor; NDC80, kinetochores complex component; CCNB1, cyclin B1; KIF20A, kinesin family member 20A; NEK2, NIMA related kinase 2; PBK, PDZ binding kinase.

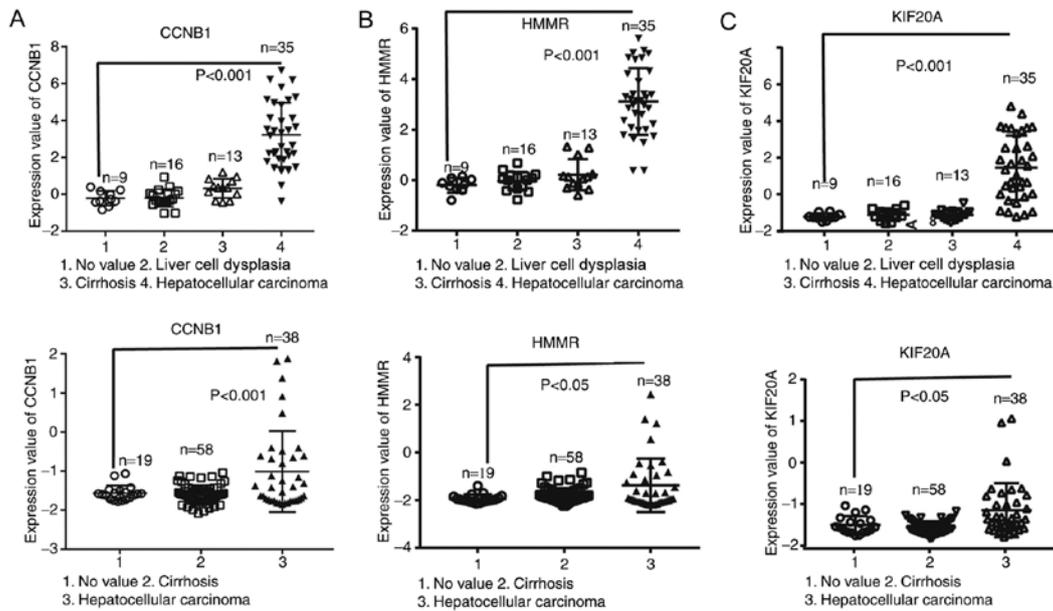


Figure 6. Comparison of CCNB1, HMMR and KIF20A gene expression between HCV-HCC and adjacent non-tumor tissues. mRNA expression of (A) CCNB1, (B) HMMR and (C) KIF20A in hepatitis C virus-induced hepatocellular carcinoma from the Wurmbach Liver dataset (top panel) and the Mas Liver dataset (bottom panel). CCNB1, cyclin B1; KIF20A, kinesin family member 20A; HMMR, hyaluronan mediated motility receptor.

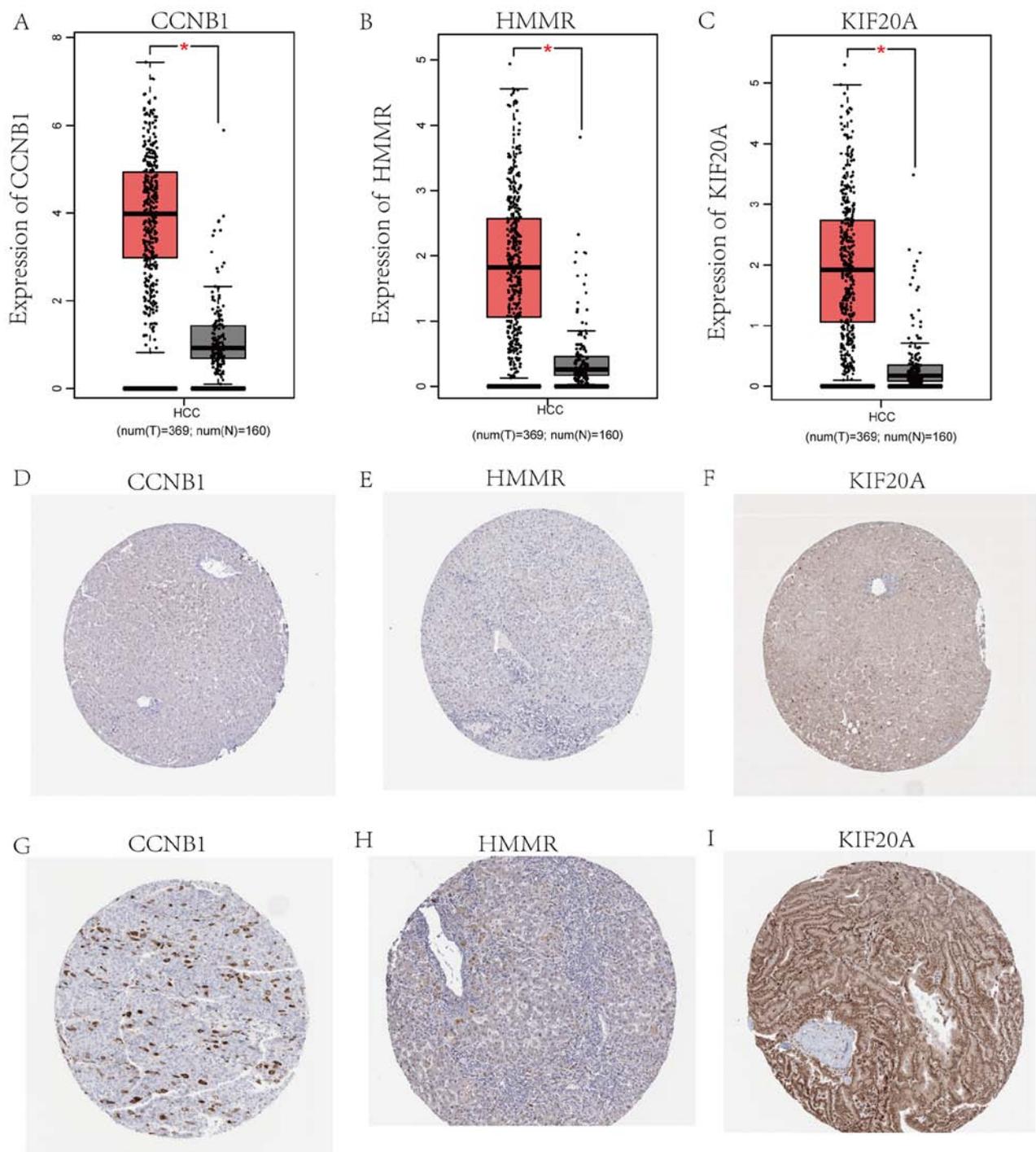


Figure 7. mRNA and protein expression of CCNB1, HMMR and KIF20A in HCV-HCC. mRNA expression of (A) CCNB1, (B) HMMR and (C) KIF20A in HCV-HCC (red; n=369) compared with normal (gray; n=160) tissues. Immunohistochemistry of the CCNB1, HMMR and KIF20A based on the Human Protein Atlas (<https://www.proteinatlas.org>). (D) Protein expression of CCNB1 in normal tissue (not detected). (E) Protein expression of HMMR in normal tissue (not detected). (F) Protein expression of KIF20A in normal tissue (staining, low; intensity, weak; quantity, >75%). (G) Protein expression of CCNB1 in tumor tissue (staining, medium; intensity, strong; quantity, <25%). (H) Protein expression of HMMR in tumor tissue (staining, medium; intensity, strong; quantity, <25%). (I) Protein expression of KIF20A in tumor tissue (staining, high; intensity, strong, quantity, >75%). *P<0.001. CCNB1, cyclin B1; KIF20A, kinesin family member 20A; HMMR, hyaluronan mediated motility receptor; HCV-HCC, hepatitis C virus-induced hepatocellular carcinoma.

Oncomine analysis showed that overexpression of CCNB1, KIF20A and HMMR mRNA were significantly associated with HCC progression, suggesting these genes are important factors in the occurrence and development of HCV-HCC. mRNA expression of CCNB1, KIF20A and HMMR from the GEPIA database was analyzed, indicating that they were all overexpressed in HCV-HCC. However, the difference

between the numbers of HCC and normal tissue samples in the GEPIA is a limiting factor in the present study. Finally, the Human Protein Atlas database, providing information on the expression of 24,000 human proteins in cells and tissues, was utilized to explore the protein expression of CCNB1, KIF20A and HMMR in HCC tissues, demonstrating significant upregulation of all of them.

In conclusion, in the present study, 368 DEGs and 10 hub genes were identified and are potential diagnostic biomarkers and therapeutic targets for HCV-HCC. Specifically, the findings suggest CCNB1, KIF20A and HMMR as candidate targets for HCV-HCC diagnosis and therapy. However, *in vitro* and *in vivo* experiments are necessary for validation of these results.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The dataset of GSE62232, GSE69715 and GSE107170 are available from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>). The dataset of Wurmbach Liver and Mas Liver are available from OncoPrint (<http://www.oncoPrint.com>).

Authors' contributions

WLL, JL and ZZM designed the study. JL analyzed the data and wrote the manuscript. ZWH, YML and LYW contributed to data collection, analysis and figure preparation. ZWH wrote parts of the manuscript and revised the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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