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Prognostic Assessment and Management of Liver Cirrhosis 2020

Lead Guest Editor: Xingshun Qi
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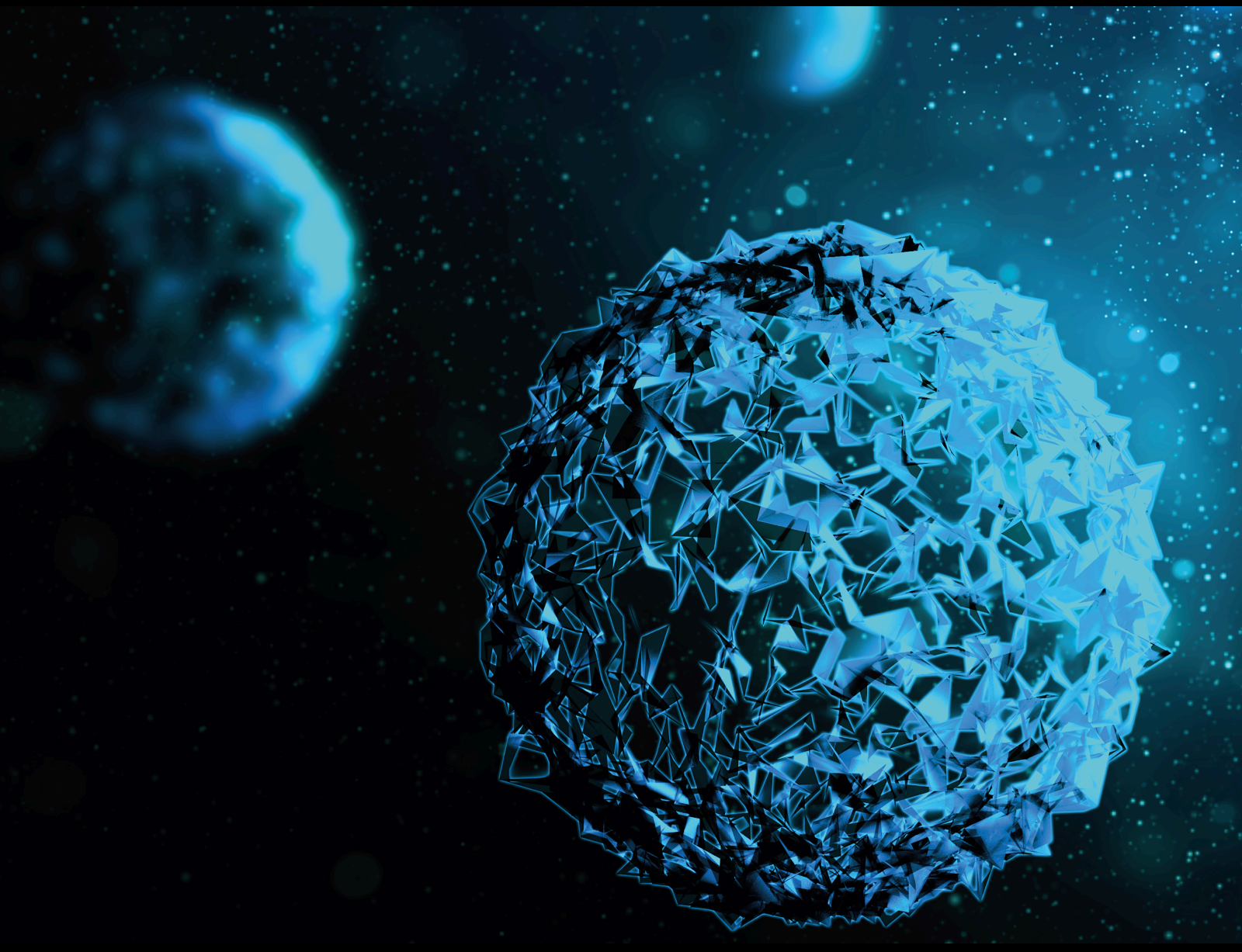

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Research Article

Microarray Data Mining and Preliminary Bioinformatics Analysis of Hepatitis D Virus-Associated Hepatocellular Carcinoma

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Several studies have demonstrated that chronic hepatitis delta virus (HDV) infection is associated with a worsening of hepatitis B virus (HBV) infection and increased risk of hepatocellular carcinoma (HCC). However, there is limited data on the role of HDV in the oncogenesis of HCC. This study is aimed at assessing the potential mechanisms of HDV-associated hepatocarcinogenesis, especially to screen and identify key genes and pathways possibly involved in the pathogenesis of HCC. We selected three microarray datasets: GSE55092 contains 39 cancer specimens and 81 paracancer specimens from 11 HBV-associated HCC patients, GSE98383 contains 11 cancer specimens and 24 paracancer specimens from 5 HDV-associated HCC patients, and 371 HCC patients with the RNA-sequencing data combined with their clinical data from the Cancer Genome Atlas (TCGA). Afterwards, 948 differentially expressed genes (DEGs) closely related to HDV-associated HCC were obtained using the R package and filtering with a Venn diagram. We then performed gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to determine the biological processes (BP), cellular component (CC), molecular function (MF), and KEGG signaling pathways most enriched for DEGs. Additionally, we performed Weighted Gene Coexpression Network Analysis (WGCNA) and protein-to-protein interaction (PPI) network construction with 948 DEGs, from which one module was identified by WGCNA and three modules were identified by the PPI network. Subsequently, we validated the expression of 52 hub genes from the PPI network with an independent set of HCC dataset stored in the Gene Expression Profiling Interactive Analysis (GEPIA) database. Finally, seven potential key genes were identified by intersecting with key modules from WGCNA, including 3 reported genes, namely, *CDCA5*, *CENPH*, and *MCM7*, and 4 novel genes, namely, *CDC6*, *CDC45*, *CDCA8*, and *MCM4*, which are associated with nucleoplasm, cell cycle, DNA replication, and mitotic cell cycle. The *CDCA8* and stage of HCC were the independent factors associated with overall survival of HDV-associated HCC. All the related findings of these genes can help gain a better understanding of the role of HDV in the underlying mechanism of HCC carcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related mortality globally [1, 2] and the second in China [3]. More than 80% of all HCC causes are associated with infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis delta virus (HDV) [4]. Approximately 292 million people worldwide are chronically infected with HBV, which causes liver injury that can progress to cirrhosis, resulting in HCC, liver failure, and eventually death [5, 6]. The HDV is known as the satellite of HBV and affects 15–20 million people

in the world [7]. Concurrent HBV and HDV infections significantly increase both the incidence and mortality of HCC among patients with chronic hepatitis B (CHB) [8]. HDV is a kind of defective RNA virus which uses HBV envelope protein for successful spread in hepatocytes [9]. Although the risk of HCC is thought to be higher when a HBV-infected patient is superinfected with HDV, the molecular mechanisms of carcinogenesis remain unclear [10]. Chronic hepatitis D (CHD) is more severe than any other type of hepatitis, but its carcinogenesis mechanism remains poorly understood. Additionally, it has been found that the intrahepatic HBV DNA levels in patients with HDV-associated HCC and non-HCC cirrhosis are significantly reduced [11]. This phenomenon of HDV-mediated inhibition of HBV replication suggests that the effects of HDV are mediated through a unique molecular mechanism. While HBV and HCV are both included in International Agency for Research on Cancer (IARC) group 1 (high evidence of carcinogenicity to humans), HDV was assigned several years ago to group 3 (not sufficient evidence of carcinogenicity) [12], due to inadequacy to support the contribution of HDV to HBV-induced HCC. Due to the dependency of HDV on HBV, there are still controversies regarding the increased risk of HCC development in chronically HDV-infected patients [4], and the available data on the particular mechanism by which HDV contributes to HCC are sparse. With the development of genomics and other "-omics" disciplines, substantial omics data from HCC specimens have been accumulated [13]. Therefore, researchers have taken advantage of gene ontology (GO) and signal pathway analysis tools to identify and characterize many differentially expressed genes (DEGs).

In the present study, the GSE55092 and GSE98383 mRNA expression profile datasets were retrieved from Gene Expression Omnibus (GEO) online [14, 15]. And the RNA-sequencing data of 371 HCC patients and their clinical data were from the Cancer Genome Atlas (TCGA). We performed DEG analysis between cancerous specimens of HBV-associated HCC and HDV-associated HCC patients and their respective paracancerous specimens using Linear Models for Microarray Data (LIMMA) [16] and other packages implemented in R/Bioconductor [17]. We aimed to investigate potential HDV carcinogenesis mechanisms by PPI network, Gene Expression Profiling Interactive Analysis (GEPIA), and Weighted Gene Coexpression Network Analysis (WGCNA), particularly to screen and identify key genes and pathways to determine their possible role in HCC pathogenesis, and to help determine their mechanism of inhibition of HBV replication and their effects in diagnosis, treatment and prognosis.

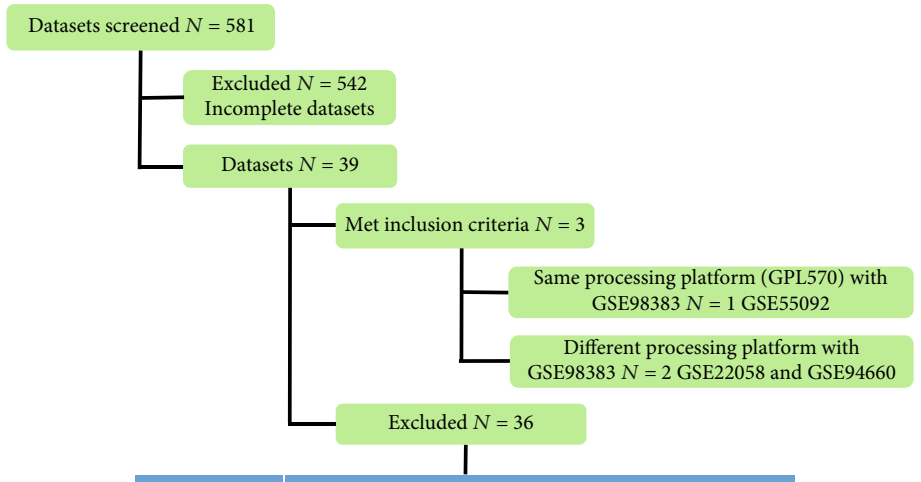
2. Methods and Materials

2.1. Acquisition of Data and Preprocessing. The RNA-sequencing data and clinical data of 371 HCC patients were downloaded from TCGA (http://cancergenome.nih.gov/. http://cancergenome.nih.gov/). The expression of genes was represented by fragments per kilobase of exon per million fragments mapped (FPKM). Microarray data were available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. The inclusion criteria for selection GEO datasets in this study were as follows: (1) hepatocellular carcinoma containing cancer and paracancer tissue; (2) HBsAg positive at least 6 months with serum HBV DNA positive; (3) anti-HDAg positive with serum HDV RNA positive (applies only to filter HDV-associated HCC dataset); and (4) sample size more than 10 with data unbiased. The exclusion criteria were as follows: (1) the second liver cancer, (2) HCV-related HCC, (3) dataset biased, and (4) no paracancer tissue and carcinoma tissue present at the same patients. We searched the GEO database using "Hepatitis D Virus," "Hepatocellular Carcinoma," and "Homo sapiens" as keywords, and there were only 2 results, between which only GSE98383 met our criteria for HDV-associated HCC. Similarly, we searched the keywords "Hepatitis B Virus," "Hepatocellular Carcinoma," and "Homo sapiens" and obtained 581 search results. The flow chart of screening HBV-associated HCC could be seen in Figure 1, and only GSE55092 was suited for an in-depth study.

Microarray data GSE55092 [18] and GSE98383 [11] were generated using the GPL570 Affymetrix HG-U133 Plus 2.0 Array platform, and we performed the analysis on the data from whole liver tissue. GSE55092 contains 39 cancer specimens and 81 paracancer specimens from 11 HBV-associated HCC patients (average age = 57.7 ± 7.7 years; 10 male patients and 1 female patient). GSE98383 contains 11 cancer specimens and 24 paracancer specimens from 5 HDV-associated HCC patients (average age = 57 ± 3 years, 5 male patients). The comparison of baseline characteristics between patients with HBV-associated HCC and HDV-associated HCC is shown in Table 1.

We downloaded the GSE55092 and GSE98383 datasets, normalized them by the Affy package of the R Bioconductor, and then converted the gene expression profile at the probe level into gene symbol level and removed the duplicated symbols. When numerous probes were mapped to one gene, the average value was defined as the expression level of that gene. According to the description of the uploader, an unsupervised multidimensional scaling (MDS) of all specimens obtained from HBV-associated HCC patients showed a clear separation between two distinct clusters that corresponded to cancer areas and paracancerous areas. A similar separation between two clusters that corresponded to cancerous areas and paracancerous areas was observed for the specimens from HDV-associated HCC patients. Therefore, all the data are available for the identification of DEGs.

2.2. Identification of DEGs. DEG analysis refers to the identification of genes with significantly different expression levels between two groups through multiple analysis modes [19]. We performed differential expression analysis using Bayes t-statistics from the LIMMA implemented in the R Bioconductor and corrected p values for multiple testing using the Benjamini-Hochberg method [20]. We identified the DEGs in primary cancerous specimens of HCC patients by comparing them with paracancerous normal specimens of the same HCC patients. The absolute value of log2-fold change (FC) was set to ≥ 1.0, and a p value of <0.01 was used as the



N	Reason for exclusion
3	Not related to HCC
15	No biopsy
13	Not have para-cancer specimens
5	No more than 10 available samples

Figure 1: Flow chart of enrolled datasets and availability of datasets.

Table 1: Comparison of baseline characteristics between patients with HBV-associated HCC and HDV-associated HCC.

	HBV-associated HCC	HDV-associated HCC	*p*
Patients	11	5	—
Age (years old)	57.7 ± 7.7	57 ± 3	0.849
Male (%)	10 [90.9]	5 [100]	1.000
ALT (U/L)	36.18 ± 17.8	87 ± 25	0.000
AST (U/L)	39.09 ± 17.0	82 ± 21	0.001
GGT (U/L)	93.9 ± 83.56	98 ± 22	0.917
PT (INR)	1.13 ± 0.14	1.4 ± 0	0.001
TB (mg/dL)	0.88 ± 0.47	2.3 ± 1.3	0.005
PLT (10^3/mL)	15.381 ± 9.373	101.4 ± 16.1	0.000
Liver pathology			
Activity grade	5.75 ± 3.06	9.1 ± 1.1	0.034
Fibrosis stage	5.1 ± 1.56	6.0 ± 0.0	0.226
F5/F6	9	5	
Tumor grade			0.107
G2	7	1	
G3	3	4	
G4	1	0	
Tumor size			0.407
<2 cm	4	0	
≥2 and ≤3 cm	4	3	
>3 cm	3	2	
Serum HDV RNA positive, no.	0	5	—
Serum HBV DNA positive, no.	11	5	—

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: γ-glutamyl transferase; TB: total bilirubin; PT: prothrombin time; PLT: Platelets.

significance criteria, and genes that met these criteria were used for further analysis. The final step is to use Venn diagrams to identify DEGs closely related to HDV-associated HCC [21].

2.3. GO Enrichment and Pathway Analysis. DAVID program (https://david.ncifcrf.gov/) [22] is a bioinformatics resource comprising a biological database and a set of annotation and analytical tool that intuitively integrates functional genomic annotations with graphics. In this study, the DEGs were submitted to DAVID for GO [23] and KEGG [24] enrichment analyses, which included biological process (BP), cellular component (CC), molecular function (MF), and related biological metabolic pathways. A p value < 0.05 was considered statistically significant.

2.4. Weighted Gene Coexpression Network Construction and Module-Clinical Characteristic Associations. We compared DEGs with the genes of 371 HCC patients downloaded from TCGA website. Expression data of the matched genes from TCGA were applied to find gene modules significantly associated with clinical trait (stage of HCC) by WGCNA [25]. In this analysis mode, the soft thresholding acts as the lowest power based on the criterion of approximate scale-free topology [26], and analogous modules would be merged together due to the similarity. The heatmap of module-clinical characteristic relationship could reveal modules significantly associated with clinical characteristics.

2.5. Construction of PPI Networks and Module Analysis. The visual protein-to-protein interaction (PPI) networks of DEGs were predicted using the web resource Search Tool for the Retrieval of Interacting Genes (STRING) [27] to search the STRING database (https://string-db.org/), which contains over 5,000 organisms as well as their over 24.6 million proteins and over 2 billion interactions. We correlated the target DEGs with the STRING database and set the significant threshold to the highest confidence level (interaction score ≥ 0.900). Subsequently, we used Cytoscape [28], a software to construct PPI networks and analyze highly interconnected modules using the built-in Molecular Complex Detection (MCODE) clustering algorithm. The parameters were set by default except for the K-core value which was equal to 8.

2.6. Validation of Module Gene. First, we uploaded the potential genes identified by PPI-network analysis to GEPIA [29] (http://gepia.cancer-pku.cn/, an online server containing TCGA/GTEx datasets) to validate the gene expression consistency between the microarray datasets (GSE55092 and GSE98383) and TCGA/GTEx HCC dataset, setting the threshold parameters as follows: |log2FC| cutoff ≥ 1.0 and p value cutoff < 0.01. Afterwards, we performed the overall survival analysis as follows: we divided the patients in TCGA/GTEx dataset into high and low expression groups with the TPM (transcripts per kilobase million) midvalue as a breakpoint; a log-rank test was used to determine significance at $p < 0.05$. Finally, we took the intersection of related genes to the OS of HCC by GEPIA and genes contained in the hub module obtained by WGCNA and got the key genes.

2.7. Univariate and Multivariate Cox Proportional Hazards Model Analysis. We performed univariate and multivariate Cox proportional hazards model analysis in patients from TCGA, to find independent factors associated with the overall survival of HCC.

3. Result

3.1. Comparison of Baseline Characteristics. GSE98383 was the only dataset of HDV-associated HCC that met our criteria, as to the screen of HBV-associated HCC dataset, overall 581 datasets were enrolled and screened for eligibility and 3 datasets met inclusion criteria. Of these 3 datasets, the data processing platform of GSE55092 was GPL570, which was the same with GSE98383, while GSE22058 and GSE94660 were different, so we took GSE55092 to stand for HBV-associated HCC for study. The number of datasets and reasons for exclusion are shown in Figure 1. The HDV-associated HCC patients in GSE98383 had higher levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), prothrombin time (PT), platelet counts (PLT), and inflammatory activity grade than the HBV-associated HCC patients in GSE55092, which is consistent with the characteristics of CHD of the most severe hepatitis. On the other hand, there was no significant difference in sex, age, tumor grade, and tumor size between the two groups.

3.2. Identification of DEGs. We identified DEGs from the microarray GSE55092 and GSE98383 datasets using the LIMMA package, setting |log2-FC| to ≥1.0 and adjusted p value to <0.01 as the criteria. By comparing the cancerous and paracancerous specimens in GSE55092 up to 1,375, DEGs were identified, comprising 518 upregulated and 857 downregulated genes (Table S1). A similar comparison in GSE98383 contains 1,605 DEGs, including 592 upregulated and 1,013 downregulated genes (Table S1). Volcano plots of the GSE55092 and GSE98383 microarrays are shown in Figures 2(a) and 2(b), respectively. We used Venn diagrams to determine the DEGs closely related to HDV-associated HCC (Figure 2(c)), and 948 DEGs (373 upregulated and 582 downregulated) were identified and selected for further analysis (Table S2).

3.3. GO Enrichment and Pathway Analysis. In order to further screen HDV-associated HCC potential target genes among these DEGs, GO and pathway analysis were performed on HDV-associated HCC DEGs using p value < 0.05 as the threshold (Figure 2(d)). The results are presented in Figure 2(d) and show the TOP-7 GO terms (BP, CC, and MF) and KEGG pathway terms significantly enriched in the DEGs. Additionally, the TOP-5 annotations of the DEGs are shown in Table 2. In the BP series, the DEGs were mostly enriched for genes related to the cellular response to chemical stimulus and organic substance, defense response, and cell adhesion. In the CC series, the DEGs were primarily enriched for genes involved in cell surface, side of membrane, membrane-bounded vesicle, external side of plasma membrane, and proteinaceous extracellular matrix. In the MF

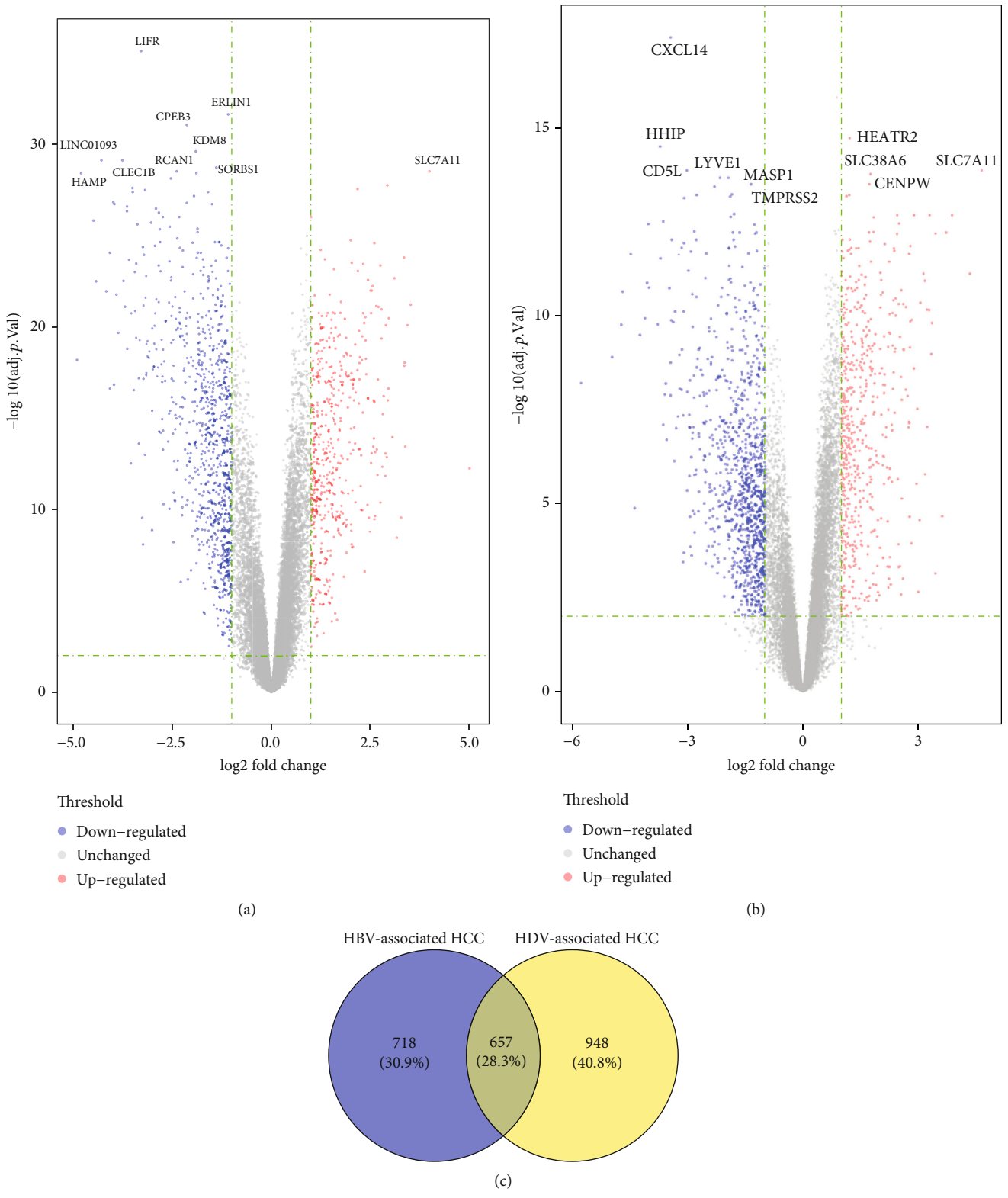
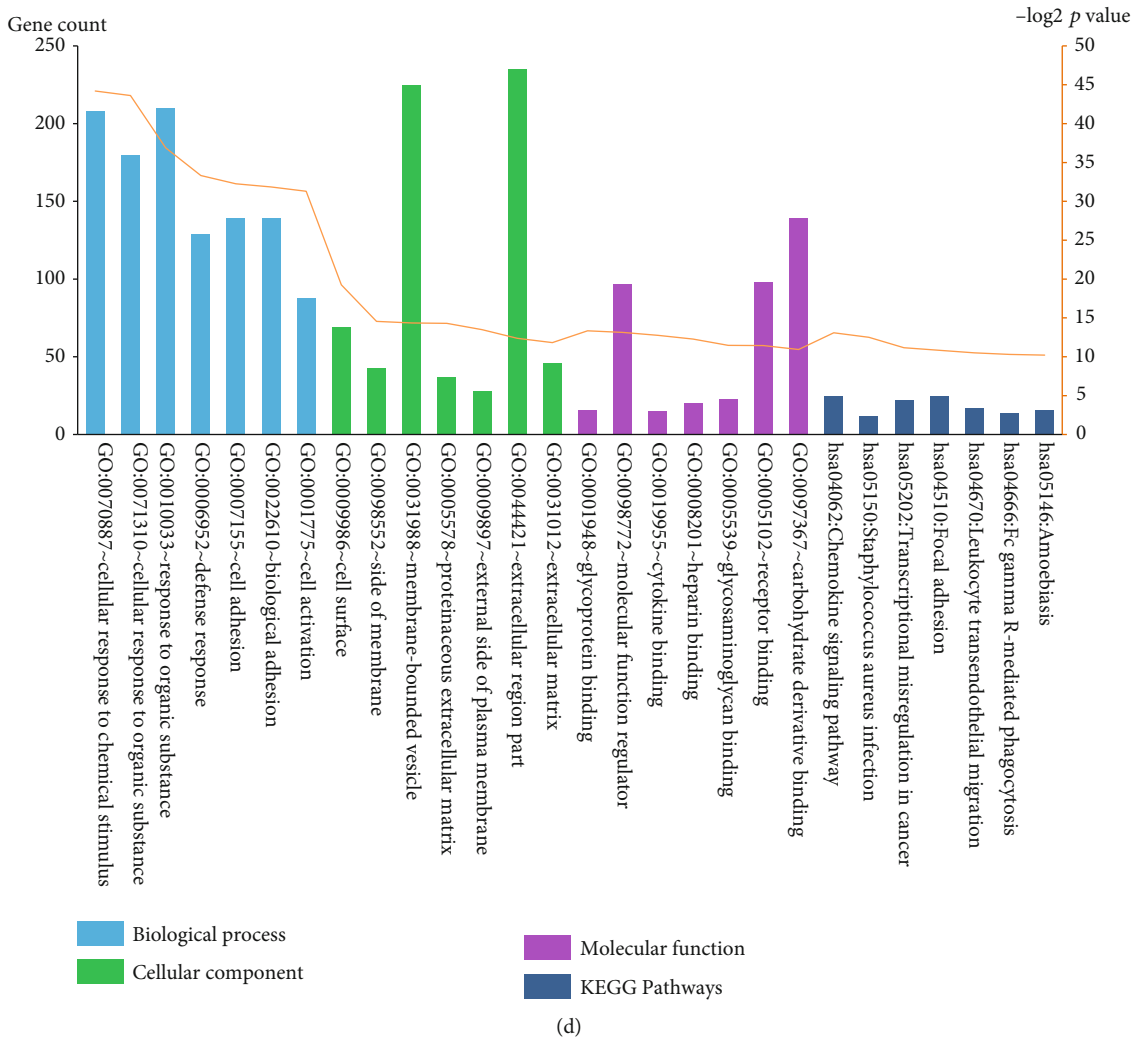


Figure 2: Continued.



(d)

FIGURE 2: Identification and filtering of DEGs closely related to HDV-associated HCC and their GO and KEGG pathway analysis. (a) Volcano plot of DEGs related to HBV-associated HCC; the DEGs with the top-10 *P* value differences are shown in the plot. (b) Volcano plot of DEGs related to HDV-associated HCC; the DEGs with the top-10 *P* value differences are shown in the plot. (c) The Venn diagram shows the intersection of DEGs between HBV-associated HCC and HDV-associated HCC. The right part (yellow) represents the DEGs closely related to HDV-associated HCC. (d) The GO terms and the KEGG pathways of DEGs significantly enriched in HDV-associated HCC. GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; HCC: hepatocellular carcinoma.

series, the DEGs were predominantly enriched for genes associated with glycoprotein binding, molecular function regulator, cytokine binding, heparin binding, and glycosaminoglycan binding. In the KEGG pathway series, the enrichment for DEGs was mainly in the chemokine signaling pathway, Staphylococcus aureus infection, transcriptional misregulation in cancer, focal adhesion, and leukocyte transendothelial migration.

3.4. Weighted Gene Coexpression Network Construction and Module-Clinical Characteristics. We compared 948 DEGs with the genes of 371 samples downloaded from TCGA datasets, matched a total of 883 genes, and performed WGCNA. As shown in Figures 3(a), 3(b), and 3(c), the soft thresholding power β was set to 3, and MEblue and MEred were merged together due to the similarity (the height = 0.25). Then, we found five coexpressed gene modules. The MEturquoise contained the most DEGs with the number of 320. The five modules and contents are stored in supplementary material Table S3. Next, we further analyzed these modules with clinical characteristics (sex, event, OS, stage, grade, and age). Obviously, the MEturquoise module was significantly associated with event (correlation coefficients $(r) = 0.19$, $p < 0.001$), OS ($r = -0.21$, $p < 0.001$), stage ($r = 0.23$, $p < 0.001$), grade ($r = 0.24$, $p < 0.001$), and age ($r = -0.14$, $p = 0.01$) (Figure 3(d)).

3.5. Construction of PPI Networks and Gene Module Analysis. We uploaded the DEGs onto the STRING online tool and analyzed them with the Cytoscape software. We then selected 353 nodes and 939 edges with the highest confidence (scores > 0.900) to construct the PPI networks (Figure 4(a)). Then, the MCODE plugin filtered out three important gene modules. Genes within module 1 and module

Table 2: The top five annotations in GO and KEGG enrichment analysis of the DEGs.

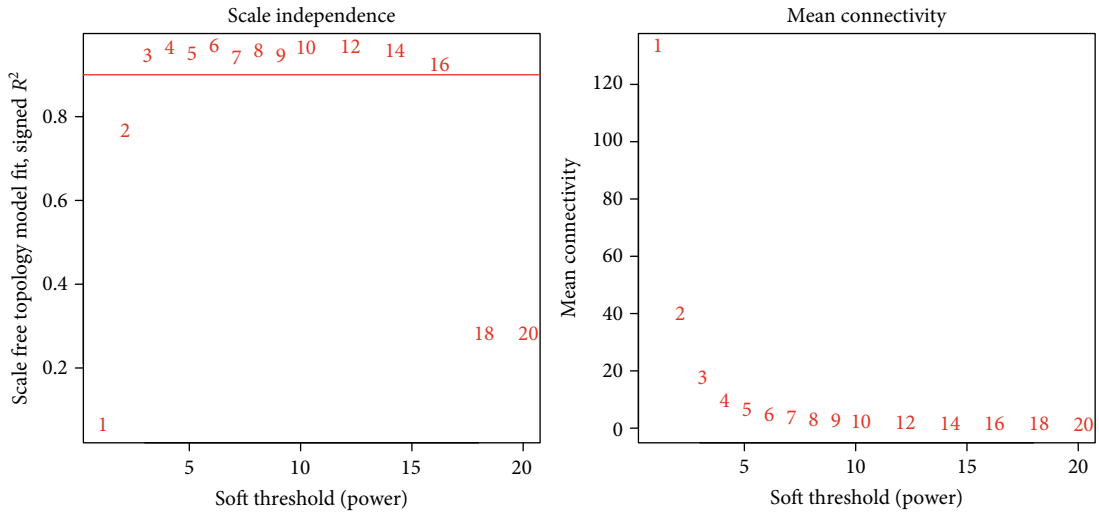
Category	Term	Count	p value
GOTERM_BP_FAT	GO:0070887~cellular response to chemical stimulus	208	$4.96E-14$
GOTERM_BP_FAT	GO:0071310~cellular response to organic substance	180	$7.46E-14$
GOTERM_BP_FAT	GO:0010033~response to organic substance	210	$7.89E-12$
GOTERM_BP_FAT	GO:0006952~defense response	129	$9.24E-11$
GOTERM_BP_FAT	GO:0007155~cell adhesion	139	$1.95E-10$
GOTERM_CC_FAT	GO:0009986~cell surface	69	$1.59E-06$
GOTERM_CC_FAT	GO:0098552~side of membrane	43	$4.16E-05$
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle	225	$4.83E-05$
GOTERM_CC_FAT	GO:0005578~proteinaceous extracellular matrix	37	$4.95E-05$
GOTERM_CC_FAT	GO:0009897~external side of plasma membrane	28	$8.75E-05$
GOTERM_MF_FAT	GO:0001948~glycoprotein binding	16	$9.68E-05$
GOTERM_MF_FAT	GO:0098772~molecular function regulator	97	$1.11E-04$
GOTERM_MF_FAT	GO:0019955~cytokine binding	15	$1.44E-04$
GOTERM_MF_FAT	GO:0008201~heparin binding	20	$2.02E-04$
GOTERM_MF_FAT	GO:0005539~glycosaminoglycan binding	23	$3.53E-04$
KEGG_PATHWAY	hsa04062: Chemokine signaling pathway	25	$1.16E-04$
KEGG_PATHWAY	hsa05150: Staphylococcus aureus infection	12	$1.73E-04$
KEGG_PATHWAY	hsa05202: Transcriptional misregulation in cancer	22	$4.38E-04$
KEGG_PATHWAY	hsa04510: Focal adhesion	25	$5.49E-04$
KEGG_PATHWAY	hsa04670: Leukocyte transendothelial migration	17	$6.91E-04$

2 are comprised of downregulated genes, while module 3 is comprised of upregulated genes, except for *PPP2R5C* and *LONRF1*. Module 1 contains 15 nodes and 105 edges (Figure 4(b)), which are mainly related to G protein-coupled receptor signaling pathway (BP), plasma membrane (CC), G protein-coupled receptor binding (MF), and chemokine signaling pathway (KEGG) (Table 3). Module 2 contains 12 nodes and 66 edges (Figure 4(c)), which are primarily related to type I interferon signaling pathway (BP), cytosol (CC), $2'$-$5'$-oligoadenylate synthetase activity (MF), and hepatitis C (KEGG) (Table 4). Module 3 contains 25 nodes and 94 edges (Figure 4(d)), which are predominantly related to the mitotic cell cycle process (BP), chromosomal part (CC), DNA replication origin binding (MF), and cell cycle (KEGG) (Table 5).

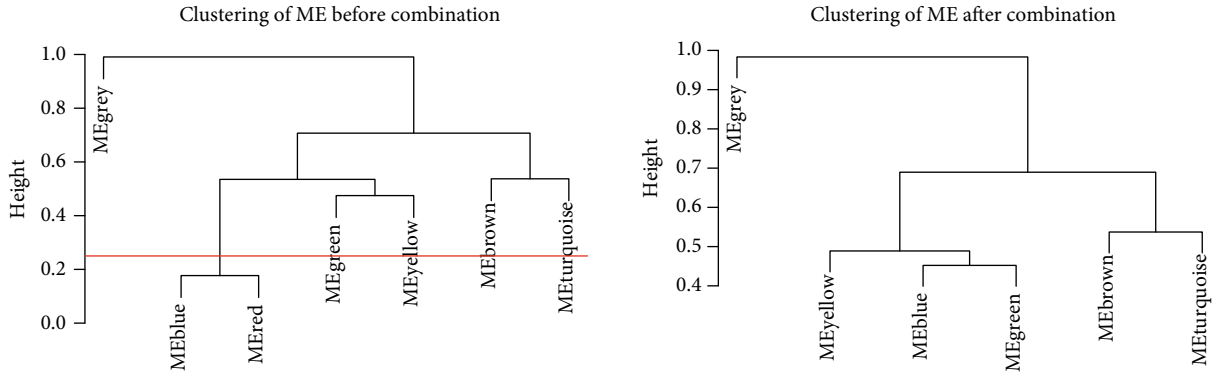
3.6. Validation of Module Genes. We compared the gene expression changes between the three module genes of HDV-associated HCC (a total of 52 genes) and the validation HCC (TCGA/GTEx) datasets in the GEPIA website to verify whether their expression in both datasets is consistent. We noticed that *CCL21* and *FPR1* in module 1 as well as *XAF1* in module 2 were downregulated in tumors compared to normal specimens in the HCC datasets, which is in accordance with the HDV-associated HCC specimens. However, *IFI6*, *IFI27*, and *ISG15* in module 2, which were downregulated in cancerous specimens compared to paracancerous normal specimens, were conversely expressed in HCC datasets. The genes in module 3, including *CDC6*, *CDC45*, *CDCA5*, *CDCA8*, *CENPH*, *MCM4*, *MCM7*, and *TCEB1*, were upregulated in tumor compared to normal specimens in the HCC datasets, which is consistent with the HDV-associated HCC patients. All the box plots comparing gene expression are shown in Figure 5(a). For further verification, 11 genes whose gene expression trends are consistent with HCC datasets were selected and used to conduct overall survival analysis. In Figure 5(b), there were the upregulated genes (*CDC6*, *CDC45*, *CDCA5*, *CDCA8*, *CENPH*, *MCM4*, *MCM7*, and *TCEB1*) which are associated with a lower survival rate in the high expression group than in the low expression group.

The reason for excluding *CCL21*, *FPR1*, *IFI6*, *IFI27*, *ISG15*, and *XAF1* is that their p value did not comply with the standards or the opposite gene expression. All the 8 retained potential genes are related to the nucleoplasm, and most of them are related to the mitotic cell cycle process, cell cycle, and DNA replication (Figure 5(c)). Taken together, the intersection of the above validated 8 genes and 320 genes in MEturquoise module by WGCNA, the potential 7 key genes (*CDC6*, *CDC45*, *CDCA5*, *CDCA8*, *CENPH*, *MCM4*, and *MCM7*) were found (Figure 5(d)).

3.7. Identification of Independent Factors of Overall Survival of HCC. We performed the univariate analysis in 371 patients from TCGA and found that *CDCA8*, stage, *CDC45*, *CDC6*, *CDCA5*, *MCM4*, *CENPH*, *MCM7*, sex, and age were significantly associated with OS of HCC. The multivariate Cox



(a)



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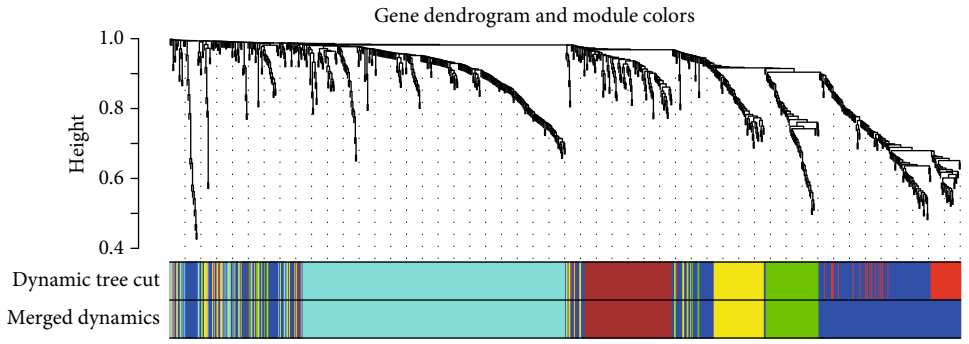


(c)

Figure 3: Continued.

FIGURE 3: The processing steps of WGCNA. (a) Analysis of the soft thresholding power ($\beta = 3$). (b) MEblue and MEred merged together due to the similarity (the height = 0.25). (c) Gene dendrogram and module colors; the MEturquoise contained the most DEGs ($n = 320$). (d) Heatmap of module-trait relationships. The MEturquoise was the most significantly associated with event, OS, stage, grade, and age. WGCNA: Weighted Gene Coexpression Network Analysis.

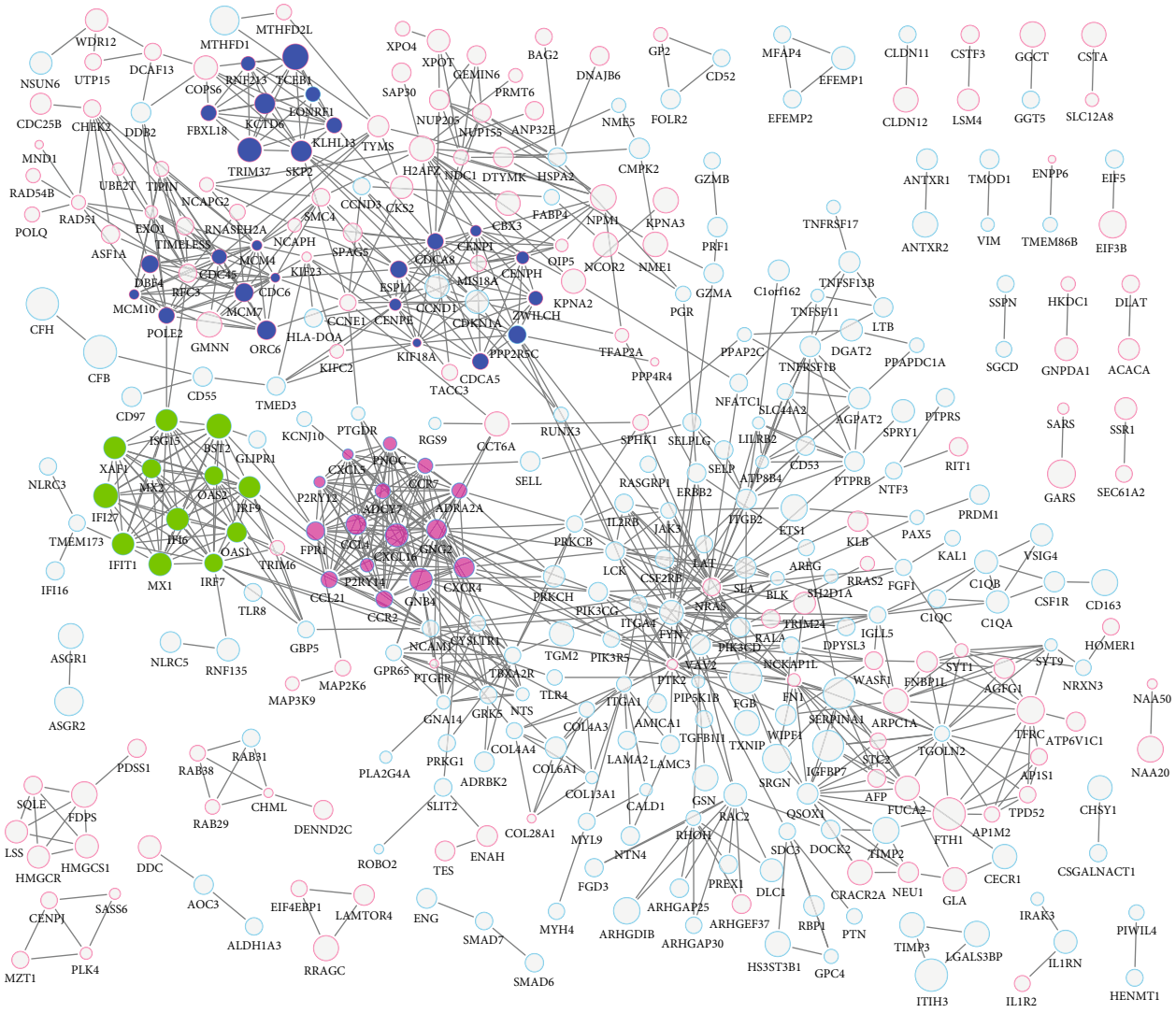
proportional hazards model showed that *CDCA8* and stage of HCC were independent factors of OS of HCC (Table 6).

4. Discussion

As the virus causing the most severe type of hepatitis, HDV affects 15-20 million people worldwide, but its specific pathogenic mechanism remains unclear. Accordingly, we undertook to find potential genes and pathways involved in the pathogenesis of this disease through text mining to help explain the underlying carcinogenic mechanism of HDV as well as the HBV inhibitory mechanism.

In this study, we compared cancerous and paracancerous specimens of patients suffering from HBV or HDV-associated HCC with the aim of identifying potential genes closely related to HDV-associated HCC. The study identified 373 upregulated DEGs and 582 downregulated DEGs. These DEGs were subjected to GO and KEGG annotation and enrichment analyses. In addition, we constructed PPI networks and sorted out 353 nodes with 939 edges, from which the three most significant modules were selected and 52 central nodes/genes were selected for validation using the GEPIA database. In the module confirmed by the WGCNA that was significantly associated with clinical features including event, OS, stage, grade, and age, only *CDC6*, *CDC45*, *CDCA5*, *CDCA8*, *CENPH*, *MCM4*, and *MCM7* were consistent with genes identified by the PPI network, which were found to be significantly correlated with nucleoplasm, cell cycle, DNA replication, and mitotic cell cycle. Univariate and multivariate Cox proportional hazards model analysis showed the stage of HCC and CDCA8 are the independent factors associated with the OS of HCC.

Cell division cycle 6 (CDC6) is thought to be significantly associated with pancreatic cancer and colorectal cancer (CRC) [30]. It has a pivotal role in regulating the process of DNA replication as well as tumorigenesis; its overexpression could interfere with the expression of tumor suppressor genes (*INK4*/*ARF*) through the mechanism of epigenetic modification [31]. During the S phase of DNA replication in eukaryotic cells, cell division cycle 45 (CDC45) is an essential component of CMG (CDC45–MCM–GINS) helicase. CDC45 acts as a hubprotein, significantly upregulated in cancerous tissues from CRC and non-small-cell lung cancer (NSCLC) patients, and promotes tumor progression [32]. It

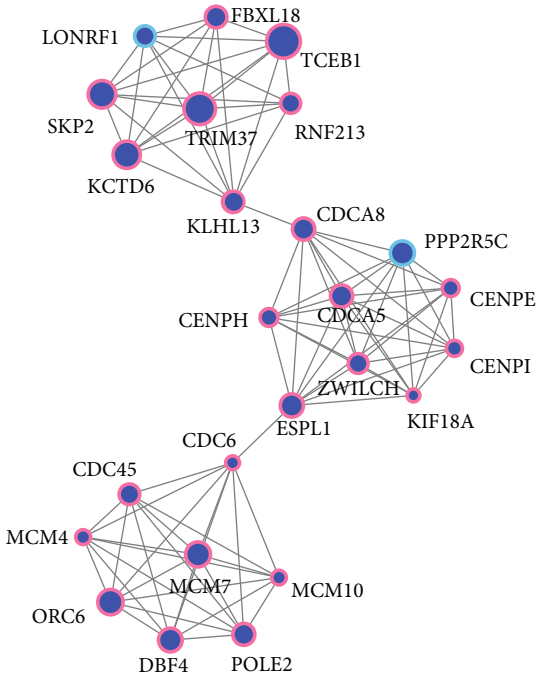


(a)

(b)

(c)

Figure 4: Continued.



(d)

Figure 4: PPI networks and the top-3 significant modules (module 1-3). (a) PPI networks constructed with the DEGs closely related to HDV-associated HCC. The red border indicates upregulation, the blue border indicates downregulation, the pink core represents module 1, the green core represents module 2, the dark blue core represents module 3, and the size of the circle represents the relative expression level of the genes; (b) module 1, the DEGs in module 1 are all downregulated; (c) module 2, the DEGs in module 2 are all downregulated; (d) module 3, the DEGs in module 3 are upregulated except for *PPP2R5C* and *LONRF1*. PPI: protein-to-protein interaction; DEGs: differentially expressed genes.

Table 3: Functional and pathway enrichment of module 1 genes.

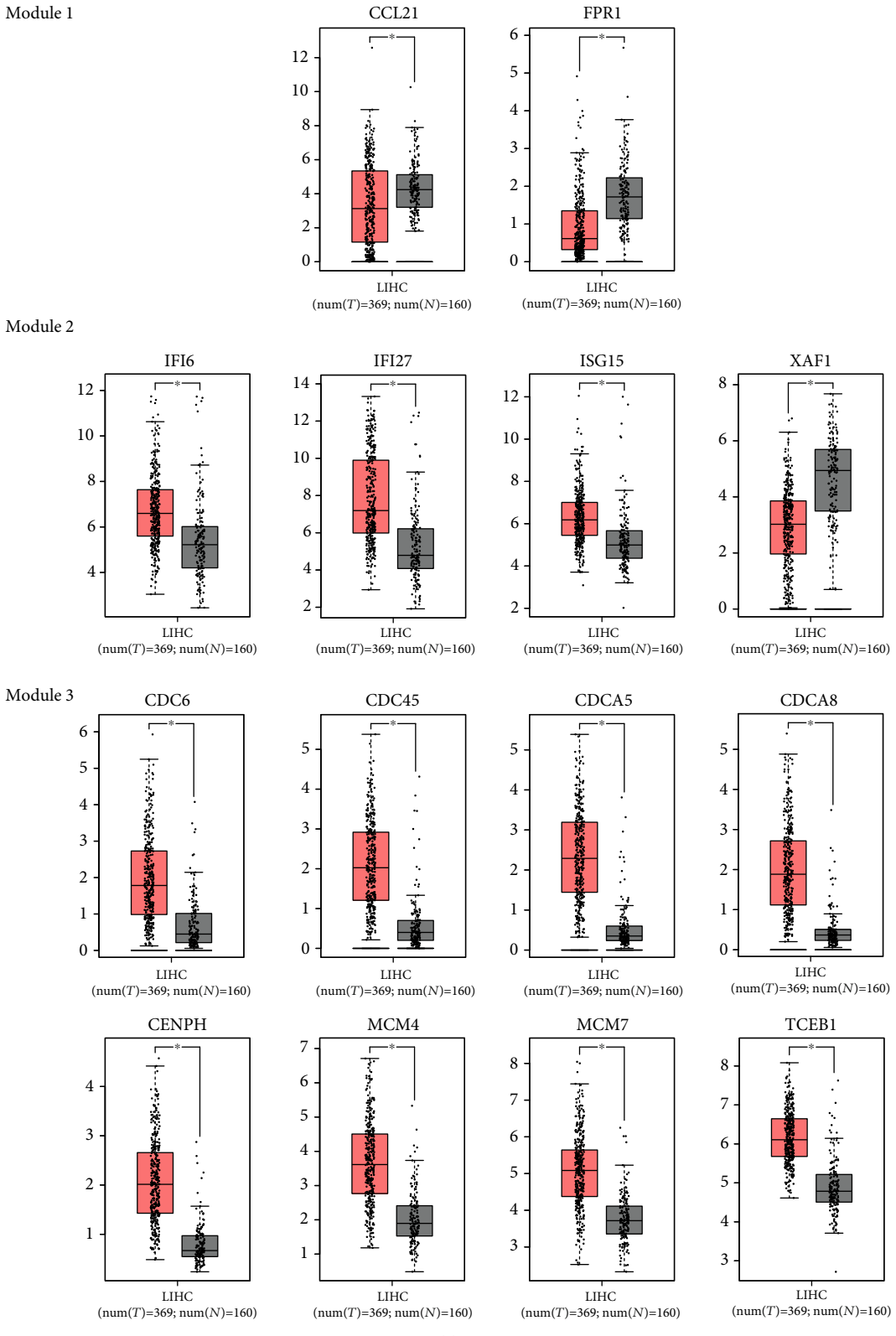
Category	Term	Count	p value	Genes
GOTERM_BP_FAT	G protein-coupled receptor signaling pathway	14	$2.79E-13$	ADCY7, ADRA2A, CCL21, CCL4, CCR2, CCR7, CXCL16, CXCL5, CXCR4, FPR1, GNG2, P2RY12, P2RY14, PNOC
GOTERM_BP_FAT	Cell chemotaxis	8	$2.16E-10$	CCL21, CCL4, CCR2, CCR7, CXCL16, CXCL5, CXCR4, FPR1
GOTERM_BP_FAT	Chemokine-mediated signaling pathway	6	$6.75E-09$	CCL21, CCL4, CCR2, CCR7, CXCL5, CXCR4
GOTERM_CC_FAT	Plasma membrane	11	0.0205	ADCY7, ADRA2A, CCR2, CCR7, CXCL16, CXCR4, FPR1, GNG2, P2RY12, P2RY14, PNOC
GOTERM_CC_FAT	External side of plasma membrane	3	0.0205	CCR2, CCR7, P2RY12
GOTERM_CC_FAT	Side of membrane	4	0.0205	CCR2, CCR7, GNG2, P2RY12
GOTERM_MF_FAT	G protein-coupled receptor binding	7	$7.55E-08$	ADRA2A, CCL21, CCL4, CCR2, CXCL16, CXCL5, PNOC
GOTERM_MF_FAT	Chemokine receptor binding	5	$7.55E-08$	CCL21, CCL4, CCR2, CXCL16, CXCL5
GOTERM_MF_FAT	Chemokine activity	4	$2.30E-06$	CCL21, CCL4, CXCL16, CXCL5
KEGG_PATHWAY	Chemokine signaling pathway	10	$1.15E-15$	ADCY7, CCL21, CCL4, CCR2, CCR7, CXCL16, CXCL5, CXCR4, GNB4, GNG2
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	7	$1.67E-08$	CCL21, CCL4, CCR2, CCR7, CXCL16, CXCL5, CXCR4
KEGG_PATHWAY	Circadian entrainment	3	0.00092	ADCY7, GNB4, GNG2

Table 4: Functional and pathway enrichment of module 2 genes.

Category	Term	Count	p value	Genes
GOTERM_BP_FAT	Type I interferon signaling pathway	12	$2.85E-27$	BST2, IFI27, IFI6, IFIT1, IRF7, IRF9, ISG15, MX1, MX2, OAS1, OAS2, XAF1
GOTERM_BP_FAT	Defense response to virus	9	$1.15E-14$	BST2, IFIT1, IRF7, IRF9, ISG15, MX1, MX2, OAS1, OAS2
GOTERM_BP_FAT	Negative regulation of viral genome replication	5	$3.48E-09$	BST2, IFIT1, ISG15, MX1, OAS1
GOTERM_CC_FAT	Cytosol	10	0.004	BST2, IFIT1, IRF7, IRF9, ISG15, MX1, MX2, OAS1, OAS2, XAF1
GOTERM_CC_FAT	Mitochondrion	6	0.0067	IFI27, IFI6, MX1, MX2, OAS1, XAF1
GOTERM_CC_FAT	Cytoplasmic part	12	0.0067	BST2, IFI27, IFI6, IFIT1, IRF7, IRF9, ISG15, MX1, MX2, OAS1, OAS2, XAF1
GOTERM_MF_FAT	$2'$-$5'$-Oligoadenylate synthetase activity	2	0.00028	OAS1, OAS2
GOTERM_MF_FAT	Double-stranded RNA binding	2	0.0229	OAS1, OAS2
KEGG_PATHWAY	Hepatitis C	5	$1.72E-07$	IFIT1, IRF7, IRF9, OAS1, OAS2
KEGG_PATHWAY	Measles	5	$1.72E-07$	IRF7, IRF9, MX1, OAS1, OAS2
KEGG_PATHWAY	Influenza A	5	$1.92E-07$	IRF7, IRF9, MX1, OAS1, OAS2

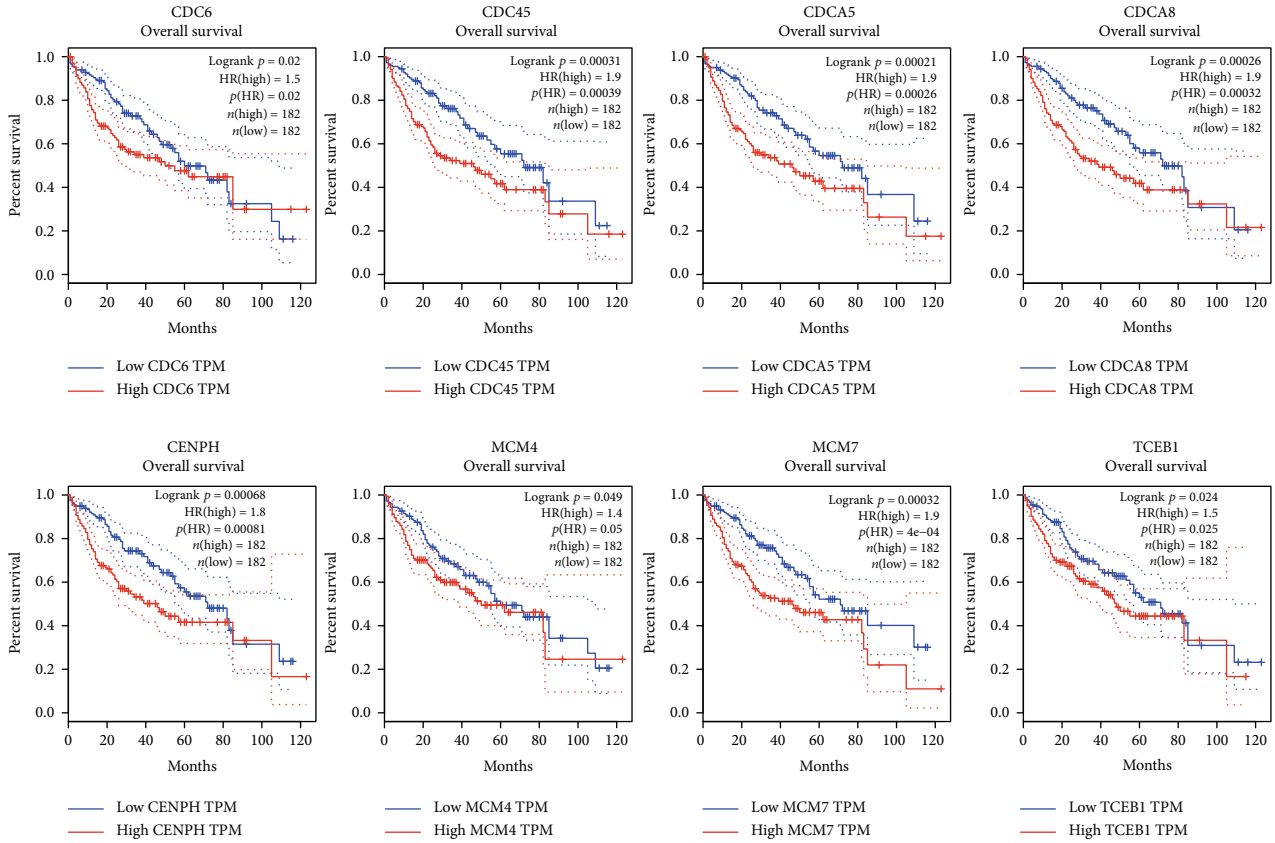
Table 5: Functional and pathway enrichment of module 3 genes.

Category	Term	Count	p value	Genes
GOTERM_BP_FAT	Mitotic cell cycle process	16	$2.69E-16$	CDC45, CDC6, CDCA5, CDCA8, CENPE, DBF4, ESPL1, KIF18A, MCM10, MCM4, MCM7, ORC6, POLE2, PPP2R5C, SKP2, ZWILCH
GOTERM_BP_FAT	Cell cycle	17	$6.32E-13$	CDC45, CDC6, CDCA5, CDCA8, CENPE, DBF4, ESPL1, KIF18A, KLHL13, MCM10, MCM4, MCM7, ORC6, POLE2, PPP2R5C, SKP2, ZWILCH
GOTERM_BP_FAT	G1/S transition of mitotic cell cycle	9	$4.80E-12$	CDC45, CDC6, DBF4, MCM10, MCM4, MCM7, ORC6, POLE2, SKP2
GOTERM_CC_FAT	Chromosomal part	13	$5.18E-10$	CDC45, CDCA5, CDCA8, CENPE, CENPH, CENPI, KIF18A, MCM10, MCM7, ORC6, POLE2, PPP2R5C, ZWILCH
GOTERM_CC_FAT	Chromosome, centromeric region	8	$3.39E-09$	CDCA5, CDCA8, CENPE, CENPH, CENPI, KIF18A, PPP2R5C, ZWILCH
GOTERM_CC_FAT	Intracellular nonmembrane-bounded organelle	18	$1.05E-06$	CDC45, CDC6, CDCA5, CDCA8, CENPE, CENPH, CENPI, ESPL1, KCTD6, KIF18A, MCM10, MCM7, ORC6, POLE2, PPP2R5C, RNF213, SKP2, ZWILCH
GOTERM_MF_FAT	DNA replication origin binding	3	0.00011	CDC45, MCM10, ORC6
GOTERM_MF_FAT	Single-stranded DNA binding	4	0.00037	CDC45, MCM10, MCM4, MCM7
GOTERM_MF_FAT	DNA helicase activity	3	0.00069	CDC45, MCM4, MCM7
KEGG_PATHWAY	Cell cycle	8	$8.55E-11$	CDC45, CDC6, DBF4, ESPL1, MCM4, MCM7, ORC6, SKP2
KEGG_PATHWAY	DNA replication	3	0.00022	MCM4, MCM7, POLE2
KEGG_PATHWAY	Ubiquitin-mediated proteolysis	4	0.00024	KLHL13, SKP2, TCEB1, TRIM37



(a)

Figure 5: Continued.



(b)

Figure 5: Continued.

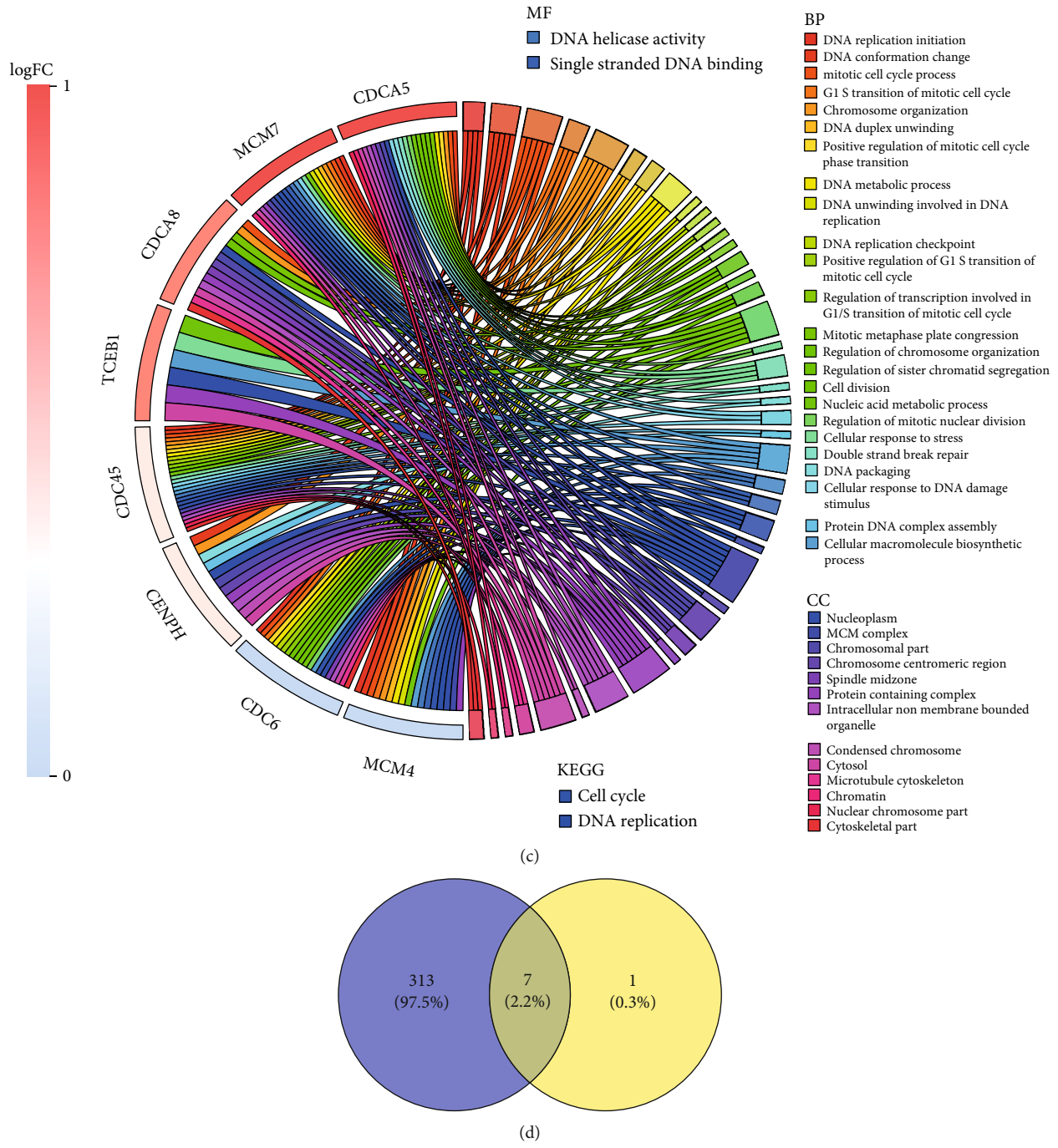


Figure 5: Validation of the expression data and survival curve of hub genes from the 3 modules using the GEPIA database and functional and pathway enrichment analysis. (a) The box plots that verify whether the expression of these DEGs is consistent with that in the LIHC datasets. Among the downregulated genes, *CCL21* and *FPR1* (module 1) as well as *XAF1* (module 2) are consistent with the HCC datasets, while *IFI6*, *IFI27*, and *ISG15* (module 2) are not. All 8 upregulated genes (module 3) are consistent with the LIHC datasets. (b) The genes are associated with overall survival whose expression is consistent with that in the LIHC datasets. All 8 upregulated genes are from module 3. (c) The chord diagram showing GO terms and KEGG pathway enrichment with the 8 hub genes involved. (d) The intersection of these 8 genes from PPI network analysis and 320 genes contained in module MEturquoise obtained by WGCNA, 7 potential key genes in the middle part. HCC: hepatocellular carcinoma; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: biological process; CC: cellular component; MF: molecular function; PPI: protein-to-protein interaction; WGCNA: Weighted Gene Coexpression Network Analysis.

is established that the MCM4/6/7 (minichromosome maintenance complex component 4/6/7) hexamer complex acts as a DNA helicase. Additionally, in endometrial cancer and skin cancer studies, it was found that MCM4 mutations may affect the interaction with MCM7, thereby disrupting the stability of the MCM4/6/7 complex [33]. In addition, MCM4 is also a member of significant predictors of poor prognosis in CRC patients [34]. Moreover, MCM7 is also a promising

Table 6: Univariate and Multivariate Cox proportional hazards model analysis of overall survival of HCC.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p value	HR	95% CI	p value
CDCA8	1.11	1.08-1.15	<0.001	1.10	1.06-1.14	<0.001
Stage I			<0.001			0.019
Stage II	1.43	0.88-2.34	0.151	1.23	0.75-2.02	0.416
Stage IIIA	2.68	1.70-4.21	<0.001	2.11	1.31-3.39	0.002
Stage IIIB	2.87	1.02-8.04	0.045	1.84	0.62-5.44	0.273
CDC45	1.13	1.07-1.19	<0.001			
CDC6	1.10	1.05-1.15	<0.001			
CDCA5	1.09	1.05-1.13	<0.001			
MCM4	1.06	1.04-1.09	<0.001			
CENPH	1.18	1.08-1.28	<0.001			
MCM7	1.01	1.00-1.01	0.029			
Gender (F vs. M)	0.82	0.57-1.16	0.257			
Age (years)	1.01	0.99-1.02	0.403			

F: female; M: male.

biomarker for early diagnosis of gastric cancer and even a predictor of meningioma recurrence after surgery [35, 36]. Another study found that high expression of MCM7 may be involved in the progression of HCC through the MCM7-cyclin D1 pathway, and MCM7 may serve as a prognostic marker for patients with HCC [37]. The cell division cycle-associated protein 5 (CDCA5) is a member of the CDCA family that comprises CDCA1-8. It plays a crucial role as a regulator of sister-chromatid cohesion and separation during cell division, and its upregulation has been shown to be associated with various cancers, including breast cancer, esophageal squamous cell carcinoma, CRC, and HCC [38, 39]. Also, a study found that the activation of the ERK and AKT pathways may be involved in the regulation of HCC cell proliferation by CDCA45 [39]. CDCA8 is an essential regulator of mitosis, and its overexpression is significantly associated with bladder cancer, cutaneous melanoma, and the progression and prognosis of breast cancer [40, 41]. Centromere protein H (CENPH) is considered to be an essential part of the active centromere complex, and its overexpression is highly related to poor prognosis in renal cell carcinoma, nasopharyngeal carcinoma, CRC, and HCC [42, 43]. Another study found that CENPH may promote the proliferation of HCC through the mitochondrial apoptosis pathway [43].

Most of the abovementioned genes are significantly associated with the cell cycle and DNA replication, and their overexpression may affect the replication of HBV DNA, thereby promoting the unique phenomenon of HDV inhibits HBV replication. All the findings related to these genes may also help us understand the mechanisms of HDV-induced liver injury and HCC. In the future, we will further verify those genes' function by performing animals, cells, and clinical trials.

5. Conclusions

In summary, 7 potential candidate genes closely related to HDV-associated HCC were identified in this study. Through comparative analysis with previous studies, these genes were found to be involved in many pathways related to tumorigenesis which provided clues to elucidate the mechanism of hepatitis D virus-induced HCC or its unique molecular mechanism in the inhibition of HBV replication. However, additional in-depth molecular biological research on these candidate genes closely related to HDV-associated HCC is necessary to confirm their functions.

Data Availability

The datasets used to support the findings of this study are available from GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA (https://portal.gdc.cancer.gov/).

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Zhe Yu, Xuemei Ma, and Wei Zhang contributed equally to this work.

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Supplementary Materials

Table S1: DEGs from microarray datasets GSE55092 and GSE98383. Table S2: 948 DEGs related to HDV-associated HCC including 373 upregulated and 582 downregulated

genes. Table S3: the five modules and contents obtained by WGCNA. *(Supplementary Materials)*

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Research Article

Oral Health-Related Quality of Life in Chronic Liver Failure Patients Measured by OHIP-14 and GOHAI

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Background. Oro-dental diseases are prevalent in chronic liver failure (CLF) patients. The aim of this study was to evaluate the quality of life associated with oral health in candidates for liver transplant surgery. *Materials and Methods*. The demographic information of 105 end-stage liver cirrhotic patients was collected. All patients were ordered a panoramic view for pretransplant dental evaluation. The DMFT (decayed-missing-filled tooth) index was calculated for dental examination. The model for end-stage liver disease (MELD) was used for the severity of liver disease. The OHIP-14 (Oral Health Impact Profile) questionnaire and GOHAI (Geriatric Oral Health Assessment Index) questionnaire were applied to evaluate the impact of oral disease on the quality of life. *Results*. A total of 79 patients thoroughly completed the questionnaires; 79.7% were male, 32.9% were over 50, and 25.3% were less than 30 years old. Further, 12.7% smoked, 2.5% were illiterate, 64.6% had not finished school, and 10.1% had university degrees. Almost half of the cirrhotic patients were suffering from the disease for more than 3 years. Most complaints reported by the patients as "very often" were becoming self-conscious (13.9%) and being uncomfortable when eating any foods (13.9%) followed by feeling tense (12.8%). There was no significant difference between gender, smoking, age, and MELD score based on quality of life (OHIP and GOHAI) ($P > 0.05$). The level of education ($P = 0.020$), duration of disease ($P = 0.017$), and DMFT index ($P = 0.039$) had a significant impact on oral health-related quality of life in CLF patients. An inverse relationship was seen between the DMFT index and the quality of life. *Conclusion*. Oral health has a high impact on the quality of life of cirrhotic patients. The psychological dimension of oral health is the most debilitating factor affecting the quality of life. This shows the importance of professional oral care, oral health, and self-care education in this group of patients.

1. Introduction

Cirrhosis or chronic liver failure (CLF) is the liver end-stage disease that is manifested by damage to the tissue and structures of this organ. The only treatment at this stage remains to be liver transplantation surgery [1]. The consequence of this disease can affect all body structures including the oral mucosa, jawbones, and teeth [2, 3].

The period of illness before transplantation is associated with increased physical, psychological, and social stress. This stress is due to complications such as impaired sexual function, change of appearance, pain, limited social interactions, and reduced job satisfaction. These complications increase emotional stress, anxiety, and depression. They also reduce adaptation and self-confidence, and above all, they decrease self-care behaviors, especially in the field of oral and dental health [3–7]. One of the important outcomes of oral diseases and poor oral health is the psychological and social impact on an individual's life [8–10].

Studies reveal that saliva production is reduced in CLF patients. This results in an increase in the rate of dental caries and opportunistic infections such as fungal-related lesions [3]. It is ascertained that oral infections affect the success of future transplant surgery. Thus, the importance of oral health in CLF patients is further elucidated [2, 11].

Health-related quality of life (HRQOL) of CLF patients is an important issue that has been addressed in many previous studies [4, 5]. HRQOL means a person's perception and

satisfaction of his physical and mental characteristics, from which he is able to perform his daily activities. This definition includes physical, mental, psychological, and social health as well as the ability to perform satisfactory daily actions [12].

Nowadays, with the importance of a patient-centered approach in clinical decisions, attention to oral health-related quality of life (OHRQOL) plays a special role in patient care [9]. OHRQOL measures the effect of various oral diseases, as well as the impact of preventive programs and dental treatment interventions on the quality of life of individuals [13]. Tooth decay and periodontal problems cause physical, functional, and biological complications. They also affect the economic, social, and psychological dimensions of patients [8, 14].

Numerous instruments have been proposed for measuring OHRQOL. Oral Health Impact Profile (OHIP-14) is a 14-item questionnaire that addresses the limitations, disabilities, and discomforts related to oral disease. Higher scores indicate a greater problem in oral health [13]. Furthermore, the GOHAI (Geriatric Oral Health Assessment Index) questionnaire is also an assessment tool for examining the relationship between oral diseases and quality of life in the elderly. This tool also addresses three main dimensions: (1) physical function, (2) psychosocial function, and (3) pain or discomfort [15].

Accordingly, considering the importance of oral health in liver transplant success and its consequent impact on the quality of life of these patients, we decided to design this study to assess the effects of oral health status and disease severity on OHRQOL of candidates for liver transplant surgery.

2. Patients and Methods

2.1. Study Group. Liver transplantation in Iran is centralized in Nemazee Hospital, Shiraz, southern Iran. This cross-sectional study enrolled eligible candidates for liver transplantation who were referred to Imam Reza Dental Clinic in Shiraz, Iran, for pretransplant dental evaluation in summer 2019. The inclusion criteria were adult patients above 18 with the initial diagnosis of chronic liver failure confirmed by pathologic evaluation and clinical examination by a member of the transplant team. Patients with a history of head and neck trauma, major systemic problem causing changes in pain sensation, fibromyalgia, edentulous subjects, any systemic disease affecting the dentition and oral structures (such as diabetes mellitus, oral lichen planus, lichenoid reactions, pemphigus vulgaris, AIDS, history of head and neck radiation, Sjogren's syndrome, and Behçet's disease), use of any medication with known effects on the oral cavity (antidepressants and tranquilizers), and individuals who were not willing to participate in the study were excluded. Further, patients were initially examined, and if any sign of oral dryness and dental anomalies were detected, they were also excluded from the study.

2.2. Ethical Considerations. Written informed consent was obtained from all patients who participated in the study. All information about individuals was coded and kept confidential. This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.DENTAL.REC.1398.071).

2.3. Data Collection Procedure

2.3.1. Sociodemographic and Disease Characteristics. Age, gender, level of education, smoking status, and duration of disease were recorded from the patients' medical records and direct interviews. Chronic liver disease patients were categorized according to the severity of liver disease using the model for end-stage liver disease (MELD) scoring system. The MELD score in the present study was calculated with the blood creatinine, bilirubin, and INR (international normalized ratio) values recorded at the time of listing for liver transplant surgery by the transplant team. MELD scores were divided into three groups: low (MELD < 10), medium (MELD 11–18), and high (MELD 19–40).

2.3.2. Dental Evaluation. Panoramic radiography was performed for all patients, along with a thorough dental examination by an oral and maxillofacial medicine specialist to record the DMFT (decayed-missing-filled tooth) index. Note that a panoramic view is ordered for all patients (dentate and edentulous) prior to transplant surgery to rule out any source of dental and bone pathologies and infections in the maxillary plus mandibular region.

2.3.3. OHRQOL Assessment. We used the OHIP-14 questionnaire and the GOHAI (Geriatric Oral Health Assessment Index) questionnaire to assess OHRQOL. The OHIP-14 questionnaire consists of 14 five-choice questions. The scores in this questionnaire are coded as follows: 5 = very often, 4 = fairly often, 3 = occasionally, 2 = hardly ever, and 1 = never. This questionnaire covers 7 aspects of OHRQOL including functional limitations, physical pain, mental distress, physical disability, mental disability, social disability, and handicap. In this questionnaire, all questions have a negative impression, so the score of all questions with good oral conditions is inverse. Thus, higher scores (range 14-70) would indicate a lower level of OHRQOL. The validity of this questionnaire has been confirmed in previous studies, and its Persian format is available [16]. GOHAI (Geriatric Oral Health Assessment Index) addresses 3 dimensions of quality of life: physical (physical), social and psychological (psychosocial), and pain and discomfort (pain and discomfort). This questionnaire has twelve items previously used for the elderly, but they are now available for all ages. The same scoring system as the OHIP-14 was used with higher scores (range 12-60) indicating a lower level of OHROQL. The validity of this questionnaire has been confirmed in previous studies, and its Persian format is available [17]. Illiterate patients were interviewed for both questionnaires.

2.4. Data Processing and Analysis. Finally, statistical data were collected with SPSS software version 24 (SPSS Inc., Chicago, IL, USA) used for data analysis. Descriptive statistics including frequency and mean levels were used to describe the data. The Spearman correlation coefficient was also employed to investigate the relationship between the DMFT indices and the quality of life. One-way ANOVA was utilized

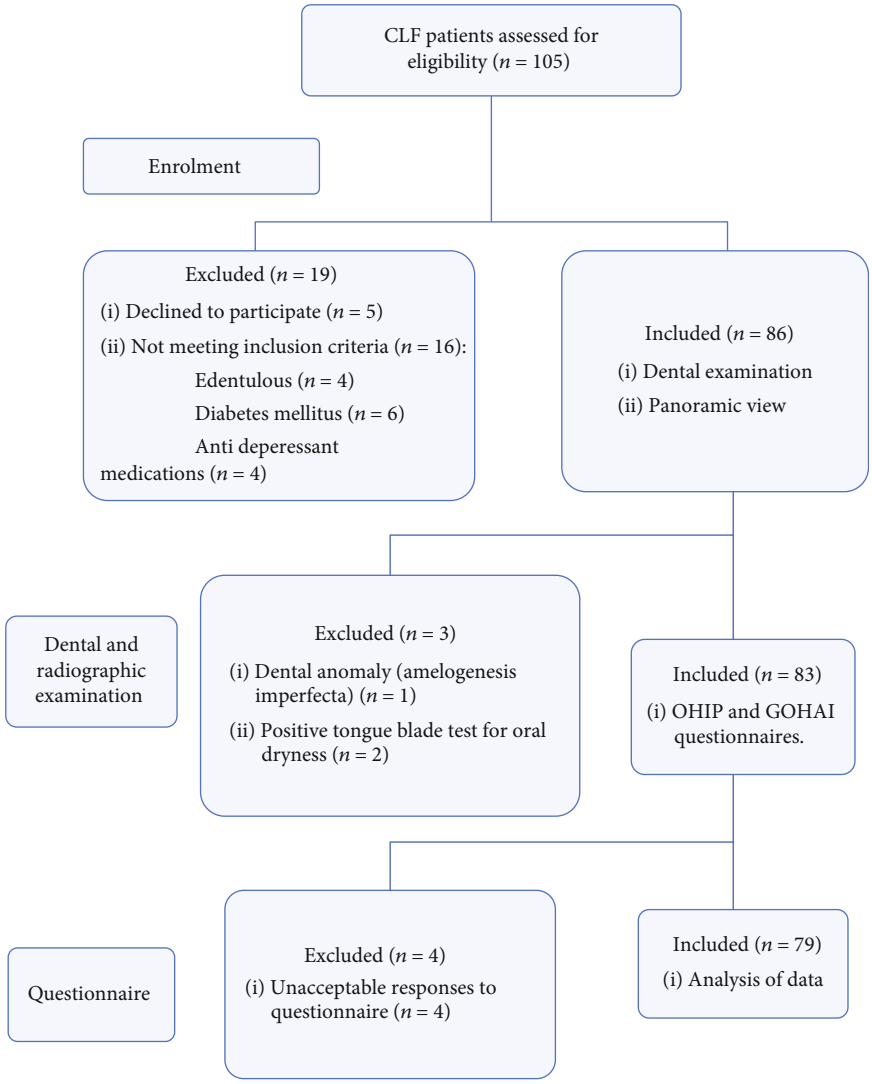

Figure 1: Flow chart of participants included in the study.

to compare the groups given the normality of the variables. A significance level of less than 5% was considered significant.

3. Results

3.1. Sociodemographic Characteristics. A total of 79 completed questionnaires were acceptable to be enrolled in the study, as seen in Figure 1. The distribution of chronic liver failure patients by gender, age, education, duration of disease, and smoking status is listed in Table 1. Based on this table, men over 50 are more likely to be candidates for liver transplant surgery. Furthermore, almost 65% of the patients had only middle school education and 87% were nonsmokers.

3.2. OHRQOL Characteristics. The mean score for the OHIP-14 questionnaire in CLF patients was 25.00 ± 10.02. This score was 23.54 ± 8.27 for the GOHAI questionnaire. The responses to the OHIP-14 items are represented in Table 2. As shown, the distribution of the patients' responses is almost uniform to all the OHIP-14 items. The mean scores for each question ranged between 1.25 for totally unable to function and 2.64 for having been self-conscious because of their teeth, mouth, or partial dentures. The major complaint reported by the patients as "never" was related to having trouble pronouncing any words (84.8%), followed by totally unable to function (83.5%) and unsatisfactory diet (83.5%). The major complaints reported by the patients as "very often" were becoming self-conscious (13.9%) and being uncomfortable when eating any foods (13.9%), followed by feeling tense (12.8%). The GOHAI distribution of answers was almost similar to the OHIP-14 questionnaire distribution.

3.3. The Effects of Different Variables on OHRQOL. The results of this study revealed that there is no significant difference between the mean quality of life based on gender, age, smoking, and MELD score according to both the OHIP-14 and GOHAI questionnaires ($P > 0.05$). This relationship, however, was significant for the level of education by the GOHAI questionnaire ($P = 0.020$). Disease duration was also significantly related to OHRQOL in this group ($P = 0.017$).

Table 1: OHRQOL according to OHIP-14 and GOHAI in CLF patients, based on gender, age, level of education, smoking, and disease duration.

Chronic liver failure patients		Number	Percentage	Mean OHIP-14	Mean GOHAI
Gender	Male	63	79.7	24.55 ± 9.15	23.93 ± 7.38
	Female	16	20.3	26.75 ± 13.08	22.0 ± 11.28
	P value (ANOVA)			0.438	0.407
Age	Below 30	20	25.3	24.65 ± 11.95	21.10 ± 8.24
	31-40	17	21.5	28.58 ± 3.12	26.82 ± 11.80
	41-50	16	20.3	24.6 ± 1.97	24.81 ± 8.03
	Above 50	26	32.9	23.50 ± 1.37	22.50 ± 4.45
	P value (ANOVA)			0.409	0.157
Education	Illiterate	2	2.5	18.50 ± 0.70	19.00 ± 7.07
	Middle school	51	64.6	25.25 ± 9.49	22.80 ± 7.39
	High school diploma	11	13.9	20.09 ± 7.48	20.09 ± 4.39
	Associate's degree	7	8.9	23.28 ± 12.12	26.57 ± 12.81
	Bachelor's degree and above	8	10.1	33.25 ± 11.75	31.50 ± 9.33
	P value (ANOVA)			0.053	0.020*
Smoking	Yes	10	12.7	25.10 ± 9.73	24.20 ± 8.98
	No	69	87.3	24.98 ± 10.13	23.44 ± 8.23
	P value (ANOVA)			0.973	0.791
Disease duration	Less than a year	20	25.3	20.90 ± 7.67	22.75 ± 7.86
	1-2 years	13	16.5	30.76 ± 10.98	28.46 ± 14.34
	2-3 years	8	10.1	29.87 ± 4.02	23.25 ± 6.08
	More than 3 years	38	48.1	24.15 ± 7.95	22.34 ± 8.91
	P value (ANOVA)			0.017*	0.132
MELD	Low	14	17.72	26.44 ± 12.18	23.47 ± 10.38
	Medium	35	44.30	23.33 ± 8.27	23.03 ± 6.77
	High	30	37.97	25.06 ± 7.54	24.73 ± 5.48
	P value (ANOVA)			0.470	0.812
Total		79	100	25.00 ± 10.02	23.54 ± 8.27

MELD = model for end-stage liver disease. *P value < 0.05 was considered significant.

The results of the Pearson correlation for the assessment of the DMFT index and OHRQOL revealed a significant relationship between the two variables based on the OHIP-14 questionnaire. This means that as the DMFT index in cirrhotic patients increased, so did the mean score of the OHIP-14 questionnaire, showing the reduction of OHRQOL ($P = 0.039$) (Table 3 and Figure 2).

4. Discussion

The results of this study revealed that most of the end-stage liver cirrhotic patients were male, were over 50 years old, and had not finished high school. Nearly half had been suffering from liver disease for more than 3 years. Interestingly, the results elucidated that oral and dental complications are effective in reducing the quality of life in patients suffering from CLF. This is also true for the level of education and duration of sickness, which can both significantly affect the OHRQOL in this group of patients. Furthermore, the major complaints reported by the patients were becoming self-conscious because of their teeth, mouth, or dentures.

There are many factors that initiate and accelerate the rate of dental caries: (1) genetic factors such as the immune system, saliva concentration, and composition, as well as teeth anatomy plus its hard tissue quality; and (2) environmental factors such as nutrition, oral hygiene, socioeconomic level, and mental status [18]. According to the mentioned factors, it should be expected that in a patient who has reached the end-stage of chronic disease, the immune system, nutritional status, and oral hygiene are highly affected, and therefore, the incidence of dental cavities, periodontal problems, and DMFT rate increased [19]. As previously shown, end-stage liver disease patients are prone to oral infections compared to healthy individuals [3]. Furthermore, periapical

Table 2: The mean and percentage of answers to the Oral Health Impact Profile (OHIP-14) questionnaire.

Dimension	Variables	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	Mean score
Functional limitation	Have you had trouble pronouncing any words because of problems with your teeth, mouth, or dentures?	84.8	1.3	10.1	3.8	—	1.32
	Have you felt that your sense of taste has worsened because of problems with your teeth, mouth, or dentures?	75.9	3.8	12.7	—	7.6	1.59
Physical pain	Have you had painful aching in your mouth?	45.6	8.9	26.6	12.7	6.3	2.26
	Have you found it uncomfortable to eat any foods because of problems with your teeth, mouth, or dentures?	55.7	2.5	24.1	3.8	13.9	2.17
Psychological discomfort	Have you been self-conscious because of your teeth, mouth, or dentures?	55.7	2.5	24.1	3.8	13.9	2.64
	Have you felt tense because of problems with your teeth, mouth, or dentures?	62.8	10.3	6.4	7.7	12.8	1.96
Physical disability	Has your diet been unsatisfactory because of problems with your teeth, mouth, or dentures?	83.5	3.8	5.1	7.6	—	1.37
	Have you had to interrupt meals because of problems with your teeth, mouth, or dentures?	64.6	11.4	11.4	11.4	1.3	1.73
Psychological disability	Have you found it difficult to relax because of problems with your teeth, mouth, or dentures?	53.2	15.2	24.1	3.8	3.8	1.89
	Have you been a bit embarrassed because of problems with your teeth, mouth, or dentures?	62.0	5.1	16.5	7.6	8.9	1.96
Social disability	Have you been a bit irritable with other people because of problems with your teeth, mouth, or dentures?	65.4	12.8	14.1	2.6	5.1	1.68
	Have you had difficulty doing your usual jobs because of problems with your teeth, mouth, or dentures?	72.2	12.7	10.1	3.8	1.3	1.49
Handicap	Have you felt that life in general was less satisfying because of problems with your teeth, mouth, or dentures?	69.6	10.1	5.1	11.4	3.8	1.69
	Have you been totally unable to function because of problems with your teeth, mouth, or dentures?	83.5	10.1	3.8	2.5	—	1.25
	Total						25.00

Never (=1), hardly ever (=2), occasionally (=3), fairly often (=4), and very often (=5).

Table 3: OHRQOL according to the DMFT index in CLF patients.

DMFT	Correlation coefficient	*P* value
OHIP-14	0.244	0.039*
GOHAI	0.110	0.359

*P value < 0.05 was considered significant.

lesions of teeth, which are the result of chronic dental infections, are more prevalent in this group [20, 21]. Hence, the existence of oral and dental problems causes pain and discomfort, disturbs the patient's nutrition, and affects appearance and esthetics, all of which can influence their quality of life [3, 20].

We found that with an increase in the DMFT index, the quality of life was significantly reduced in CLF patients. Contrary to the results of this study, in a study conducted by Schmalz et al., OHRQOL was evaluated before and after liver transplant surgery. This study indicated a reduced OHRQOL compared to healthy individuals but not related to oral complications in this group of patients. They concluded that further studies with a larger population are warranted to confirm this matter [22]. Likewise, in the study of Mohammadzadeh et al., the components of the DMFT index by D, M, and F did not show a difference in terms of quality of life among the patients [14]. However, this study did not consider patients with systemic disease. A study with a similar result to the present study showed that factors such as tooth decay and bad breath in patients with oral complications can reduce the quality of life, as well as physiological and mental ability [23]. This is similar to our study which shows that the psychological aspects of oral health are the most debilitating factor in CLF patients. It is also proved that the reduced rate of caries and improved oral hygiene augment the quality of life [8].

Additionally, regarding the psychological aspect of oral health, our results are similar to other studies which found that becoming self-conscious claimed the largest score for answering "very often" [23]. Prior to the transplant procedure, a multidisciplinary evaluation is performed to assess the patient's suitability for this surgery. In this evaluation,

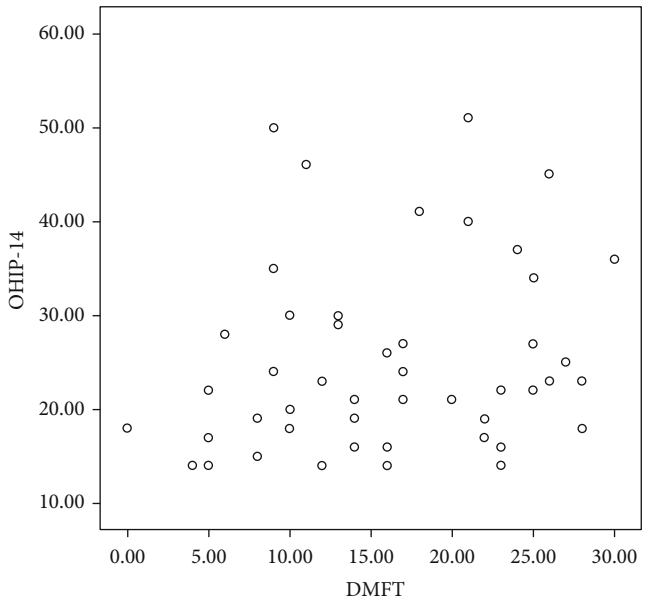

Figure 2: Correlation of OHIP-14 with the DMFT index in CLF patients.

the presence of psychological factors that could compromise the patient or graft survival must be ruled out. This highlights the importance of psychological health in CLF patients [24]. Our findings support the need for oral health education and oral hygiene instructions to reduce the psychological burden of oral complications in CLF patients.

The MELD score is used in CLF patients to show the severity of the disease and predict the overall prognosis. Many countries use it for the allocation of patients for liver transplant surgery. We did not find any relations between the MELD score and the OHRQOL. This is similar to the results of other studies which did not find any significant relations regarding MELD scores and oral health status [3, 21]. However, there are studies that did find a relationship between severity of liver disease (MELD score) and oral health status [24]. Note that neither of these studies evaluated OHRQOL.

In relation to patients suffering a systemic disease and quality of life, a study conducted by Cervino et al. elucidated that oral complications affect the quality of life in diabetic individuals [25]. Helenius-Hietala et al. and Zwiech and Bruzda-Zwiech in two separate studies examined the effect of oral and dental infections on the quality of life and course of disease of two groups of kidney and liver patients. Both studies showed that oral infections had a negative effect on disease improvement as well as on the quality of life [2, 11]. It is important to note that the presence of oral lesions in cirrhotic patients as the focus of infection can affect the prognosis of the future transplant procedure and would cause serious problems for patients.

In regard to the level of education, we found that chronic liver failure patients who had finished high school and patients who had bachelor's degrees and above had the lowest level of OHRQOL according to the GOHAI questionnaire. In general, it is stated that low levels of education have a negative impact on oral health-related quality of life [26, 27]. Other researchers have also shown that with higher education, patients' awareness of chronic diseases and their ability to cope with its complications increase, and hence, the quality of life will improve [28]. These findings are contrary to the results of the present study. We suggest that although educated patients are more aware of the complications and problems of the disease which in some cases helps improve their condition, the expectations of such people from life and its quality are far higher. Thus, in the case of a chronic disease with no permanent cure, such patients are driven away from their desired life expectations, which in turn directly reduces their quality of life. Nevertheless, note that the distribution of the education level was not homogenous in our study. We propose a larger sample size with sufficient participants in each group in future studies.

The present study showed that there is a significant difference between the duration of illness and the quality of life (OHIP). In a study conducted in 2017 by Busija et al., they also found a significant relationship between disease duration and quality of life of patients, which was also related to their age and the significant effect of disease duration on quality of life [29]. Our results revealed that patients who have been ill for less than a year or have had the disease for more than three years report a higher quality of life. This may be because in the first year patients are not yet fully aware of their disease and its complications. Further, they are not yet seriously involved in the side effects of the disease and medications, and there are still minor oral and dental problems. However, during the second year, patients are more entangled with the complications of the disease, and they are more driven away from social and individual activities, all inducing more anxiety, stress, sadness, and fear. On the other hand, patients whose disease duration has been extended are somehow more familiar with the treatment course of their disease and have become more adaptable with the complications. In other words, they have become more accustomed to the disease and have accepted it.

Since CLF patients suffer from a chronic disease and its complications, encouraging the patients to participate in the study was somehow difficult. So, further studies with larger sample sizes are suggested. It is also suggested that, in future studies, the effects of oral hygiene habits and nutritional status of the patients should be considered on OHRQOL.

5. Conclusion

Finally, we can conclude that the quality of life related to oral health in candidates for liver transplant surgery is affected by their education and the duration of the disease as well as the DMFT index. The psychological dimensions of oral health are the most debilitating aspect. This can affect the outcomes of the transplant procedure. Thus, we support the importance of oral hygiene instructions with emphasis on self-care in end-stage liver cirrhotic patients to reduce its psychological aspect and its impact on the success of the transplant surgery.

Data Availability

Other data used to support the findings of this study are available upon request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Protective Role of Probiotic Supplements in Hepatic Steatosis: A Rat Model Study

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Background. Treating nonalcoholic fatty liver disease (NAFLD) is considered one of the public health priorities in the past decade. So far, probiotics have represented promising results in controlling the signs and symptoms of NAFLD. However, attempts to find the ideal probiotic strain are still ongoing. The present study is designed to find the best strain amongst suitable probiotic strains according to their ability to ameliorate histopathological and oxidative stress biomarkers in hepatic steatosis-induced rats. *Methods*. Initially, four probiotics species, including *Lactobacillus* (*L.*) *acidophilus*, *L. casei*, *L. reuteri*, and *Bacillus coagulans*, were cultured and prepared as a lyophilized powder for animals. The experiment lasted for fifty days. Initially, hepatic steatosis was induced by excessive ingestion of D-fructose in rats for eight weeks, followed by eight weeks of administering probiotics and D-fructose concurrently. Forty-two six-week-old male rats were alienated to different groups and were supplemented with different probiotics ($1 * 10^9$ CFU in 500 mL drinking water). After eight weeks, blood and liver samples were taken for further evaluation, and plasma and oxidative stress markers corresponding to liver injuries were examined. *Results*. Administration of probiotics over eight weeks reversed hepatic and blood triglyceride concentration and blood glucose levels. Also, probiotics significantly suppressed markers of oxidative stress in the liver tissue. *Conclusions*. Although some of the single probiotic formulations were able to mitigate oxidative stress markers, mixtures of probiotics significantly ameliorated more symptoms in the NAFLD animals. This enhanced effect might be due to probiotics' cumulative potential to maintain oxidative stress and deliver improved lipid profiles, liver function markers, and inflammatory markers.

1. Background

Nonalcoholic fatty liver disease (NAFLD) is amongst the most prevalent origins of chronic liver disease, which happens to be one of the public health priorities in this century [1]. Despite many efforts and the introduction of several molecular-targeted therapeutic agents, no effective treatments for NAFLD have been provided so far. To provide a new strategy, much attention has been focused on the relationships between NAFLD and the gastrointestinal microbiome [2]. Previous studies have stated that two main risk factors related to NAFLD, e.g., diabetes and obesity, are associated with alterations in the gut microbiome and overgrowth of pathogens in the small intestine [3]. Although the association between the pathology of NAFLD and the gut microbiota is still unknown, it has been found that the microbial overgrowth and their metabolites can lead to overwhelming inflammation due to liver damage [4]. When encountering enteric pathogens, the intestinal epithelium releases inflammatory and pro-inflammatory cytokines [5]. Also, according to previous

studies, an increase in oxidative stress, inflammatory response, and prolipogenic status is observed in NAFLD [6].

Gut microbiota helps the host organisms against pathogens by making a protective barrier and preventing the disruption or loss of intestinal microflora. It is assumed that any alterations in lifestyle and certain dietary habits may trigger some severe disorders such as NAFLD [7, 8]. As stated before, an increase in the intake of energy or refined carbohydrates such as sugar or syrups rich in fructose is observed in most NAFLD cases [9].

The presence of probiotics in appropriate amounts contributes to the general health of the host by creating symbiotic relationships [10]. Probiotics as living and safe organisms which endow many beneficial effects to their hosts and increase their immune system are widely accepted as a natural treatment against metabolic syndromes, diabetes [11], osteoporosis [12], and other related disorders. Literature reviews showed that certain probiotic strains, such as *Lactobacillus* and *Bifidobacterium*, can protect mice against the onset of fructose-induced NAFLD [13]. *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* decrease the levels of faecal TNF-α by inhibiting the pathogens binding to the gastrointestinal tract (GI) linings. Also, consistent effects were observed in obese patients with NAFLD who were treated by these two organisms [14]. The effectiveness of probiotics on certain diseases generally depends on many factors, including the bacterial strain, concentration of probiotics, route of administration, age, and diet of the host [15]. As the data about the effectiveness of different types of probiotics are highly diverse and controversial [16], investigating appropriate probiotics for the prevention or treatment of NAFLD can be helpful for both health professionals and the general public.

The consumption of high fructose can cause insulin resistance (IR), excessive production of reactive oxygen species (ROS), hepatic steatosis, liver malfunction, and depletion of the hepatocyte population [17, 18]. Oxidative stress, induced by a high-energy diet, builds up to the genesis and progression of steatohepatitis from steatosis [19]. Based on previous studies, oxidative stress is implicated in the pathogenesis of NAFLD [20]. There have been several animal and clinical studies that clarify correlations between conducted tests, including oxidative stress biomarkers and liver pathogenicity [21].

This research is aimed at examining the impacts of some probiotic species on fatty acid profile and liver functions in the hepatic steatosis rats in order to understand the protective role of probiotics in the prevention and genesis of liver dysfunctions, especially in NAFLD. We were mainly focused on the effects of single and mixture formulations of these probiotics in NAFLD. Different results regarding the administration of probiotics and oxidative stress markers are highlighted and discussed. This research may help answer questions regarding the role of the abovementioned probiotics in the prevention of NAFLD in rats.

2. Methods

2.1. Chemicals. A list of the chemicals used is provided in the supplementary table (Table S1). Kits used for the examination of the hepatic malfunction biomarkers such as triglyceride (TG), alkaline phosphatase (ALT), and glucose were purchased from Pars Azmun® Co. (Iran). Materials used for the buffer preparations were purchased from Merck KGaA (Darmstadt, Germany). All cultures used for bacterial inoculum preparation, including De Man, Rogosa, and Sharpe (MRS) agar, L-S differential (LS), trypticase soy powders were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microbial Identification. Initially, 20 different samples of traditional fermented yoghurt were gathered from the north coast of the Persian Gulf. The samples were kept at 4°C. Ten grams of each yoghurt sample was homogenized using a laboratory mixer after being diluted in peptone solution (4%) and sterilized water. LS medium, as a differential medium for the growth of *Streptococcus*, and *Lactobacillus* were used. For the specific isolation of lactobacilli, MRS agar was employed.

After the incubation of plates under anaerobic conditions for three days (at 37°C), the isolates were identified according to their biochemical, cultural, and morphological properties based on Bergey's Manual of Systematic Bacteriology [22]. Several biochemical tests, including the Voges-Proskauer (VP) test, nitrate reduction, resistance to bile salts, sugar fermentation, and motility were conducted on the probiotics in order to confirm the bacterial strains (Table 1) [23].

Also, 16S rRNA gene sequence analysis was done for the molecular identification of isolates. The purified isolates were diluted in a saline solution. Then, the isolates were centrifugated at 4500 g for 10 min using a refrigerated laboratory centrifuge and washed several times before preparing for PCR amplification. Heat shock method was used for DNA extraction, and two universal forward and reverse primers were used for amplification of 16SrDNA sequence of bacteria with the following sequences:

(i) F: 5′-ACGGGCGGTGTGTAC-3′

(ii) R: 5′-CAGCCGCGGTAATAC-3′

Amplification buffer contains 4 ng whole genome of bacteria, 2.5 units of DNA polymerase (Taq), 400 nM of each primer, and excess dNTP which finally reaches to 50 μL. Routine PCR protocol, according to Gholami et al. [24], was applied for the amplification process during 20 cycles. The PCR products were then placed in a Tris/borate/ethylene-diamine tetra-acetic acid (EDTA) buffer containing 1 μg/mL ethidium bromide and electrophoresed using 1% agarose gel and visualized by a UV apparatus. The DNA sequence, which was about 800 bp in length, was retrieved by a DNA gel purification kit (AccuPrep®, Bioneer, Korea) and sent to the CinnaGen Co. for sequencing determination [24]. The obtained sequence was analyzed using bioinformatic tools in the NCBI database and finally submitted after performing the steps. Accordingly, the isolated strains were identified, and four probiotic strains, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans* were selected for animal studies.

Table 1: The biochemical and physiological characteristics of the probiotics used in this study.

Test	*B. coagulans*	*L. reuteri*	*L. acidophilus*	*L. casei*
Motility	*Yes*	*No*	*No*	*No*
Catalyze	*Yes*	*No*	*No*	*No*
Oxidase	*No*	*Yes*	*No*	*Yes*
Lactose	*No*	*Yes*	*No*	*Yes*
Fructose	*Yes*	*No*	*Yes*	*Yes*
Glucose	*Yes*	*Yes*	*Yes*	*Yes*
Galactose	*Yes*	*No*	*No*	*Yes*
Cellobiose	*Yes*	*No*	*Yes*	*Yes*
Sorbose	*No*	*No*	*No*	*No*
Maltose	*Yes*	*Yes*	*Yes*	*Yes*
Sucrose	*Yes*	*Yes*	*Yes*	*Yes*
Mannose	*Yes*	*No*	*Yes*	*Yes*
Cellulose	*No*	*Yes*	*No*	*No*
Trehalose	*No*	*No*	*Yes*	*Yes*
Xylose	*No*	*Yes*	*No*	*No*
Melezitose	*Yes*	*No*	*Yes*	*Yes*
Melibiose	*No*	*Yes*	*No*	*Yes*
Arabinose	*Yes*	*Yes*	*Yes*	*No*
Ribose	*Yes*	*Yes*	*Yes*	*Yes*
Raffinose	*No*	*No*	*Yes*	*No*
VP	*Yes*	*Yes*	*No*	*No*
Nitrate reduction	*No*	*No*	*No*	*No*
Gas production from glucose	*No*	*Yes*	*No*	*Yes*
Resistance to bile salts	*Yes*	*Yes*	*Yes*	*Yes*
Growth at 15°C	*Yes*	*Yes*	*No*	*Yes*
Growth at 45 °C	*Yes*	*Yes*	*No*	*Yes*
Motility	*Yes*	*No*	*No*	*No*
Catalyze	*Yes*	*No*	*No*	*No*
Oxidase	*No*	*Yes*	*No*	*Yes*
Lactose	*No*	*Yes*	*No*	*Yes*
Fructose	*Yes*	*No*	*Yes*	*Yes*
Glucose	*Yes*	*Yes*	*Yes*	*Yes*
Galactose	*Yes*	*No*	*No*	*Yes*
Cellobiose	*Yes*	*No*	*Yes*	*Yes*
Sorbose	*No*	*No*	*No*	*No*
Maltose	*Yes*	*Yes*	*Yes*	*Yes*
Sucrose	*Yes*	*Yes*	*Yes*	*Yes*
Mannose	*Yes*	*No*	*Yes*	*Yes*
Cellulose	*No*	*Yes*	*No*	*No*
Trehalose	*No*	*No*	*Yes*	*Yes*
Xylose	*No*	*Yes*	*No*	*No*
Melezitose	*Yes*	*No*	*Yes*	*Yes*
Melibiose	*No*	*Yes*	*No*	*Yes*
Arabinose	*Yes*	*Yes*	*Yes*	*No*
Ribose	*Yes*	*Yes*	*Yes*	*Yes*
Raffinose	*No*	*No*	*Yes*	*No*
VP	*Yes*	*Yes*	*No*	*No*
Nitrate reduction	*No*	*No*	*No*	*No*

Table 1: Continued.

Test	*B. coagulans*	*L. reuteri*	*L. acidophilus*	*L. casei*
Gas production from glucose	*No*	*Yes*	*No*	*Yes*
Resistance to bile salts	*Yes*	*Yes*	*Yes*	*Yes*
Growth at 15 °C	*Yes*	*Yes*	*No*	*Yes*
Growth at 45 °C	*Yes*	*Yes*	*No*	*Yes*

2.3. Formulations. Four different probiotic strains, namely, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bacillus coagulans*, and a mixture of these strains were cultured, centrifugated, freeze-dried, and designed at a concentration of 1×10^9 probiotics/mL. The probiotic strains were freshly prepared every day and dispersed in drinking water containing 20% of D-fructose.

2.4. Experimental Animals. Forty-two, male, 42-day-old Sprague-Dawley rats (av. weight of about 90 g) were obtained from the Razi Institute in Shiraz, Iran. Rats were observed for several days after their arrival to the laboratory and given seven days to familiarize themselves with their new setting. They were placed alone in metal cages at controlled room temperature, and their living conditions were considered as a 12-hour light-dark cycle with a humidity of around 50%. The animals were then randomly divided into the following seven experimental groups:

(i) Group 1: received *Lactobacillus acidophilus* + high-fructose regimen

(ii) Group 2: received *Bacillus coagulans* + high-fructose regimen

(iii) Group 3: received *Lactobacillus casei* + high-fructose regimen

(iv) Group 4: received *Lactobacillus reuteri* + high-fructose regimen

(v) Group 5: received a mixture of the mentioned probiotics inclusive of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans* + high-fructose regimen

(vi) Group 6: received high-fructose regimen

(vii) Group 7: received drinking water

Group 6 was considered a positive control and group 7 a negative control.

All rats were exposed to standard diet and water concurrently with no limitations. Food and water did not differ between different groups and were monitored. This study was conducted at the Center of Comprehensive Experimental Medicine, Shiraz, Iran, from November 2018 until March 2019.

2.5. Diet Preparations. Initially, daily water consumption was measured, and the average was calculated. Each rat drank about 70 mL of water daily. In the beginning, 15 mg D-fructose was allocated to each rat and diluted in the drinking water once a day, freshly made every day. As the rats got more massive, the amount was calculated based on the weight/volume formula. Each rat received a minimum of $1 * 10^9$ CFU/mL of probiotics (single or mixed) in drinking water containing 20% of D-fructose freshly prepared every day before noon. D – fructose > 99% (Merck KGaA, Darmstadt, Germany) was used in drinking water to induce NAFLD. Drinking water containing fructose was freshly prepared and administered every day based on the weight/volume formula.

Rats had free access to a standard pellet diet consisting of 20% protein; 6.0% fat; 10.0% crude fibre; 5.0% crude ash; 0.6% calcium; 0.4% phosphorus; 0.9% sodium; and 0.5-1% moisture and other nutritional additives inclusive of vitamin A, vitamin D3, manganese, zinc, and selenium. A 15-gram portion of this pellet has 60 calories.

2.6. Surgical Protocol. This experiment lasted for sixteen weeks, and on the last day, rats were euthanized after being anaesthetized by injection of 80 mg/kg thiopental intraperitoneally. It was ensured that unconsciousness persisted until death occurred with minimum pain and distress. All animals were behaved and sacrificed following the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The experimental procedures were all performed according to the approval of the Ethical Committee of Shiraz University of Medical Sciences under code no. 97-01-33-15624.

2.7. Blood and Tissue Sampling. The liver and blood samples were gathered from rats anaesthetized by intraperitoneal injection of thiopental at the end of the experiment. The blood samples, obtained from the inferior vena cava, were put in the gel separator/clot activator vacuum tubes. To obtain blood serum, all blood samples were centrifuged (15 min at 4°C, 5000 g). A standard diagnostic kit (Pars Azmun® Co., Iran) and an autoanalyzer (Mindray BS-200®, China) were applied for measuring ALT, TG, and glucose levels [25]. During the experiment, each animal's body weight was monitored every week, and any weight change was recorded.

2.8. Oxidative Stress Assays

2.8.1. The Antioxidant Power of the Liver. Ferric-reducing/antioxidant power (FRAP) assay was applied to calculate the antioxidant capacity of the animal liver after the consumption of probiotics [26]. FRAP reagent is composed of three solutions, including the TPTZ solution, the 20 mmol/L ferric chloride solution, and the 300 mmol/L acetate buffer (pH = 3.6), that are combined at a ratio of 1/1/10,

respectively. The TPTZ solution was prepared by mixing 10 mmol/L TPTZ in 40 mmol/L hydrochloric acid. All solutions were freshly made just before the assay was conducted.

A total of 500 mg liver tissues was dissolved in Tris-HCl buffer. After homogenization, 50 μL of homogenates was dissolved in 150 μL deionized water and transferred to 1.5 mL of the FRAP solution and incubated in a dark environment (37°C for 5 min). In this study, absorbance was measured using a microplate ELIZA reader (BioTek®, US) at 593 nm.

2.8.2. Liver Glutathione Assay. To measure the liver glutathione content, initially, 200 mg of liver tissue samples was added to the EDTA solution (8 mL, 40 mmol/L) and conducted to the homogenization process [27]. A total of 5 mL samples was added to 4 mL of distilled water and TCA (1 mL, 50% *w*/*v*) at 4°C. After vortexing and centrifugating at 10000 g for 15 min using a refrigerated centrifuge, this was followed by mixing the supernatant with a solution consisting of Tris-HCl buffer (4 mL), DTNB (100 μL), and 1 mL methanol. A microplate ELIZA reader (BioTek®, USA) was used to measure the absorbance of the solution at 412 nm.

2.8.3. Peroxidation of Liver Lipids. A total of 500 mg homogenate liver samples was dissolved in potassium chloride solution at 4°C and transported to a solution containing thiobarbiturate and phosphoric (V) acid at a ratio of 1 : 3 *v*/*v*. After boiling the mixture for 45 minutes, vigorous mixing was applied to add 2 mL n-butanol, and the liver specimens were centrifugated at 10000 g for 5 min. A microplate ELIZA reader (BioTek®, USA) was used to measure the absorbance of the sample at 532 nm [28].

2.8.4. Liver ROS. According to Jamshidzadeh et al., 200 mg of liver specimens was dissolved in Tris-HCl buffer at a ratio of 1 : 10 *w*/*v* at 4°C; then, this was added to 5 μL DCFH-DA (10 mmol/L) and incubated for 30 minutes at 37°C. A FLUOstar Omega® Microplate Reader (Germany) was used to measure the intensity of fluorescence of the samples at $\lambda_{\text{excitation}} = 485$ nm and $\lambda_{\text{emission}} = 525$ nm [29].

2.9. Carbonylation of Liver Proteins. To measure the carbonylated protein level of the hepatic tissues, according to Colombo et al., a spectrophotometric test was applied when the liver proteins were carbonylated by the assistance of 2,4-dinitrophenyl hydrazine (DNPH) [30]. A total of 0.5 g liver specimens was homogenized in 0.1 M sodium phosphate buffer (containing 0.1% Triton X-100, pH = 7.4) and a sample of 0.5 mL of the liver homogenate was added to 0.5 mL of 0.1% DNPH (*w*/*v* in 2.0 N HCl). This mixture was incubated for 60 minutes in the dark at 24°C. Total liver proteins were precipitated by the addition of 0.5 mL TCA, and then, this mixture was quickly centrifugated at 10000 g for 5 min. The biomass was collected, and the supernatant was washed several times and discarded using a 1 mL ethanol : ethyl acetate solution; then, the pellets were dispersed again in 1 mL Tris buffer. An Ultrospec 2000® Spectrophotometer (Pharmacia Biotech, Sweden) was used to record the absorbance at 370 nm.

2.10. Statistical Analysis. The data were statistically analyzed using a one-way analysis of variance (one-way ANOVA), followed by the Tukey method for post hoc analysis, using SPSS IBM software version 23, and the results were displayed as mean ± SEM. Values were considered significant when $P < 0.05$.

3. Results

3.1. Biochemical and Molecular Tests for Bacterial Identification. After conducting several identification tests, the results indicated that the nitrate reduction test was negative for all the strains, and the test for determining the resistance to bile salts showed positive results for all. The VP test was positive for *Lactobacillus reuteri* and *Bacillus coagulans* strains and negative for all the other strains. The *Bacillus coagulans* strain showed positive results for the motility test (Table 1). Also, the PCR sequences were examined and then submitted in the NCBI databases under accession numbers MN658702, MN658703, MN658704, and MN658705. The similarity of the sequences was analyzed using the BLAST bioinformatics tools. According to all identification tests, four microbial probiotic isolates, including *L. acidophilus*, *L. casei*, *L. reuteri*, and *B. coagulans*, were found. Other strain designation assays including physiological and molecular analyses as well as probiotics and safety property assays such as acid and bile tolerance, antibacterial activity, antibiotic susceptibility testing, catalase, and hemolytic test and MTT assay were previously tested and reported by our team [12, 23].

3.2. Animal Studies. According to Figure 1, the weight of the animals increased by 77.2 ± 6% in the high-fructose diet (HFD) group (positive group) which had been significantly increased compared to the negative control group ($P < 0.05$). Mean body mass in groups 1, 2, 3, and 4 with a high-fructose plus probiotic diet were lower with 62.5 ± 6%, 80 ± 7%, 68.8 ± 5%, and 69 ± 6% differences, respectively, which had been significantly decreased compared to the HFD-only group ($P < 0.05$). Also, group 5, which received a mixture of probiotics, had a much lower mean body mass with a difference of 85% compared to the HFD-only group ($P < 0.05$).

As indicated in Figure 2(a), serum ALT levels had a 67.5 ± 5% increase in the high-fructose regimen group compared to the negative control group ($P < 0.05$). Serum ALT levels in groups 1, 2, 3, 4, and 5 receiving probiotics plus HFD-diet were significantly lower with a difference of 67.5 ± 5%, 69.5 ± 4%, 70.2 ± 5%, 69.5 ± 3%, and 67.5 ± 2%, respectively, compared to the HFD-only rats ($P < 0.05$).

As has been demonstrated in Figure 2(b), the serum triglyceride levels in the HFD-only group had a 31.8 ± 4% increase compared to the negative control group ($P < 0.05$). Serum triglyceride levels were significantly lower in the HFD plus probiotic groups compared to the HFD-only group and had differences of 32 ± 4%, 32.8 ± 5%, and 33 ± 2% in groups 1, 4, and 5, respectively ($P < 0.05$). The changes in groups 2 and 3 were nonsignificant ($P < 0.05$).

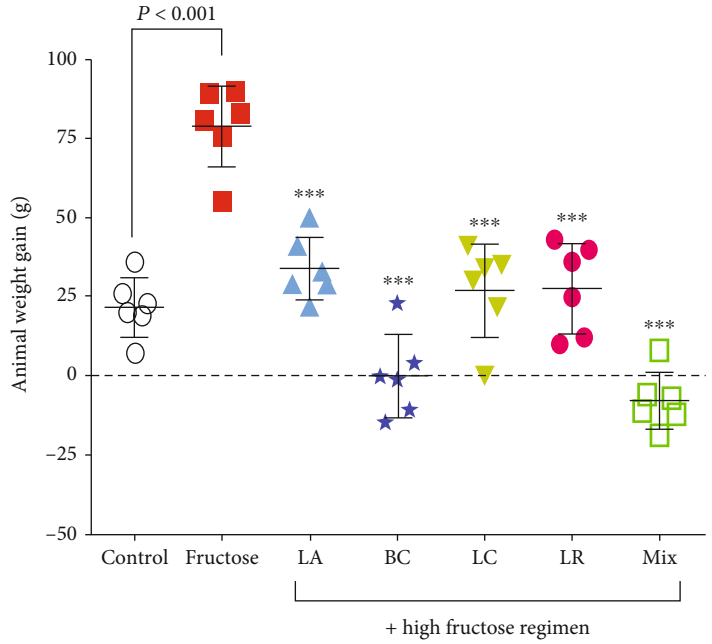


Figure 1: Animal weight gain in NAFLD rats and the effect of probiotic administration. Data are demonstrated as mean ± SEM ($n = 6$). Ctrl: control; LA: *Lactobacillus acidophilus*; BC: *Bacillus coagulans*; LC: *Lactobacillus casei*; LR: *Lactobacillus reuteri*; Mix: a mixture of probiotics including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans*. *** indicates a significant difference from the fructose control group ($P < 0.001$).

The serum glucose level in the HFD-only group was significantly increased by 80 ± 4% in comparison to the negative control group and had nonsignificant results in groups 1, 2, 3, and 4 ($P < 0.05$). However, the serum glucose level in the fifth group, which received a mixture of probiotics plus an HFD, was significantly lower with a difference of 33.3 ± 4% compared to the HFD-only group (Figure 2(c), $P < 0.05$).

The total antioxidant level in the liver tissue of group 5 showed a 36 ± 2% increase compared to the HFD-only group which had been statistically significant; also, the total antioxidant level in the HFD-only group was significantly lowered by 45 ± 4% in comparison to the negative control group ($P < 0.05$). No significant modifications occurred in all the other testing groups (Figure 3(a), $P < 0.05$).

According to Figure 3(b), the formation of reactive oxygen species (ROS) was significantly lowered with a difference of 20.8 ± 2%, 33.3 ± 3%, 37.5 ± 2%, and 50 ± 3% in groups 1, 2, 3, and 5, respectively, compared to the HFD-only group ($P < 0.05$). No significant modifications were noted in group 4, and an increase of 82.5 ± 2% in ROS formation occurred in the HFD-only group in comparison to the negative control group ($P < 0.05$).

The protein-carbonylation level of the liver tissue in groups that received probiotics plus an HFD was significantly decreased by 67.1 ± 3%, 47.8 ± 3%, 56.5 ± 1%, 57.3 ± 0.5%, and 78.2 ± 3% in groups 1, 2, 3, 4, and 5, respectively, in comparison to the HFD-only group ($P < 0.05$). This item was increased by 67.3 ± 5% in the HFD-only group compared to the control group (Figure 3(c), $P < 0.05$).

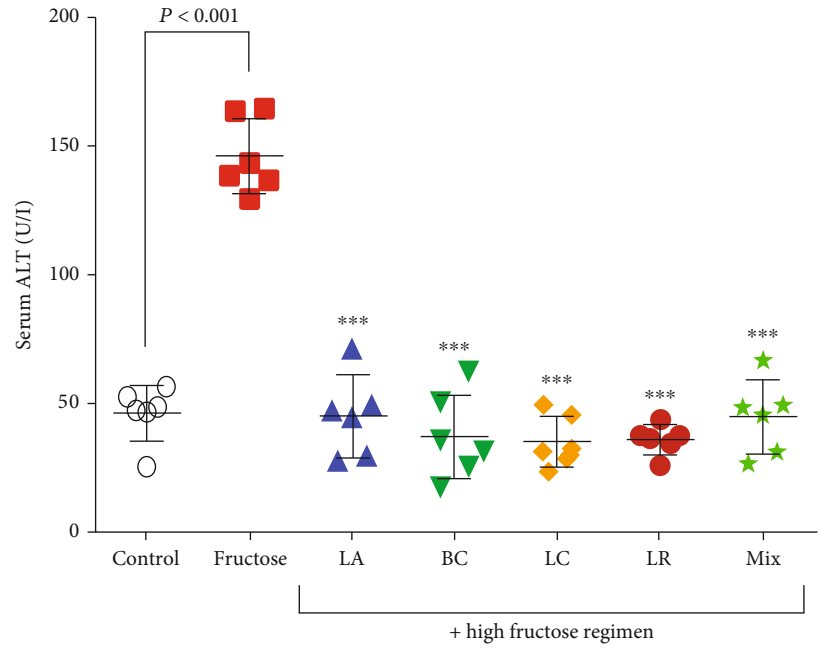
The lipid peroxidation levels were significantly lowered by 31 ± 1.5%, 32.8 ± 1%, 46.6 ± 0.5%, 31 ± 1%, and 62 ± 1%, respectively, in groups 1, 2, 3, 4, and 5, which received an HFD plus probiotic strains, compared to the HFD-only group ($P < 0.05$). This item was significantly increased by 82.8 ± 4% in the HFD-only group in comparison to the negative control group (Figure 3(d), $P < 0.05$).

The liver glutathione content in the HFD-only group was significantly decreased by 29.5 ± 3% compared to the negative control group and increased by 21.4 ± 2%, 25.6 ± 2%, and 23.6 ± 2% in groups 1, 3, and 5, respectively, compared to the HFD-only group ($P < 0.05$). The glutathione content did not have any significant changes in groups 2 and 4 (Figure 3(e), $P < 0.05$).

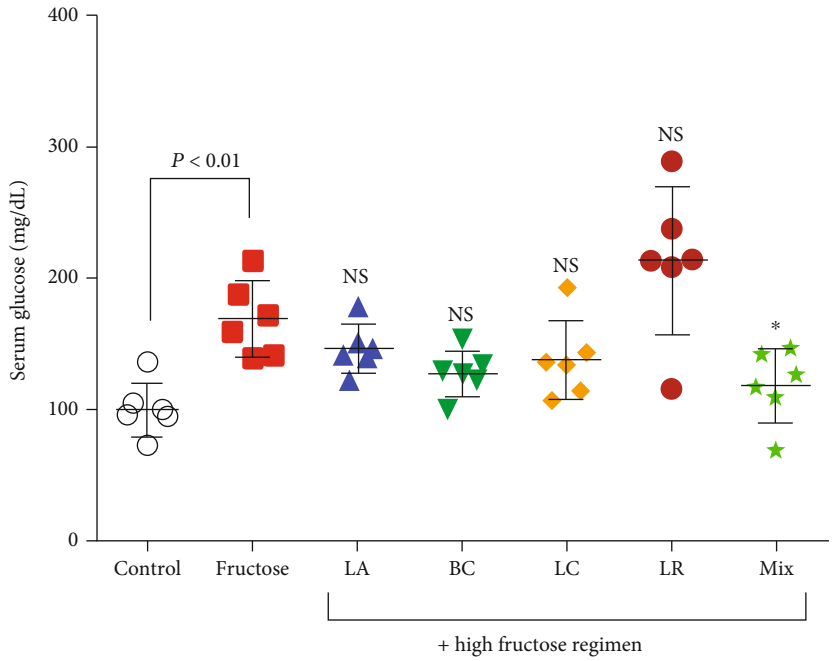
The liver tissue triglyceride levels were lowered with differences of 52 ± 2%, 61.6 ± 3%, and 60 ± 2% in groups 1, 4, and 5, compared to the HFD-only group, respectively, which was statistically significant (Figure 3(f), $P < 0.05$). Liver tissue triglyceride levels increased by 92 ± 6% in the HFD-only group compared to the negative control group ($P < 0.05$). The changes in groups 2 and 3 were nonsignificant ($P < 0.05$).

4. Discussion

In the past decade, research on probiotics has attracted much attention due to their protective role in NAFLD. The results of a research conducted by Yadav et al. showed that the probiotic "Dahi" containing *Lactobacillus acidophilus* and *Lactobacillus casei* improved parameters such as blood glucose and triglyceride levels and decreased the high-density lipoprotein cholesterol in animals suffering from metabolic syndrome [31]. Probiotics used in our study were gathered from organic yoghurts made in unique, organic dishes made from clay. Yoghurts were gathered from areas around the Persian Gulf and villages

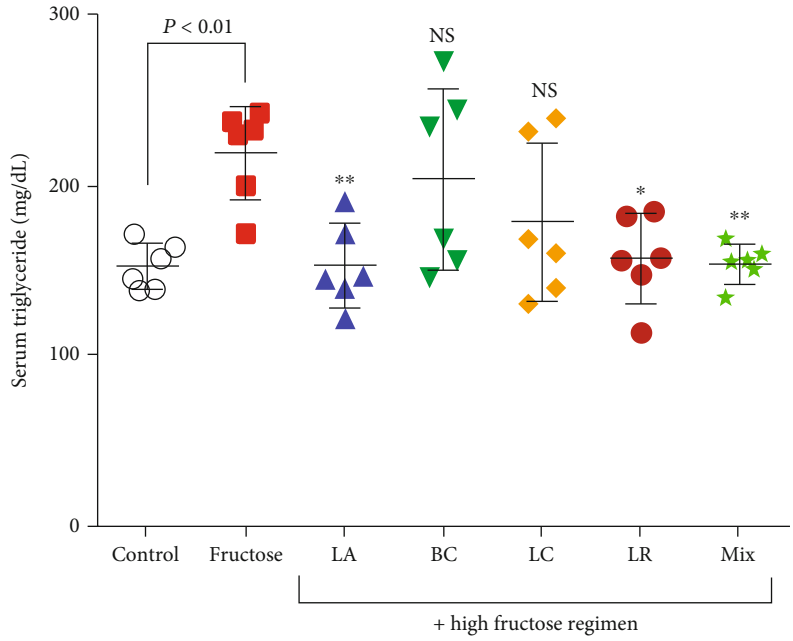


(a)



(b)

Figure 2: Continued.



(c)

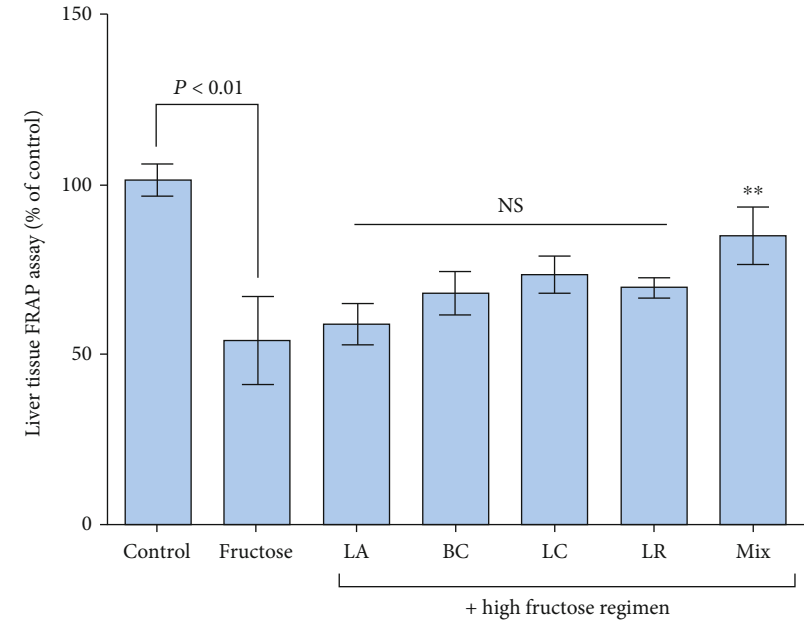
Figure 2: Serum biochemical changes in NAFLD rats and the effects of probiotic administration. (a) Serum alanine aminotransferase (ALT) test, (b) serum triglyceride level, and (c) serum glucose level. Data are demonstrated as mean ± SEM ($n = 6$). Ctrl: control; LA: *Lactobacillus acidophilus*; BC: *Bacillus coagulans*; LC: *Lactobacillus casei*; LR: *Lactobacillus reuteri*; Mix: a mixture of probiotics including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans*; ns: not significant. ∗ indicates a significant difference from the fructose control group ($P < 0.05$). ∗∗ indicates a significant difference from the fructose control group ($P < 0.01$). ∗∗∗ indicates a significant difference from the fructose control group ($P < 0.001$).

of Fars province, which have always been famous for having beneficial health effects, and people have been using them for years for different health complications such as diarrhoea, fatty liver diseases, or weight loss [32]. A majority of the locals believe that this type of yoghurt can prevent illnesses, but no experiments had supported this idea up until now [33]. On that account, initially, the isolated strains were characterized and confirmed for possessing probiotic features [12, 23, 34]. In these studies, the properties of isolated and selected probiotics were rigorously examined. These tests were inclusive of morphological, physiological, and biochemical properties as well as 16SrDNA, acid and bile tolerance, antimicrobial activity, hemolytic activity, protease activity, cell surface hydrophobicity, and autoaggregation [23]. When samples were gathered, probiotic strains were isolated and used solely or as a mixture in this study. It is worth adding that these probiotics were tested before use, and none of them showed any characteristics of being pathogenic or harmful to humans/mammals. Animal and human studies showed that the composition of gut microbiota was significantly changed in NAFLD, and the beneficial effects of probiotic supplements in compensating were frequently approved [35–38].

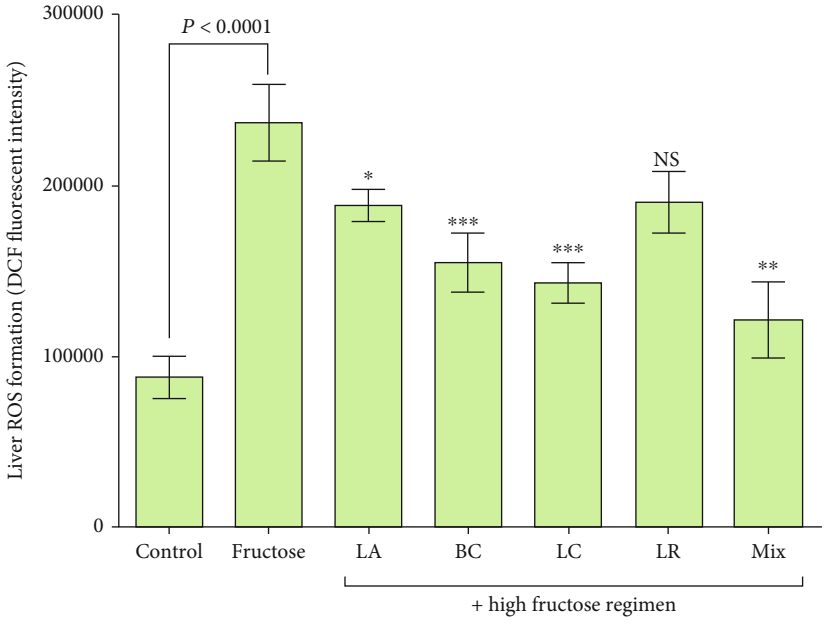
Several systematic reviews and meta-analysis have mentioned that the potential of probiotics is disease- and strain-specific [16, 39, 40]. Up to now, most studies have focused on *Lactobacillus* and *Bifidobacterium* strains. In this study, the effects of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans* were compared together and with the mixture of the mentioned probiotics in NAFLD-induced male Sprague-Dawley rats.

This study examined some of the critical serum and histopathological markers related to NAFLD, including mean body weight, serum ALT, serum TG, serum glucose, liver TG content, liver glutathione content and FRAP, ROS, protein carbonylation, and lipid peroxidation of the liver tissue. According to our results, probiotics were able to change the mentioned parameters during the experiment. Upon the administration of probiotics, improved disease markers such as serum TG, serum glucose, and ALT levels and improved oxidative stress markers were observed. Conservation can probably explain improved liver triglyceride levels in the expression and activity of the transcription factor peroxisome proliferator-activated receptor-α (PPAR-alpha). There are some studies indicating that substances binding to PPAR-alpha induced its activation to bind to DNA, and stimulated the expression of proteins involved in the metabolism of fatty acids [41, 42].

Also, oxidative stress has been reportedly associated with the pathogenesis of NAFLD [43, 44], and the improved results reveal the critical role of probiotics in the prevention of NAFLD. Based on previous studies, oxidative stress may lead to the genesis of steatohepatitis from steatosis caused by a high-calorie diet [19]. Therefore, amelioration of oxidative stress markers along with the reduction of free radicals and inflammation in the liver tissue could be the factors that contribute to liver pathology based on previous experiments

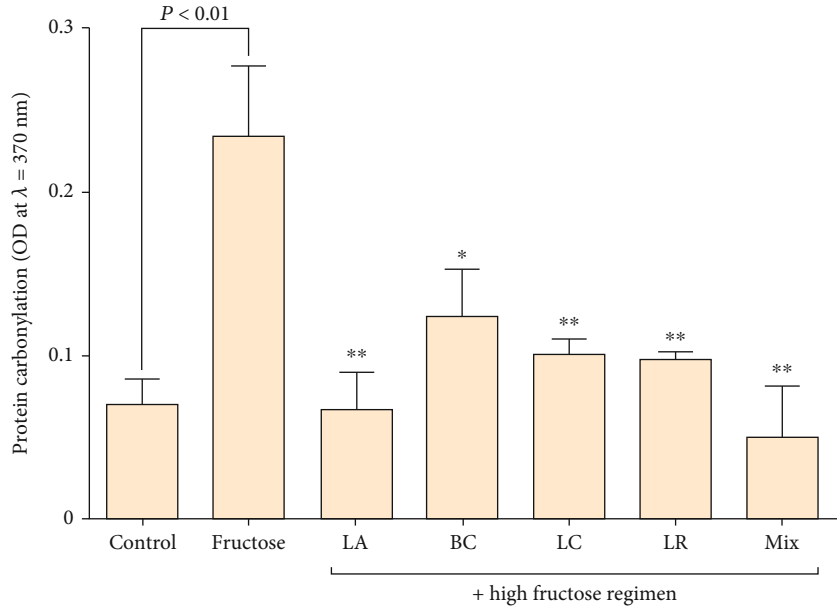


(a)

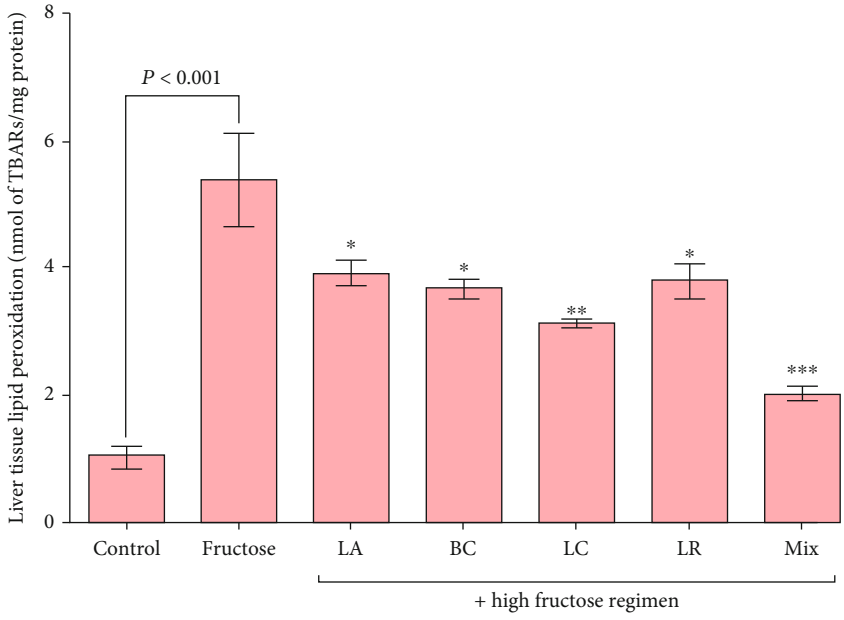


(b)

Figure 3: Continued.



(c)



(d)

Figure 3: Continued.

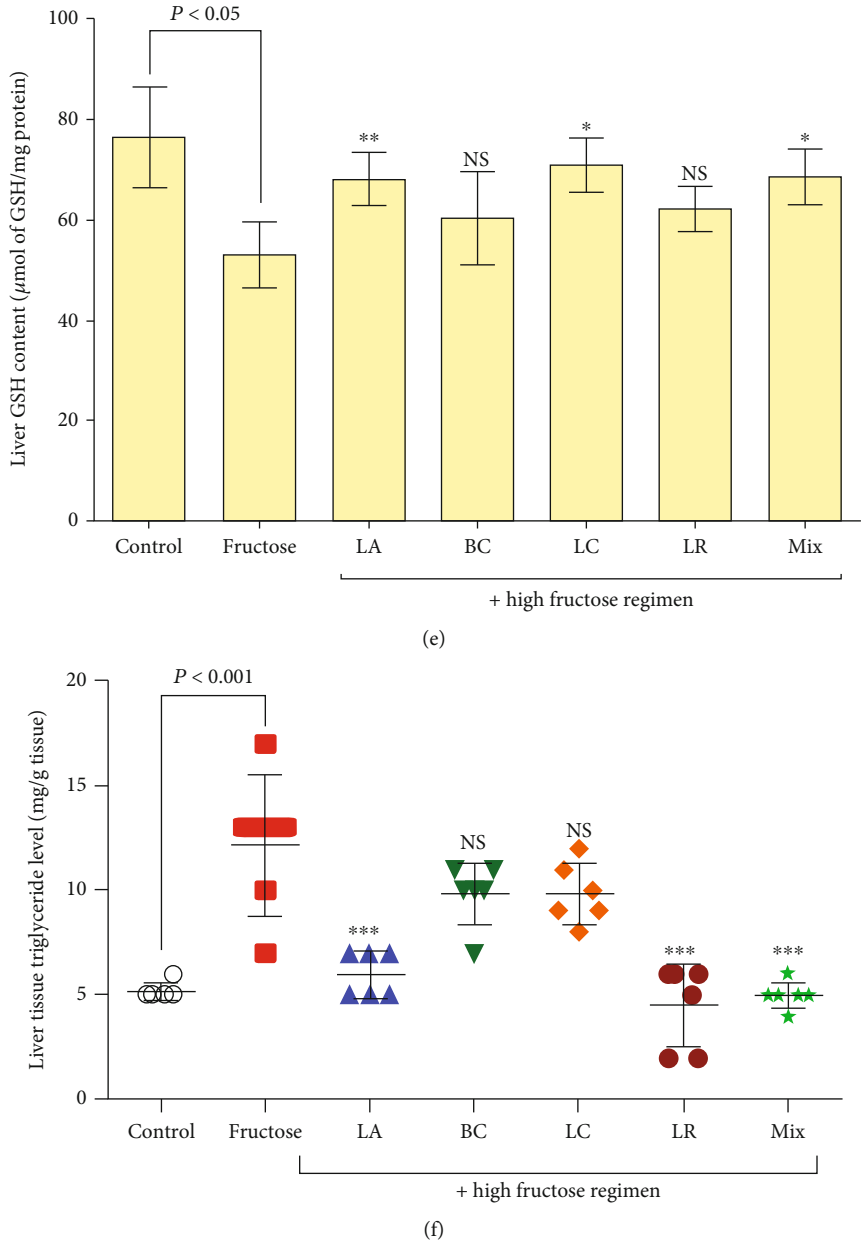


Figure 3: Oxidative stress markers in the liver tissue of NAFLD rats and effects of probiotic administration. Data are demonstrated as mean ± SEM ($n = 6$). (a) Liver tissue ferric-reducing/antioxidant power (FRAP) assay, (b) liver reactive oxygen species (ROS) formation, (c) liver protein carbonylation, (d) liver tissue lipid peroxidation, (e) hepatic glutathione content, and (f) liver tissue triglyceride level. Ctrl: control; LA: *Lactobacillus acidophilus*; BC: *Bacillus coagulans*; LC: *Lactobacillus casei*; LR: *Lactobacillus reuteri*; Mix: a mixture of probiotics inclusive of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans*; ns: not significant. ∗ indicates a significant difference from the fructose control group ($P < 0.05$). ∗∗ indicates a significant difference from the fructose control group ($P < 0.01$). ∗∗∗ indicates a significant difference from the fructose control group ($P < 0.001$).

[45, 46]. As stated in the previous studies, the changes observed in the antioxidant response, the hepatoprotective effects, could be due to an increase in the expression and activity of the transcription factor Nrf2 [47].

Based on previous studies [48], animals fed with a high-fructose diet had elevated serum lipid profiles such as TG, serum ALT, and serum glucose compared to animals with a standard diet. The increased levels of lipids may be linked to the accumulation of fat droplets in animals receiving a high-fructose diet, leading to the genesis of hepatic steatosis and different stages of NAFLD [49]. High-fructose diets can alter the gut microbiota, leading to gut permeability, decreased bacterial LPS removal, and increased metabolic endotoxemia [50]. According to previous studies [51], endotoxemia can lead to various metabolic dysfunctions resulting in disturbance of fat metabolism. This incidence may eventually cause an increase in fatty acid uptake, fat disposition in the hepatic tissues, and hepatic steatosis. Also, a *Bacillus*

species called *Bacillus coagulans*, primarily considered as a probiotic strain, had interestingly attenuated the development of NAFLD. This strain significantly prevented the weight gain in NAFLD-induced rats and substantially lowered free radicals that may contribute to inflammatory and metabolic diseases. *Bacillus coagulans* is a sporogenic bacteria which is essential from an industrial point of view because of its resistance to strong gastric acid and high temperatures. Recent studies on the probiotic effects of *Bacillus* species have been focused on the prevention and treatment of metabolic disorders [52]. Some probiotic-containing supplements controlled weight gain and hyperglycemia induced in animals by a high-fructose diet [53]. Moreover, a combination of soya pulp and *Bacillus coagulans* demonstrated improved bile acid levels in metabolic dysfunctions and NAFLD diseases [54].

This study primarily focuses on answering the critical question of which probiotic strains to use in the prevention of NAFLD. Based on recent studies, probiotics have been shown to have diverse effects on different organs and hosts and proven to have the potential to be disease- and strain-specific, as mentioned in the manuscript [16, 55]. It is believed that isolated strains of probiotics from different origins may have different results. Although each strain used in this study was able to overcome some implications of nonalcoholic fatty liver disease, our results indicated that a mixture of these probiotic strains had an overall better outcome in all the tested markers and was considered as the best-treating group. Using the mixture of probiotic strains seemed to help to maintain or to preserve the lipid profiles and to reduce the chance of hepatic steatosis in group 6, which received a high-fructose regimen. It has been proved that using a mixture of probiotic strains is highly beneficial for the host's health rather than a single-strain probiotic in many cases [56]. Animals with NAFLD displayed improvement in several disease markers and amelioration of the metabolic syndrome after receiving a mixture of probiotic strains [57]. This might be due to their presumed complementary and synergistic effects, especially within the gut [58].

Moreover, the observed hepatoprotective effects may be influencing mitochondrial functions, which is one of the most relevant causes in the prevention of hepatic steatosis [59]. We observed the same results regarding the consumption of a mixture of probiotics. In this case, we had used four different species, inclusive of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bacillus coagulans* that had not been used together in any other studies.

Therefore, these results showed that a cocktail mixture of probiotics could be considered a promising combination for the prevention of NAFLD. Although rat models with a nutritional deficiency to develop NAFLD are usually preferred due to their natural preference and vital importance in illuminating the pathophysiological mechanisms of NAFLD, it is essential to note that translation of animal-study results to the human population has failed frequently [60]. The results of this study have provided significant evidence for the role of probiotics in the prevention of NAFLD; nevertheless, more randomized clinical trials are needed to confirm their role in humans. On the whole, due to probiotics' relatively low production cost, low side effects, availability, and limitations that are not difficult to overcome, further clinical studies can illuminate the effects of probiotics in the human body.

5. Conclusion

Since different probiotic strains exert diverse effects on metabolic disorders, future studies should include an emphasis on the interactions between probiotics and NAFLD. This experiment used four different probiotic strains, and the results showed that the mixture of the mentioned probiotic strains was more effective in the prophylaxis of NAFLD than single-strain therapy. Improved lipid profiles, liver function markers, and inflammatory marker levels supported our theory. Having nutritional and therapeutic potentials, probiotics were able to control and prevent hepatic steatosis and similar disorders.

We believe a more detailed investigation regarding specific microbiome profiles of the GI in NAFLD patients may allow better-individualized modulation of the disease by probiotics. Further studies must be focused on the potentially suitable probiotic mixtures, concentrations, intervals, and algorithms of administration in human clinical trials.

Abbreviations

NAFLD: Nonalcoholic fatty liver disease
GI: Gastrointestinal tract
TG: Triglyceride
ALT: Alkaline phosphatase
TCA: Trichloroacetic acid
DTNB: 5,5-Dithionitro-benzoic acid
DNPH: 2,4-Dinitrophenylhydrazine
DCF-DA: 2,7-Dichlorofluorescein
HCl: Hydrochloric acid
EDTA: Ethylene-diamine tetra-acetic acid
TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine
Tris-HCl: 2-Amino-2-hydroxymethyl-propane-1,3-diol-hydrochloride
MRS: De Man, Rogosa, and Sharpe
LS: L-S differential medium
VP: Voges-Proskauer
FRAP: Liver ferric-reducing antioxidant power
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS: Reactive oxygen species.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

This work was approved by the Ethics Committee (No. 2225b125) of Shiraz University of Medical Sciences, Shiraz, Iran.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

AA collaborated in methodology, investigation, writing, and original draft preparation. OF collaborated in software, data curation, writing (preparation), and resources. MMO collaborated in the investigation and draft writing (review and editing). AJ collaborated in the investigation, validation of data, draft writing, and financial acquisition. RH collaborated in the investigation, validation, software and formal analysis, writing, and editing. SNA collaborated in conceptualization, draft preparation, and review and editing. AG collaborated in conceptualization, data curation, visualization, resources, writing (review and editing), supervision, project administration, and funding acquisition. All authors read and approved the final manuscript.

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Supplementary Materials

Supplementary Table 1: list of the chemicals and manufacturing companies *(Supplementary Materials)*

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Research Article

Association between Circulating Growth Differentiation Factor 15 and Cirrhotic Primary Biliary Cholangitis

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Primary biliary cholangitis (PBC) is a common condition that usually shows a progressive course towards cirrhosis without adequate treatment. Growth differentiation factor 15 (GDF15) plays multiple roles in various pathological conditions. The overall role of circulating GDF15 in cirrhotic PBC requires further investigation. Twenty patients with cirrhotic PBC, 26 with non-cirrhotic PBC, and 10 healthy subjects were enrolled between 2014 and 2018, and the serum levels of GDF15 were measured via enzyme immunoassay. The correlations between serum GDF15, weight, biochemical parameters, and the prognosis were analysed. Serum levels of GDF15 were significantly higher in cirrhotic PBC patients than in non-cirrhotic PBC patients or healthy controls ($p = 0.009$ and $p < 0.001$, respectively). The circulating GDF15 levels strongly correlated with weight changes ($r = -0.541$, $p = 0.0138$), albumin ($r = -0.775$, $p < 0.0001$), direct bilirubin ($r = -0.786$, $p < 0.0001$), total bile acids ($r = 0.585$, $p = 0.007$), and C-reactive protein ($r = 0.718$, $p = 0.0005$). Moreover, circulating GDF15 levels strongly correlated with the Mayo risk score ($r = 0.685$, $p = 0.0009$) and Model for End-stage Liver Disease score ($r = 0.687$, $p = 0.0008$). Determined by the area under the receiver operating characteristic curves, the overall diagnostic accuracies of GDF15 were as follows: cirrhosis = 0.725 (>3646.55 pg/mL, sensitivity: 70.0%, specificity: 69.2%), decompensated cirrhosis = 0.956 (>4073.30 pg/mL, sensitivity: 84.62%, specificity: 100%), and cirrhotic biochemical non-responders = 0.835 (>3479.20 pg/mL, sensitivity: 71.43%, specificity: 92.31%). GDF15 may be a useful and integrated biochemical marker to evaluate not only the disease severity and prognosis but also the nutrition and response to treatment of cirrhotic PBC patients, and its overall performance is satisfactory. Therapy targeting GDF15 is likely to benefit cirrhotic PBC patients and is worth further research.

1. Introduction

Primary biliary cholangitis (PBC) is an immune-mediated inflammatory cholestatic liver disease characterized by non-suppurative destructive cholangitis and interlobular bile duct destruction. It is a chronic progressive condition leading to end-stage liver disease including liver cirrhosis (LC) and hepatocellular carcinoma and their associated complications that commonly require liver transplantation [1–3]. It has been reported that without effective therapy, the median time of progression to extensive liver fibrosis is 2 years with about one-third of the patients remaining in early-stage disease over a follow-up period of 4 years [4–6]. Conversely, several early-stage studies have demonstrated that the incidence of progression to LC after 6 years of follow-up was 1 in 2 for patients who received penicillamine or placebo (compared to 1 in 10 for patients who received ursodeoxycholic acid) [7]. Cirrhosis is a great burden on public health care. In 2010, it was the twelfth leading cause of mortality worldwide, responsible for approximately 1 million deaths [8]. Vibration-controlled transient elastography (VCTE) and liver biopsy were commonly used to diagnose cirrhosis. The VCTE is recommended as the initial assessment for significant liver fibrosis and cirrhosis, and it is a quick, portable

point-of-care test. But the reliability of VCTE may be influenced by operator experience, obesity, ascites, narrow intercostal spaces, hepatic inflammation, cholestasis, and hepatic congestion. It is only a surrogate marker for the diagnosis [9]. Liver biopsy can diagnose the cirrhosis accurately but is an invasive method and not feasible in all patients and can pose complications of pain, haemorrhage, infection, perforation of a neighbouring organ, or even death. Moreover, small specimen's size, sampling error, and variability with inter- and intraobserver reliability may lead to poor reproducibility for liver biopsies. These disadvantages limit its broad application in cirrhosis diagnosis [9]. Early serum biomarker screening in patients at a high risk of developing LC may reduce the morbidity and mortality rates and decrease medical costs. However, the sensitivity and specificity of currently available serum biomarker for cirrhosis diagnosis are unsatisfactory. Optimal diagnostic serum biomarkers for cirrhosis are needed.

Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine 1 (MIC-1), is a stress-responsive cytokine belonging to the transforming growth factor beta (TGF-β) superfamily which includes several proteins involved in tissue homeostasis, differentiation, remodelling, and repair [10, 11]. GDF15 has been demonstrated to play multiple roles in various pathological conditions such as cancer, inflammatory diseases, cardiovascular diseases, lung diseases, kidney injury, and metabolic disorders [12–16]. Recent studies have found that GDF15 can induce anorexia and fat and lean body mass loss [17, 18], and there appears to be a consistent correlation between an increase in the serum levels of GDF15 and a decrease in the markers of nutrition [19]. In addition, it was reported that an elevated serum GDF15 level is detected during hepatitis C virus infection, which is potentially caused by either viral agents or host stress/injury, or by both. GDF15 may contribute to HCV pathogenesis by altering the signalling and growth of host and represents a potential diagnostic serum biomarker and interventional target for viral hepatitis [10]. Measuring serum levels of GDF15 is a noninvasive and simple-to-use test. However, the clinical relevance of the relationship between circulating GDF15 and end-stage liver diseases, such as in PBC patients with cirrhosis, has not been reported.

The aim of the present study was to measure the serum levels of GDF15 in cirrhotic PBC patients and examine the relationship between serum GDF15 and changes in the body weight and clinical parameters to determine the role of GDF15 in cirrhotic PBC patients. Illustrating the biological function of circulating GDF15 in cirrhosis will help promote its potential application in the diagnosis and targeted therapy of cirrhotic PBC patients.

2. Materials and Methods

2.1. Patients. All enrolled patients were diagnosed and followed up at the Third Affiliated Hospital of Sun Yat-Sen University between 2014 and 2018. The diagnosis of PBC was based on the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines [20]. Cirrhosis was diagnosed using either an imaging technique such as ultrasonography, magnetic resonance imaging, or computed tomography or via liver biopsy. PBC patients received ursodeoxycholic acid (UDCA) at a standard dose of 13-15 mg/kg daily. Decompensated cirrhosis was defined based on the complications that the patient had such as variceal bleeding, ascites, encephalopathy, and jaundice [21]. Ten individuals with no abnormal clinical (according to previous medical records), physical, or biochemical findings were included as healthy controls in this research. The standard clinical laboratory methods were used to measure the biochemical parameters. The "Paris criteria" were employed to define the biochemical response to UDCA treatment [22], and the Model for End-stage Liver Disease (MELD) score [23] and Mayo risk score (MRS) [24] were employed to evaluate the prognosis of the patients.

Patients' body weights were measured on admission to the hospital and compared with the body weights described in their previous medical records. Malnutrition was evaluated according to the Global Leadership Initiative on Malnutrition (GLIM) criteria for the diagnosis of malnutrition [25].

The exclusion criteria were applicable to patients who had any of the following: (a) heart failure, renal disease, or pulmonary disease; (b) other hepatological pathologies such as viral hepatitis, primary sclerosing cholangitis, alcoholic liver disease, and fatty liver disease; (c) any carcinoma; and (d) long-term usage of diuretics for ascites or oedema before admission to the hospital.

The study protocol was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University. Blood samples were acquired after obtaining written consent from the patients.

2.2. Measurement of Serum GDF15 Levels. We used enzyme-linked immunosorbent assay (ELISA) (Abcam, UK) to measure the levels of serum GDF15 as per the manufacturer's instructions for all patients at the time of hospital admission.

2.3. Statistical Analysis. The baseline demographic and clinical characteristics are listed as means with standard error of the mean (SEM) or percentage. Student's t test or the Mann–Whitney U test was employed to estimate continuous data whereas the chi-square test or Fisher's exact test was employed to estimate categorical data. Pearson's correlation coefficient or Spearman's rank correlation was employed to assess the correlation of data. All parameters exhibiting strong correlations in the univariate analysis as covariates were subjected to multiple linear regression. Multiple linear regression analysis was conducted to detect independent relationships and adjust the effects of covariates. Receiver operating characteristic (ROC) curves were used to compare the diagnostic values of GDF15. The areas under the curves were calculated by selecting clinically relevant threshold levels to optimize the sensitivity and specificity. SPSS version 19 (IBM, Armonk, NY, USA) was used for the statistical analyses. All analyses were two-sided, and differences were defined as statistically significant when $p < 0.05$.

Table 1: Clinical and laboratory parameters of PBC patients and healthy controls.

Feature	Healthy controls ($n = 10$)	Non-cirrhotic PBC patients ($n = 26$)	Cirrhotic PBC patients ($n = 20$)
Age (years)	53.20 ± 4.13	52.12 ± 2.11	53.95 ± 2.63
Gender (male)	4	3	7
Weight change (kg)	0.300 ± 0.44	−0.962 ± 0.51	−1.50 ± 0.43$^{*\#}$
Weight change (%)	0.47% ± 0.71%	−1.49% ± 0.77%	−2.71% ± 0.74%$^{*\#}$
>5% within past 6 months	0	2	4
BMI (kg/m^2)	23.27 ± 2.51	21.39 ± 2.09*	19.82 ± 2.12$^{*\#}$
Low BMI (<18.5 if <70 years)	0	2	6
CRP (mg/L)	3.37 ± 0.49	8.89 ± 1.87	15.99 ± 1.75*
CRP > 5 mg/L	0	13*	12*
Malnutrition (n, %)	0	4 (15.38%)	9 (45%)$^{*\#}$
GDF15 (pg/mL)	656.58 ± 146.13	3037.41 ± 568.91*	4926.44 ± 662.84$^{*\#}$
ALT (U/L)	15.80 ± 1.98	113.00 ± 17.37*	85.85 ± 15.27*
AST (U/L)	21.80 ± 1.29	114.12 ± 15.64*	102.10 ± 14.09*
TBIL (μmol/L)	8.56 ± 1.34	59.85 ± 12.49*	95.69 ± 18.58*
DBIL (μmol/L)	2.91 ± 0.56	43.71 ± 10.78*	69.87 ± 14.58*
GGT (U/L)	25.80 ± 4.62	474.08 ± 84.86*	309.60 ± 71.96*
ALP (U/L)	60.30 ± 5.66	308.08 ± 41.13*	269.95 ± 25.71*
TBA (μmol/L)	3.27 ± 0.34	81.18 ± 16.80*	132.54 ± 19.57$^{*\#}$
ALB (g/L)	43.98 ± 0.52	38.39 ± 0.85*	34.24 ± 1.02$^{*\#}$
GLB (g/L)	27.13 ± 1.16	34.75 ± 1.28*	35.86 ± 2.25*
INR	0.95 ± 0.01	1.04 ± 0.06*	1.28 ± 0.09$^{*\#}$
MELD score	N/A	5.73 ± 1.07	10.14 ± 1.47$^{\#}$
Mayo risk score	N/A	5.53 ± 0.29	6.58 ± 0.35$^{\#}$
ANA positive	0	25	19
Anti-SP100	0	1	1
Anti-GP210	0	1	4
AMA positive	0	22	16
AMA-M2 positive	0	9	6

$^{*}p < 0.05$ compared to corresponding values in healthy controls. $^{\#}p < 0.05$ compared to corresponding values in non-cirrhotic PBC patients. PBC: primary biliary cholangitis; BMI: body mass index; GDF15: growth differentiation factor 15; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TBIL: total bilirubin; DBIL: direct bilirubin; GGT: gamma-glutamyl transpeptidase; ALP: alkaline phosphatase; TBA: total bile acids; ALB: albumin; GLB: globulin; INR: international normalized ratio; CRP: C-reactive protein; MELD score: Model for End-stage Liver Disease score.

3. Results

3.1. Demographic and Clinical Characteristics of Patients with PBC and Healthy Controls. Forty-six PBC patients (26 without cirrhosis and 20 with cirrhosis) and 10 healthy controls were included in this study (see Table 1). The age was not different in non-cirrhotic and cirrhotic PBC patients and healthy controls (52.12 ± 2.11 vs. 53.95 ± 2.63 vs. 53.20 ± 4.13 years, $p > 0.05$). Serum liver enzyme (alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT)) levels, bilirubin (total bilirubin (TBIL) and direct bilirubin (DBIL)) levels, and total bile acid (TBA) levels were significantly higher in both non-cirrhotic and cirrhotic PBC patients than in healthy controls. The international normalized ratio (INR) was significantly higher in patients with PBC (both non-cirrhotic and cirrhotic) than in healthy controls. In contrast, serum albumin (ALB) levels were significantly lower in PBC patients regardless of cirrhosis than in healthy controls. In addition, the TBA, INR, MELD score, and Mayo risk score of cirrhotic PBC patients were significantly higher than those of non-cirrhotic PBC patients. Serum levels of C-reactive protein (CRP) were significantly higher in cirrhotic PBC patients than in healthy controls (see Table 1).

The weight changes were 0.300 ± 0.44 kg (0.47% ± 0.71%) in healthy controls, −0.962 ± 0.51 kg (−1.49% ± 0.77%) in non-cirrhotic PBC patients, and −1.50 ± 0.43 kg (−2.71% ± 0.74%) in cirrhotic PBC patients. Weight loss was significantly higher in cirrhotic PBC patients than in non-cirrhotic PBC patients ($p = 0.015$) and healthy control individuals ($p = 0.011$). The BMI was significantly lower in

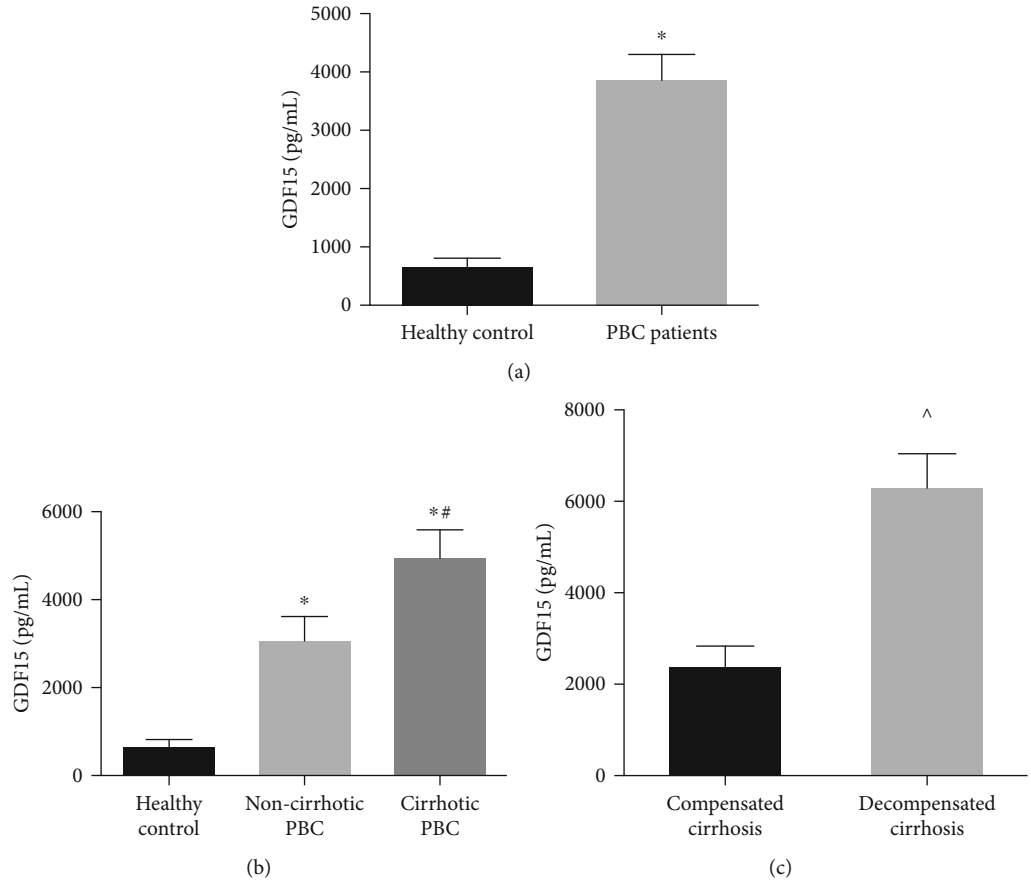

Figure 1: Serum GDF15 concentrations in PBC patients with and without cirrhosis and healthy controls.

cirrhotic PBC patients than in non-cirrhotic PBC patients (19.82 ± 2.12 vs. $21.39 \pm 2.09\,kg/m^2$, $p = 0.016$) and healthy controls (19.82 ± 2.12 vs. $23.27 \pm 2.51\,kg/m^2$, $p < 0.001$). The incidence of malnutrition was 45% (9/20) in cirrhotic PBC patients and 15.38% (4/26) in non-cirrhotic PBC patients. None of the healthy controls presented with malnutrition. The incidence of malnutrition was higher in cirrhotic PBC patients than in non-cirrhotic PBC patients ($p = 0.046$) and healthy controls ($p = 0.013$).

3.2. Serum Levels of GDF15 in Patients with PBC (Cirrhotic and Non-cirrhotic) and Healthy Controls. Serum levels of GDF15 were significantly higher in PBC patients than in healthy controls (3858.73 ± 449.19 vs. 656.58 ± 146.13 pg/mL; $p < 0.001$) (see Figure 1(a)). Serum levels of GDF15 were significantly higher in non-cirrhotic PBC patients than in healthy controls (3037.41 ± 568.91 vs. 656.58 ± 146.13 pg/mL; $p = 0.002$) (see Figure 1(b)), and serum levels of GDF15 were significantly higher in cirrhotic PBC patients than in healthy controls (4926.44 ± 662.84 vs. 656.58 ± 146.13 pg/mL; $p < 0.001$) or non-cirrhotic PBC patients (4926.44 ± 662.84 vs. 3037.41 ± 568.91 pg/mL; $p = 0.009$) (see Figure 1(b)). Moreover, serum levels of GDF15 were significantly higher in PBC patients with decompensated cirrhosis than in PBC patients with compensated cirrhosis (6679.31 ± 828.27 vs. 2784.04 ± 477.06 pg/mL; $p < 0.001$) (see Figure 1(c)).

3.3. Clinical and Laboratory Parameters Related to GDF15 in Cirrhotic PBC Patients. Serum bilirubin (TBIL, DBIL) and TBA levels are typical markers of cholestasis. Prominently, positive correlations were detected between serum GDF15 levels and TBIL ($r = 0.733$, $p = 0.0002$), GDF15 and DBIL ($r = 0.786$, $p < 0.0001$), and GDF15 and TBA ($r = 0.585$, $p = 0.007$) in cirrhotic PBC patients (see Figures 2(a)–2(c)). There was a negative correlation between ALB and GDF15 ($r = -0.775$, $p < 0.0001$) in cirrhotic PBC patients (see Figure 2(d)). We also detected a negative correlation between the serum levels of GDF15 and weight changes ($r = -0.541$, $p = 0.0138$) in cirrhotic PBC patients (see Figure 2(e)). Serum levels of CRP, which is an acute-phase protein expressed in the liver, rise in response to inflammation. CRP is a typical marker of the response to inflammation. A positive correlation was detected between serum GDF15 levels and CRP ($r = 0.718$, $p = 0.0005$) (see Figure 2(f)).

The MELD score (based on a calculation including the INR and bilirubin and creatinine levels) is commonly employed to assess disease severity and outcomes in patients with liver diseases [21]. In the present study, GDF15 levels strongly correlated with the MELD score ($r = 0.687$, $p = 0.0008$) (see Figure 2(g)).

The Mayo risk score (based on a series of potential risk factors including age, albumin and bilirubin levels, prothrombin

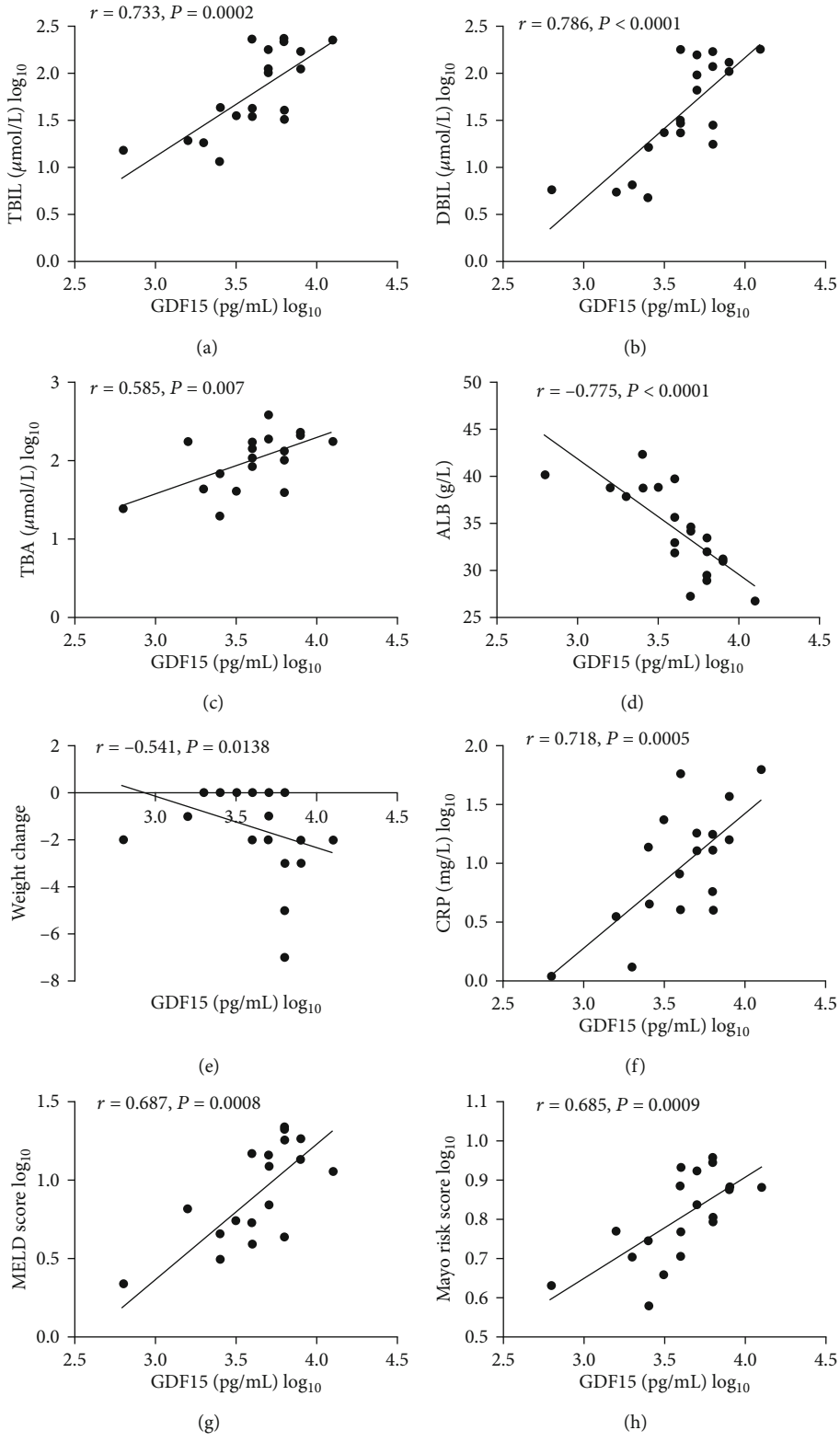

Figure 2: Laboratory and clinical parameters associated with GDF15 in cirrhotic PBC patients (μmol/L).

time, and the presence of peripheral oedema and diuretic treatment) is typically employed to assess the outcomes in PBC patients [22]. In the present study, GDF15 levels strongly correlated with the Mayo risk score ($r = 0.685$, $p = 0.0009$) (see Figure 2(h)).

GDF15 levels did not differ significantly in cirrhotic PBC patients with different ANA titres ($p = 1.000$) and ANA patterns ($p = 0.114$) (see Figure 3).

Univariate regression analysis showed significant positive correlations between GDF15 and bilirubin (TBIL and DBIL)

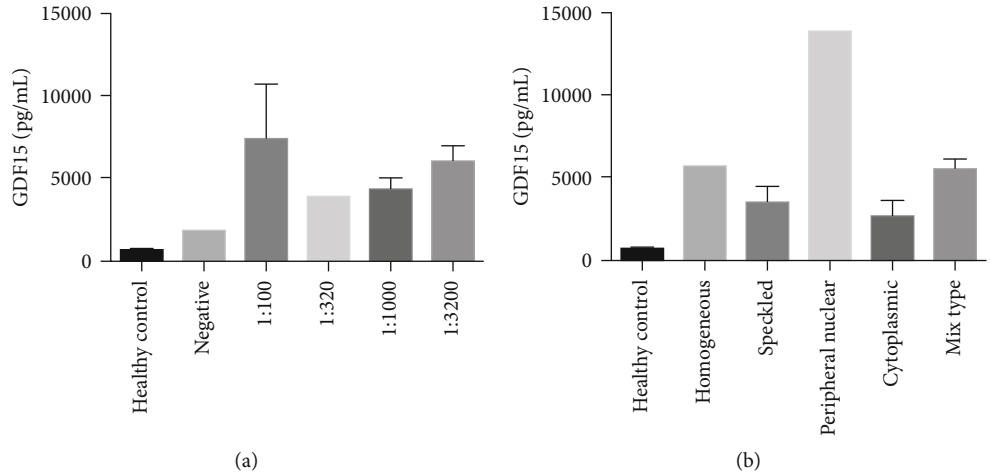

Figure 3: Serum GDF15 concentrations in cirrhotic PBC patients with different ANA titres and ANA patterns.

levels and GDF15 and CRP and TBA levels but significant negative correlations between GDF15 and ALB levels (see Table 2). We also detected a significant positive relationship between serum GDF15 levels and patients' MELD and Mayo risk scores (see Table 2). Multivariate analysis of these data revealed that ALB was an independent variable of serum GDF15 levels ($p = 0.038$) in cirrhotic PBC patients (see Table 3).

3.4. Serum GDF15 Levels, Patient Prognosis, and Biochemical Responsiveness in Cirrhotic PBC Patients. There were 7 biochemical responders and 13 biochemical non-responders among the 20 cirrhotic PBC patients. Prominently, serum levels of GDF15 were significantly higher in biochemical non-responders (patients that failed to respond to treatment) than in biochemical responders (5972.83 ± 809.45 vs. 2983.14 ± 757.12 pg/mL, $p = 0.014$) (Table 4). Serum bilirubin (TBIL and DBIL), INR, CRP, and the Mayo risk scores were significantly higher in biochemical non-responders than in biochemical responders. The clinical and laboratory features of biochemical responders and non-responders are presented in Table 4.

ROC curve analysis was used to define the optimal cut-off to determine the sensitivity and specificity of serum GDF15 for categorizing cirrhotic PBC patients versus non-cirrhotic PBC patients. The area under the ROC curve (AUROC) was 0.725 (95% CI 0.578-0.872), with a sensitivity of 70.0% (95% CI 0.457-0.881), specificity of 69.2% (95% CI 0.482-0.857), and an optimal cut-off value of 3646.55 pg/mL (see Figure 4). The results showed that the serum levels of GDF15 could be effectively used to differentiate cirrhotic patients from other patients in the cohort with PBC.

ROC curve analysis was used to define the optimal cut-off to determine the sensitivity and specificity of serum GDF15 for categorizing PBC patients with decompensated cirrhosis versus PBC patients with compensated cirrhosis. The area under the ROC curve (AUROC) was 0.956 (95% CI 0.873-1.000), with a sensitivity of 84.62% (95% CI 0.546-0.981), specificity of 100% (95% CI 0.590-1.000), and an optimal cut-off value of 4073.30 pg/mL (see Figure 5). The results showed that the serum levels of GDF15 could be effectively used to differentiate patients with decompensated cirrhosis among cirrhotic PBC patients.

ROC curve analysis was used to define the optimal cut-off to determine the sensitivity and specificity of serum GDF15 for categorizing biochemical responders versus biochemical non-responders in cirrhotic PBC patients. The AUROC was 0.835 (95% CI 0.633-1.000) with a sensitivity of 71.43% (95% CI 0.290-0.963), specificity of 92.31% (95% CI 0.639-0.998), and an optimal cut-off value of 3479.20 pg/mL (see Figure 6). The results showed that the serum levels of GDF15 could be effectively used to differentiate biochemical non-responders among cirrhotic PBC patients.

Table 2: Univariate regression analysis of clinical and laboratory parameters associated with GDF15 in cirrhotic PBC patients.

Variables	*B*	SE	*p* value	95% CI	R^2
TBIL (μmol/L)	0.517	0.108	<0.001	0.290-0.745	0.558
DBIL (μmol/L)	0.435	0.078	<0.001	0.272-0.599	0.635
TBA (μmol/L)	0.522	0.163	0.005	0.179-0.865	0.362
ALB (g/L)	-0.052	0.009	<0.001	-0.072 to -0.032	0.629
CRP (mg/L)	0.455	0.101	<0.001	0.243-0.668	0.547
MELD score	0.577	0.140	0.001	0.283-0.872	0.485
Mayo risk score	1.950	0.460	<0.001	0.983-2.917	0.499

TBIL: total bilirubin; DBIL: direct bilirubin; TBA: total bile acids; ALB: albumin; CRP: C-reactive protein; MELD score: Model for End-stage Liver Disease score; CI: confidence interval.

Table 3: Multivariate regression analysis of clinical and laboratory parameters associated with GDF15 in cirrhotic PBC patients.

Variables	*B*	SE	*p* value	95% CI	R^2
ALB (g/L)	-0.036	0.016	0.038	-0.070 to -0.002	0.671

ALB: albumin; SE: standard error; CI: confidence interval.

Table 4: Clinical and laboratory parameters of biochemical responders and non-responders in cirrhotic PBC patients.

Feature	Biochemical responders ($n = 7$)	Biochemical non-responder ($n = 13$)	p value
Age (years)	53.29 ± 6.01	54.31 ± 2.66	0.859
Gender (male)	3	4	0.651
GDF15 (pg/mL)	2983.14 ± 757.12	5972.83 ± 809.45	0.014
Weight changes (kg)	−0.67 ± 0.33	−2.00 ± 0.66	0.209
ALT (U/L)	79.29 ± 19.16	89.39 ± 21.59	0.877
AST (U/L)	76.71 ± 12.28	115.77 ± 19.95	0.211
TBIL (μmol/L)	44.16 ± 22.34	123.44 ± 22.87	0.003
DBIL (μmol/L)	31.25 ± 20.68	90.66 ± 17.30	0.008
GGT (U/L)	220.43 ± 73.73	357.62 ± 102.96	0.536
ALP (U/L)	225.71 ± 40.25	293.77 ± 32.23	0.249
TBA (μmol/L)	88.33 ± 27.50	156.35 ± 24.38	0.097
ALB (g/L)	36.80 ± 1.47	32.87 ± 1.23	0.059
GLB (g/L)	35.10 ± 4.10	36.26 ± 2.79	0.819
INR	1.07 ± 0.07	1.40 ± 0.13	0.040
CRP (mg/L)	7.20 ± 3.05	21.12 ± 5.71	0.038
MELD score	7.36 ± 2.67	11.63 ± 1.68	0.135
Mayo risk score	5.23 ± 0.42	7.30 ± 0.35	0.002

PBC: primary biliary cholangitis; GDF15: growth differentiation factor 15; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TBIL: total bilirubin; DBIL: direct bilirubin; GGT: gamma-glutamyl transpeptidase; ALP: alkaline phosphatase; TBA: total bile acids; ALB: albumin; GLB: globulin; INR: international normalized ratio; CRP: C-reactive protein; MELD score: Model for End-stage Liver Disease score.

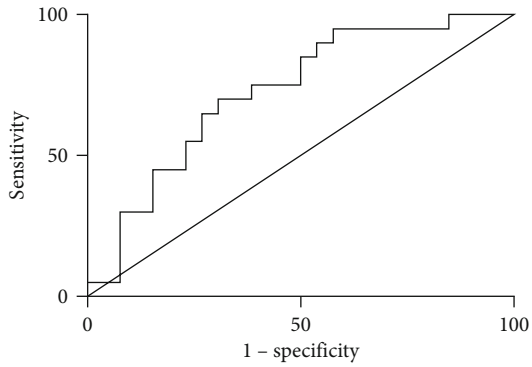

Figure 4: GDF15 ROC curve shows the comparison between cirrhotic PBC and non-cirrhotic PBC patients.

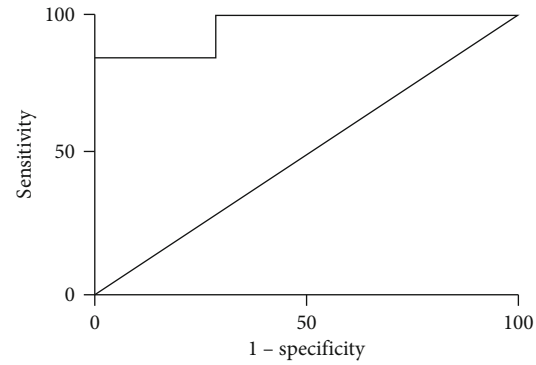

Figure 5: GDF15 ROC curve shows the comparison between PBC patients with decompensated cirrhosis and PBC patients with compensated cirrhosis.

4. Discussion

GDF15 is involved in the stress response program of different cell types after cellular injury and regulates inflammation [16, 26]. Previous studies have reported that GDF15 expression is rapidly induced following injury to hepatocytes and bile duct epithelial cells [16, 27]. In patients with PBC, bile duct lesions, biliary secretion impairment, and hepatocellular accumulation of toxic endogenous bile acids result in cellular damage and necroinflammatory lesions and fibrosis of the liver. The present study showed that the increase in the GDF15 levels was positively correlated with the degree of cholestasis and inflammation in cirrhotic PBC patients. The increase in GDF15 is most likely a response to cell stress/damage and inflammation caused by PBC. As it is a circulating cytokine, it is logical to hypothesize that GDF15 has a paracrine, autocrine, or endocrine action in PBC patients. Our findings are in accord with those of previous studies which suggested that serum GDF15 levels were elevated in patients with chronic liver diseases such as nonalcoholic fatty liver disease (NAFLD) and chronic hepatitis B or C virus infection [28, 29] and supplement those of previous studies. In addition, the present study also showed that serum levels of GDF15 were markedly increased in cirrhotic PBC patients, especially in decompensated cirrhotic PBC patients. Our

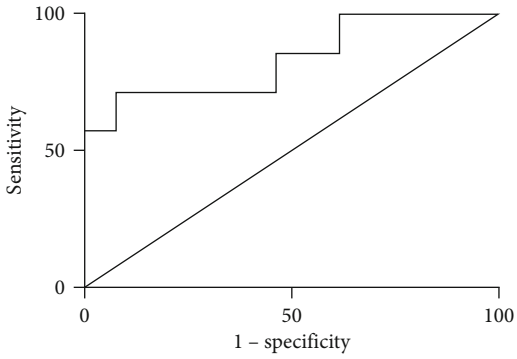

Figure 6: GDF15 ROC curve shows the comparison between biochemical responders and biochemical non-responders among cirrhotic PBC patients.

findings are in accord with those of previous studies which suggested that the increase in GDF15 levels depends on the severity of fibrosis rather than hepatic cell injury/inflammation. Thus, GDF15 may be a good indicator of the severity of the liver fibrosis.

It has been reported that GDF15 is associated with multiple organ fibrosis such as atrial, renal, and pulmonary fibrosis [12–14]. Recently, the association between GDF15 and liver fibrosis has attracted increasing attention. Elevated GDF15 levels were found to be associated with advanced liver fibrosis in chronic liver diseases [28, 29]. Chronic and repetitive hepatocyte injury results in the overexpression of GDF15, and a dysregulation of GDF15 release may lead to prolonged stimulation of hepatic stellate cells (HSCs) and promote the progression of liver cirrhosis [28, 29]. GDF15 has been reported to not only directly stimulate transforming growth factor beta 1 (TGF-β1) expression [30] but also induce the phosphorylation of SMAD2 and SMAD3 proteins to activate human HSCs and induce fibrosis [31]. Serum GDF15 levels may be a potential biomarker of advanced fibrosis in chronic liver diseases.

The present study showed that serum GDF15 levels in cirrhotic PBC patients were markedly increased. Serum GDF15 levels of non-cirrhotic PBC patients were also moderately higher than those of healthy controls, but significantly lower than those of cirrhotic PBC patients. The ROC curve comparing cirrhotic PBC patients and non-cirrhotic PBC patients in the cohort suggested that GDF15 could differentiate LC with an AUROC of 0.725. These results demonstrated that GDF15 could serve as a serum biomarker of LC. Decompensated LC often has a high mortality rate, and it is essential to distinguish between compensated and decompensated cirrhosis when predicting patients' prognosis. Decompensated LC patients cannot tolerate the reliable but highly invasive diagnostic modality of liver biopsy. As to the noninvasive modality, current models including MELD scores cannot distinguish between compensated and decompensated cirrhosis and conventional radiological modalities for fibrosis assessment can only provide the morphological evaluation of liver fibrosis, so improved tools for early and noninvasive diagnosis of LC are urgently needed. The present study showed that serum GDF15 levels in PBC patients with decompensated cirrhosis were markedly increased. Serum GDF15 levels of PBC patients with compensated cirrhosis were also moderately higher than those of healthy controls, but significantly lower than those of PBC patients with decompensated cirrhosis. The ROC curve comparing PBC patients with decompensated cirrhosis and PBC patients with compensated cirrhosis in the cohort suggested that GDF15 could differentiate decompensated LC with an AUROC of 0.956. These results demonstrated that GDF15 could serve as a serum biomarker of decompensated LC.

Patients with cirrhosis are exceptionally vulnerable to developing malnutrition and some degree of cachexia because of the key role played by the liver in regulating the nutritional state and energy balance. It has been reported that the prevalence of malnutrition in LC ranges from 10% to 100% depending on the severity of the disease [32, 33]. In the present study, we found that there were negative correlations between ALB and GDF15 ($r = -0.775$, $p < 0.0001$) (see Figure 2(d)) and GDF15 and weight changes ($r = -0.541$, $p = 0.0138$) (see Figure 2(e)) in cirrhotic patients with PBC. Recently, the role of GDF15 in body weight regulation has been reported. In humans with chronic diseases and malignancies, GDF15 can suppress appetite and induce weight loss even in cachexia [34–36]. GDF15 may contribute to malnutrition in patients with cirrhotic PBC. The elevated circulating GDF15 levels in cirrhotic PBC patients may suppress appetite and reduce food intake, thus influencing nutrient intake. As a result, the synthesis of ALB and maintenance of body weight were influenced by serum GDF15 levels in this study.

Studies have shown that compared to the outcomes of well-nourished patients, malnourished patients with liver disease have poorer outcomes and higher morbidity rates due to major complications requiring hospitalization (71.3% vs. 38.2%, $p = 0.002$) as well as higher mortality rates (41.1% vs. 18.2%, $p = 0.001$) [37]. Early detection and treatment of malnutrition are imperative to improve patient outcomes [38, 39]. Identification of patients in the anorexia–cachexia spectrum who could gain clinical benefits from nutritional support and other therapies is a clinical problem that needs to be solved urgently. However, no well-validated biomarkers for predicting malnutrition and cachexia are available thus far. In the present study, we found that there were negative relationships between ALB and GDF15 ($r = -0.775$, $p < 0.0001$) (see Figure 2(d)) and GDF15 and weight changes ($r = -0.541$, $p = 0.0138$) (see Figure 2(e)) in cirrhotic PBC patients but a positive relationship between CRP and GDF15 ($r = 0.718$, $p = 0.0005$) (see Figure 2(f)). The ALB levels < 32 g/L and CRP levels > 5 mg/L form part of the diagnostic criteria for malnutrition and cachexia [40]. Thus, GDF15 may be useful as a biochemical marker to predict malnutrition and cachexia.

The primary characteristics of malnutrition and cachexia are inadequate nutrient intake, decreased or absent physical activity, and altered metabolism, partly due to a pathological systemic inflammatory response [41]. To improve malnutrition and cachexia, adequate nutrition should be provided to preserve and restore muscle mass and limit systemic inflammation [41]. Malnutrition and cachexia are the focus of

many ongoing studies, but the optimum means for diagnosis or early detection have not been definitely identified thus far. It also appears that, in some patients, the dominant cause of weight loss, loss of muscle mass, and cachexia is a systemic inflammatory response, which emphasizes the importance of systemic inflammation as a target for therapeutic intervention [41]. Such targets should have the direct or indirect potential to stimulate anabolism and/or improve appetite [42]. GDF15 is involved in inflammation regulation [16, 26] and can suppress appetite [17, 18]. In murine models of tumours, mice overexpressing GDF15 showed weight loss, and the degree of weight loss was proportional to the elevation of serum levels of GDF15 [17]. This phenomenon of weight loss in murine models of tumours could be reversed by the utilization of monoclonal antibodies to GDF15 and reproduced by the utilization of recombinant GDF15 [17]. Moreover, it has been reported that serum levels of GDF15 might be associated with all-cause mortality in multiple diseases, and it is possible that disease-specific therapeutic interventions that decrease serum levels of GDF15 may also reduce the risk of mortality and increase longevity [43]. Treatment targeting GDF15 may improve symptoms as well as the nutrient status, thereby improving the outcome of cirrhotic PBC patients.

Recently, the role of serum GDF15 levels in predicting advanced liver fibrosis and severity of chronic liver disease in NAFLD, alcoholic liver diseases, and chronic hepatitis B and C was reported [28, 29]. However, there are limited data on the role of serum levels of GDF15 in patients with PBC. The MELD score is commonly employed to assess the disease severity and prognosis in patients with liver disease [23]. In the present study, a positive correlation was detected between serum levels of GDF15 and MELD scores ($r = 0.687$, $p = 0.0008$) (see Figure 2(g)) in cirrhotic PBC patients. This result demonstrated that serum levels of GDF15 reflect the disease state, and GDF15 could serve as a serum biomarker to indicate the severity of the disease in cirrhotic PBC patients.

The Mayo risk score is typically employed to assess the outcomes of PBC patients [24]. In the present study, a positive correlation was detected between serum levels of GDF15 and the Mayo risk score ($r = 0.685$, $p = 0.0009$) (see Figure 2(h)) in cirrhotic PBC patients. Assessment of the biochemical response indicated that serum GDF15 levels were significantly elevated in biochemical non-responders (see Table 4). The ROC curve comparing biochemical responders and non-responders in cirrhotic PBC patients suggested that GDF15 levels could be used to differentiate biochemical non-responders to UDCA treatment among cirrhotic PBC patients with an AUROC of 0.835. These results demonstrated that GDF15 could serve as a serum biomarker of treatment response to UDCA in cirrhotic PBC patients and potentially indicate the prognosis of cirrhotic PBC patients. This is a novel finding about the role of GDF15 in chronic liver diseases.

Despite these novel findings, this study has some limitations. Data regarding the muscle mass were lacking. This investigation was a small-scale single-centre cohort study and too limited in size to arrive at any definite conclusion. Large-scale multicentre cohort studies are needed to construct more accurate associations.

5. Conclusions

In conclusion, the present study is important as a supplement to previous studies on the role of GDF15 in chronic liver diseases [28, 29]. These findings provide evidence that GDF15 can predict liver fibrosis, severity response to UDCA treatment, and malnutrition in chronic liver disease. Measuring serum GDF15 levels, a noninvasive and simple-to-use test, could be potentially useful in evaluating the disease severity and prognosis of cirrhotic PBC patients, and it has its advantages over the existing prediction models. Serum levels of GDF15 have high sensitivity and specificity in differentiating between compensated and decompensated LC when compared to MELD score, and serum levels of GDF15 have high sensitivity and specificity in differentiating between cirrhotic PBC patients and non-cirrhotic PBC patients when compared to Mayo risk scores. In addition, it can predict malnutrition and cachexia that are associated with the disease severity and prognosis in cirrhotic PBC patients. No well-validated biomarkers for predicting malnutrition and cachexia in end-stage liver disease are available thus far, and improved tools for early and noninvasive diagnosis of LC are urgently needed. GDF15 may be a useful and integrated biochemical marker to evaluate not only the disease severity and prognosis but also the nutrition and response to treatment of patients with chronic liver diseases, and its overall performance is satisfactory. Therapy targeting GDF15 is likely to benefit cirrhotic PBC patients and is worth further research.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Yu-Tian Chong and Qiwan Mo were responsible for the conceptualization. Zhan-Yi Li, Yu Liu, Fangji Yang, and Yuan-Kai Wu were responsible for the data curation. Zhan-Yi Li, Yu Liu, Yuan-Kai Wu, Fang-Ji Yang, and Qiwan Mo were responsible for the data analysis and interpretation. Yu-Tian Chong, Xiang-Yong Li, and Zhanyi Li were responsible for funding acquisition. Zhan-Yi Li, Yu Liu, Fang-Ji Yang, and Xiang-Yong Li were responsible for the investigation. Yu Liu, Zhan-Yi Li, Yu-Tian Chong, and Qiwan Mo were responsible for the methodology. All authors were involved in manuscript writing. All authors gave final approval of the manuscript. All authors are accountable for all aspects of the work. Zhanyi Li and Yu Liu contributed equally to this work.

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Research Article

Risk Factors for Hepatocellular Carcinoma Recurrence and Survival after Liver Transplantation in Patients with HCV-Related Cirrhosis

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Purpose. We aimed to identify prognostic factors for survival and recurrence of hepatocellular carcinoma (HCC) after liver transplantation (LT) for patients with HCC and hepatitis C virus-related cirrhosis (HCV-cirrhosis). *Methods*. This retrospective cohort study followed all adult patients with HCV-cirrhosis who underwent LT because of HCC or had incidental HCC identified through pathologic examination of the explanted liver at a university hospital in Rio de Janeiro, Brazil, over 11 years (1998-2008). We used Cox regression models to assess the following risk factors regarding HCC recurrence or death after LT: age, Model for End-stage Liver Disease score, Child-Pugh classification, alpha-fetoprotein (AFP), whether patients had undergone locoregional treatment before transplantation, the number of packed red blood cell units (PRBCU) transfused during surgery, the number and size of HCC lesions in the explanted liver, and the presence of microvascular invasion and necrotic areas within HCC lesions. *Results*. Seventy-six patients were followed up for a median (interquartile range (IQR)) of 4.4 (0.7-6.6) years. Thirteen (17%) patients had HCC recurrence during the follow-up period, and 26 (34%) died. The median survival time was 6.6 years (95% CI: 2.4-12.0), and the 5-year survival was 52.5% (95% CI: 42.3-65.0%). The final regression model for overall survival included four variables: age (hazard ratio (HR): 1.02, 95% CI: 0.96-1.08, $P = 0.603$), transplantation waiting time (HR: 1.00, 95% CI: 1.00-1.00, $P = 0.190$), preoperative AFP serum levels (HR: 1.01, 95% CI: 1.00-1.02, $P = 0.006$), and whether >4 PRBCU were transfused during surgery (HR: 1.15, 95% CI: 1.05-1.25, $P = 0.001$). The final cause-specific Cox regression model for HCC recurrence included only microvascular invasion (HR: 14.86, 95% CI: 4.47-49.39, $P < 0.001$). *Conclusion*. In this study of LT for HCV-cirrhosis, preoperative AFP levels and the number of PRBCU transfused during surgery were associated with overall survival, whereas microvascular invasion with HCC recurrence.

1. Introduction

Hepatocellular carcinoma (HCC) represents the third cancer-related cause of death in the world and has an estimated incidence of roughly 750,000 new cases each year [1, 2]. It is associated with high recurrence rates of up to 80% after surgical resection and with lower survival rates when compared with other cancers. That picture is even worse for patients with HCC associated with hepatitis C virus (HCV) infection [3]. Additionally, more than 90% of patients with HCV and HCC also have liver cirrhosis.

Liver transplantation (LT) is considered the best modality of treatment for HCC because it has the potential to clear both the tumor and the underlying liver cirrhosis [4]. In a landmark article published in 1996, the adoption of the Milan criteria for the selection of adult patients with HCC for LT was associated with an improvement in overall survival rates from about 35% in five years to 75% in four years and recurrence rates below 10% [5]. Importantly, these criteria involve only the following: single tumor ≤ 5 cm, or up to 3 foci of the tumor, each ≤ 3 cm, and no evidence of gross vascular invasion or extrahepatic metastasis [6].

Because of the growing demand for LT, several authors advocated the expansion and refinement of prognostic criteria for the selection of eligible patients with HCC [6–9]. Even when the Milan criteria are strictly applied, in real-life cases of LT, it is not rare to find an explanted liver with tumors whose size or number exceeds the limits established by those criteria [10, 11]. In this regard, other variables besides the number and size of tumors in the liver, such as the presence of microvascular invasion, and levels of alpha-fetoprotein (AFP) have also been associated with increased recurrence of HCC after LT [8, 12–16]. A recent systematic review about HCC recurrence after LT concluded that the quality of the studies on this subject was low and that more longitudinal studies providing external validation of risk prediction models in diverse populations are highly needed [17]. Hence, the present study is aimed at examining prognostic factors for mortality and HCC recurrence after LT in a real-life population of patients with HCV-related HCC in Brazil, using the Least Absolute Shrinkage and Selection Operator (LASSO) method [18] to select the most influential variables for survival regression models.

2. Materials and Methods

This was a retrospective cohort study based on the review of medical records of all patients undergoing LT at Clementino Fraga Filho Hospital in Rio de Janeiro, Brazil, between January 1, 1998, and December 31, 2008. Our study was approved by the Research Ethics Review Committee of the Faculty of Medicine of the Federal University of Rio de Janeiro under #147/10.

Inclusion criteria involved patients aged 18 years or older who had HCV-cirrhosis and underwent LT because of HCC or had incidental HCC identified through pathologic examination of the explanted liver. For this study, patients were followed up to June 1, 2012.

Preoperative diagnosis of HCC was performed according to the 2000 guidelines of the European Association for the Study of the Liver (EASL) standards [19] and required coincident findings in at least two different radiological examinations (ultrasonography, computed tomography, or magnetic resonance imaging), showing hepatic nodules > 2 cm with evidence of arterial hypervascularization. Alternatively, the EASL guidelines also allowed the noninvasive diagnosis of HCC to be made by a single imaging finding of a focal lesion > 2 cm with arterial hypervascularization when associated with AFP levels > 400 ng/ml. Additionally, according to the standard for selection of patients for transplantation at the time of the study, only patients passing the Milan criteria were considered eligible for LT. All cases of HCC diagnosed preoperatively were confirmed by pathological examination of the explanted liver.

We extracted the following data from patients' medical records: sex, date when the transplantation occurred, age at the date of LT, Model for End-stage Liver Disease (MELD) score (which ranges from 6 to 40, with higher values indicating more severe liver disease), Child-Pugh classification (A, B, and C, with higher levels meaning more severe liver disease), AFP levels before the transplantation (ng/dl), whether patients had undergone locoregional treatment before transplantation (transarterial chemoembolization, radiofrequency ablation, or surgical resection), and the total number of packed red blood cell (PRBC) units that were transfused during surgery. We also reviewed the reports of the pathological examination of the explanted liver for the number and size of HCC lesions, the presence of microvascular invasion, and necrotic areas within HCC lesions. The number of HCC lesions was categorized in the following four groups: single lesion, 2 to 3 lesions, 4 to 5, and more than 5 lesions. Total tumor size was classified as <5 cm, between 5 and 9 cm, and >9 cm. Cases of multicentric HCC were classified as having total tumor size larger than 9 cm and more than 5 lesions.

Additionally, we extracted data from medical records regarding dates of death and when diagnoses of HCC recurrence were made. All cases for which there was not a record of death in their medical records were contacted by phone to confirm they were alive by June 2012.

2.1. Statistical Analysis. We described categorical data as absolute numbers and proportions and continuous data as mean and standard deviation (SD) or median and interquartile ranges (IQR), as appropriate [20]. We used a Cox proportional hazards model to assess risk factors for death after LT [21]. We did not use the Fine and Gray subdistribution hazards method to assess risk factors for HCC recurrence accounting for the competing risk of death because that approach is not considered ideal for such purposes [22, 23]. Instead, we used a cause-specific Cox proportional hazards model to assess risk factors for HCC recurrence, as recommended for studies aiming at assessing risk factors for outcomes for which there are one or more competitive events [24]. For that last model, we only included patients who had survived at least one month after LT, as performed by others [25], because it is unlikely that HCC recurrence would be diagnosed in the first month after transplantation.

We used the LASSO method [18] for the selection of variables for both multivariable models, an approach that is considered superior to stepwise methods and that is particularly useful for research contexts where there are a relatively large number of variables in comparison to the total number of observations.

We assessed the proportional hazards assumption of the Cox proportional hazards models through the examination of Schoenfeld residual plots [21].

Six patients with missing data were excluded from the LASSO analyses and from the Cox models that included any variable with missing data.

We adopted a two-sided alpha level of 0.05 for statistical significance and used the R software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria) for all statistical analyses.

3. Results

Between 1998 and 2008, 373 patients underwent LT at the study hospital. Out of that total, HCV-cirrhosis with HCC was the primary reason for LT for 53 patients. Additionally, 23 patients undergoing LT due to HCV-cirrhosis had their explanted livers diagnosed with incidental HCC. Hence, 76 patients were included in this study.

Fifty-three (70%) patients were male, and the mean (SD) age overall was 56 (7.1) years. Nineteen (25%) patients were classified as Child-Pugh stage C, and the median (IQR) time from inclusion in the transplantation waitlist to surgery was 533 (332 to 846) days. The median (IQR) and maximum duration of follow-up were 4.4 years (0.7 to 6.6 years) and 12 years, respectively. Table 1 presents further details regarding the demographic and clinical characteristics of patients.

Thirteen (17%) out of 76 patients had HCC recurrence during the follow-up period, and 26 (34%) died during the timespan of the study. The median survival time was 6.6 years (95% CI: 2.4 to 12.0), and the 5-year survival was 52.5% (95% CI: 42.3% to 65.0%). Figure 1 shows the Kaplan-Meier overall survival curve after LT. The 5-year cumulative incidence of HCC recurrence was 14.5% (95% CI: 7.7% to 23.5%).

The results of simple (univariable) Cox regressions for overall survival are shown in Table 2. Table 3 shows the results of the cause-specific simple Cox regressions for HCC recurrence. The LASSO procedure selected only four variables for the multivariable Cox regression for overall mortality: age, transplantation waiting time, preoperative AFP serum levels, and whether >4 PRBC units were transfused during surgery. The multivariable Cox regression for the overall survival outcome including those four variables (Table 4) showed that intraoperative transfusion of >4 PRBC units was associated with a 15% increased hazard of death (hazard ratio [HR]: 1.15, 95% CI: 1.05 to 1.25, $P = 0.001$) and that every increase of 100 ng/ml of AFP was associated with a 1% increased hazard of death (HR: 1.01, 95% CI: 1.00 to 1.02, $P = 0.006$). Neither age nor transplantation waiting time was significantly associated with the overall survival outcome in the multivariable Cox regression. Figure 2 depicts the overall survival curves according to the number of PRBC transfused during surgery.

Table 1: Clinical and demographic characteristics of the study subjects.

	N (%)
Sex	
Female	23 (30.3%)
Male	53 (69.7%)
Age	
Mean (SD)	56 (7.1)
Child-Pugh stage	
A	28 (36.8%)
B	29 (38.2%)
C	19 (25.0%)
MELD	
Median (IQR)	14 (9.8 to 16)
Transplantation waiting time (days)	
Median (IQR)	533 (332 to 846)
Missing data	5 (6.6%)
Locoregional therapy before transplantation*	47 (61.8%)
Transarterial chemoembolization	37 (48.7%)
Radio frequency ablation	5 (6.6%)
Liver resection	5 (6.6%)
Number of red blood cell units transfused during transplantation	
Median (IQR)	2.0 (1.0 to 4.0)
Alpha-fetoprotein (ng/ml)	
Median (IQR)	17.2 (5.6 to 67)
Missing data	3 (3.9%)
Number of HCC tumors	
Single tumor	23 (30.3%)
2-3 tumors	19 (25.0%)
4-5 tumors	7 (9.2%)
>5 tumors	27 (35.5%)
Total tumor size	
<5 cm	37 (48.7%)
5-9 cm	10 (13.2%)
>9 cm	29 (38.2%)
Presence of tumor necrosis	
Yes	37 (48.7%)
Missing data	1 (1.3%)
Microvascular invasion	
Yes	11 (14.5%)

SD: standard deviation; IQR: interquartile range; MELD: Model for End-stage Liver Disease; HCC: hepatocellular carcinoma.

The LASSO procedure selected only the microvascular invasion variable for the cause-specific Cox regression examining HCC recurrence. The presence of microvascular invasion in the pathological examination of the explanted liver was associated with an increase of almost 15 times in the hazard of HCC recurrence (HR: 14.86, 95% CI: 4.47 to 49.39, $P < 0.001$). Figure 3 shows the cumulative incidence

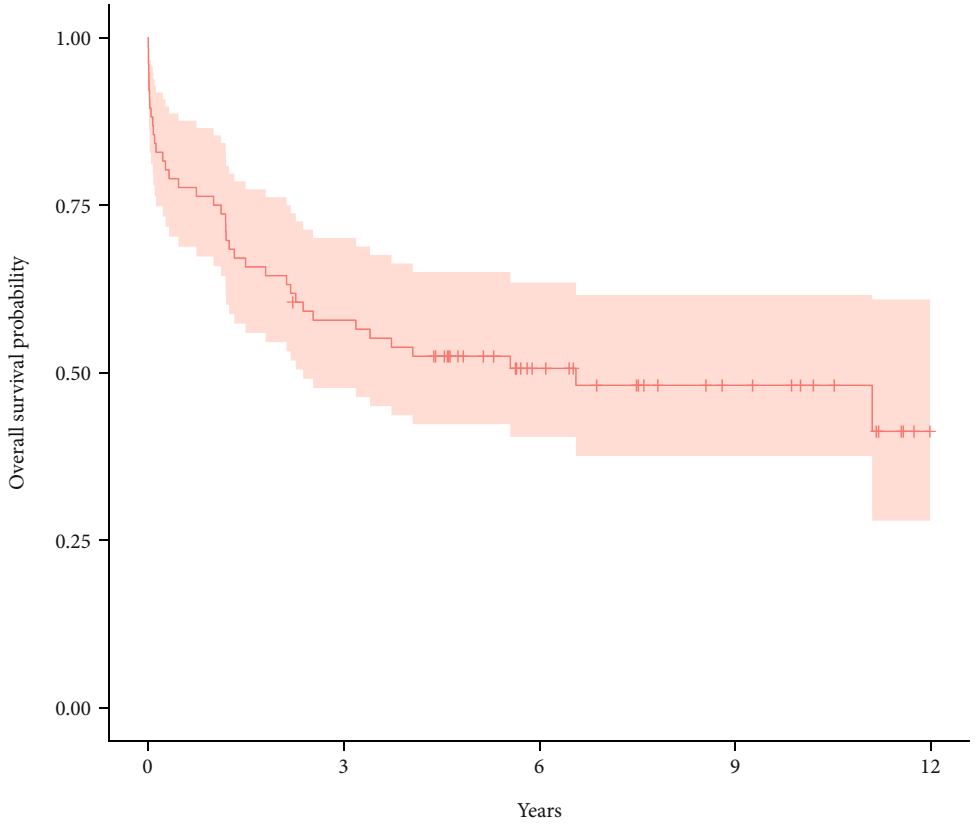

Figure 1: Overall survival with 95% confidence interval.

curves of HCC recurrence according to the presence or absence of microvascular invasion. Schoenfeld residual plots were consistent with the proportional hazards assumption.

4. Discussion

The outcomes after LT for patients with liver cirrhosis and HCV depend on variables related not only to the transplantation, such as organ availability, donor selection, allocation strategies, liver disease severity, and local expertise but also on factors associated with HCC survival, such as AFP levels and tumor characteristics. Still, HCV recurrence was a big concern some years ago, when HCV treatment was hardly performed after LT. Therefore, it is vital to analyze the outcomes according to the main underlying liver diseases, such as HCV in the Western world, where HCC accounts for approximately 30% of liver transplants [26].

When LT is proposed for a patient with HCC, one of the main concerns involves the possibility of multicentric recurrence and intrahepatic distant recurrence, which often occur in the first 2 years and are particularly common in HCV-related HCC, contributing to the worse outcomes in this population [3, 26, 27]. Although the need for PRBC transfusion in LT had already been associated with length of hospital stay and acute rejection [28], a recent study found that patients with HCC had a 5 times higher chance of requiring massive intraoperative transfusion of 10 or more PRBC units than patients without HCC [29]. In that study, Danforth et al. [29] evaluated a sample of 124 patients undergoing LT, in whom HCC was the main etiology of liver disease in only 16 (12.9%). Of note, half of our 76 patients with HCV-related HCC required intraoperative transfusions of at least 2 PRBC units and a quarter received 4 or more PRBC. Our results showed that transfusions of more than 4 PRBC units were associated with lower survival in this population, a finding that is consistent with results from previous studies involving other populations of patients undergoing LT [30] and that likely reflects a range of possible factors such as the degree of difficulty of the surgical procedure, the occurrence of intraoperative complications leading to blood loss, and adverse immune effects related to PRBC transfusions [31]. Importantly, our results point towards a possible role of interventions aimed at preventing blood loss and minimizing the need for intraoperative transfusions as a means to improve the outcomes of patients undergoing LT.

AFP is widely recognized as a prognostic predictor of survival for patients with HCC undergoing LT based on studies of patients with heterogeneous underlying causes of liver disease [32]. Our study found that in a population of patients with HCV-cirrhosis, every increase of 100 ng/ml in AFP levels was associated with a 1% increase in the hazard of death and contributes to the literature with information concerning this specific subgroup of patients with HCC.

Rates of HCC recurrence after LT usually vary from 5% to 15% [13, 26, 33]. However, those estimates were derived from heterogeneous samples in hospitals where most patients had an early diagnosis and were submitted to LT with small tumors. For instance, in a long-term study

Table 2: Results of univariable Cox regressions for overall survival.

	HR	95% CI	*P* value
Age	1.02	0.97-1.08	0.378
Sex			
Female	—	—	—
Male	0.68	0.31-1.50	0.336
Transplantation waiting time	1.00	1.00-1.00	0.483
Child-Pugh classification			
Child-Pugh stage A	—	—	—
Child-Pugh stage B	1.27	0.55-2.95	0.572
Child-Pugh stage C	0.50	0.16-1.59	0.240
MELD score	1.00	0.92-1.09	0.990
Alpha-fetoprotein (ng/ml) × 100[a]	1.01	1.00-1.02	0.018
Previous locoregional treatment for HCC	1.55	0.67-3.57	0.307
Number of HCC lesions			
Single tumor	—	—	—
2-3 tumors	1.59	0.58-4.40	0.370
4-5 tumors	1.54	0.40-5.99	0.530
>5 tumors	1.05	0.38-2.89	0.931
Total tumor size			
<5 cm	—	—	—
5-9 cm	0.88	0.25-3.10	0.846
>9 cm	1.05	0.46-2.39	0.913
Incidental HCC diagnosed postoperatively	0.59	0.24-1.48	0.265
Microvascular invasion	0.53	0.13-2.27	0.395
Presence of tumor necrosis	1.44	0.66-3.15	0.355
>4 red blood cells units transfused during transplantation	4.06	1.86-8.86	<0.001

[a]The results reported here for alpha-fetoprotein levels correspond to increases of 100 units of alpha-fetoprotein levels; i.e., the hazard ratio of 1.01 means that every increase of 100 ng/ml is associated with a 1% increase in the hazard of death. HCC: hepatocellular carcinoma; MELD: model for end-stage liver disease.

performed by Doyle et al., patients had only a 7% incidence of HCC recurrence, but their sample had different underlying liver diseases and very small tumors (2.3 ± 1.3 cm) [27]. In our study, which was restricted to patients whose HCC was related to HCV-cirrhosis, we found a recurrence rate of 17% after LT, which was lower than the 32.4% recurrence rate described by Bozorgzadeh et al. [34] in their cohort of 37 patients with HCC due to HCV-cirrhosis after a mean follow-up of 37 months after LT.

Although AFP levels, the number of tumor lesions, and their size are considered well-established risk factors for HCC recurrence after LT, we did not find significant associations between those variables and HCC recurrence in our study [35]. The most probable explanation for that lack of association is insufficient statistical power related to our limited sample size. On the other hand, our results showed an almost 15 times higher hazard of HCC recurrence in patients whose pathological examination of their explanted livers revealed microvascular invasion than when that feature was

Table 3: Results of simple cause-specific Cox regressions for hepatocellular carcinoma recurrence.

	HR	95% CI	*P* value
Age	0.96	0.89-1.05	0.403
Sex			
Female			
Male	3.71	0.47-28.97	0.212
Transplantation waiting time	1.00	1.00-1.00	0.573
Child-Pugh classification			
Child-Pugh stage A			
Child-Pugh stage B	2.41	0.62-9.32	0.202
Child-Pugh stage C	0.50	0.05-4.81	0.549
MELD score	0.92	0.80-1.06	0.262
Alpha-fetoprotein (ng/ml) × 100*	1.00	0.99-1.01	0.746
Received previous locoregional treatment for HCC	1.28	0.37-4.37	0.695
Number of HCC lesions			
Single tumor			
2-3 tumors	2.58	0.23-28.49	0.439
4-5 tumors	9.05	0.81-100.45	0.073
>5 tumors	5.81	0.70-48.30	0.103
Total tumor size			
<5 cm			
5-9 cm	3.76	0.62-22.64	0.149
>9 cm	3.04	0.76-12.15	0.116
Incidental HCC diagnosed postoperatively	1.13	0.33-3.87	0.842
Microvascular invasion	14.86	4.47-49.39	<0.001
Presence of tumor necrosis	1.44	0.41-5.12	0.569
>4 red blood cells units transfused during transplantation	1.30	0.35-4.92	0.695

[a]The results reported for alpha-fetoprotein levels correspond to increases of 100 units of alpha-fetoprotein levels; i.e., the hazard ratio of 1.01 means that every increase of 100 ng/ml is associated with a 1% increase in the hazard of hepatocellular carcinoma recurrence. HCC: hepatocellular carcinoma; MELD: model for end-stage liver disease.

Table 4: Results of multivariable Cox regressions for overall survival.

	HR	95% CI	*P* value
Age	1.02	0.96-1.08	0.603
Transplantation waiting time	1.00	1.00-1.00	0.190
Alpha-fetoprotein (ng/ml) × 100[a]	1.01	1.00-1.02	0.006
>4 red blood cells units transfused during transplantation	1.15	1.05-1.25	0.001

[a]The results reported here for alpha-fetoprotein levels correspond to increases of 100 units of alpha-fetoprotein levels; i.e., the hazard ratio of 1.01 means that every increase of 100 ng/ml is associated with a 1% increase in the hazard of hepatocellular carcinoma recurrence.

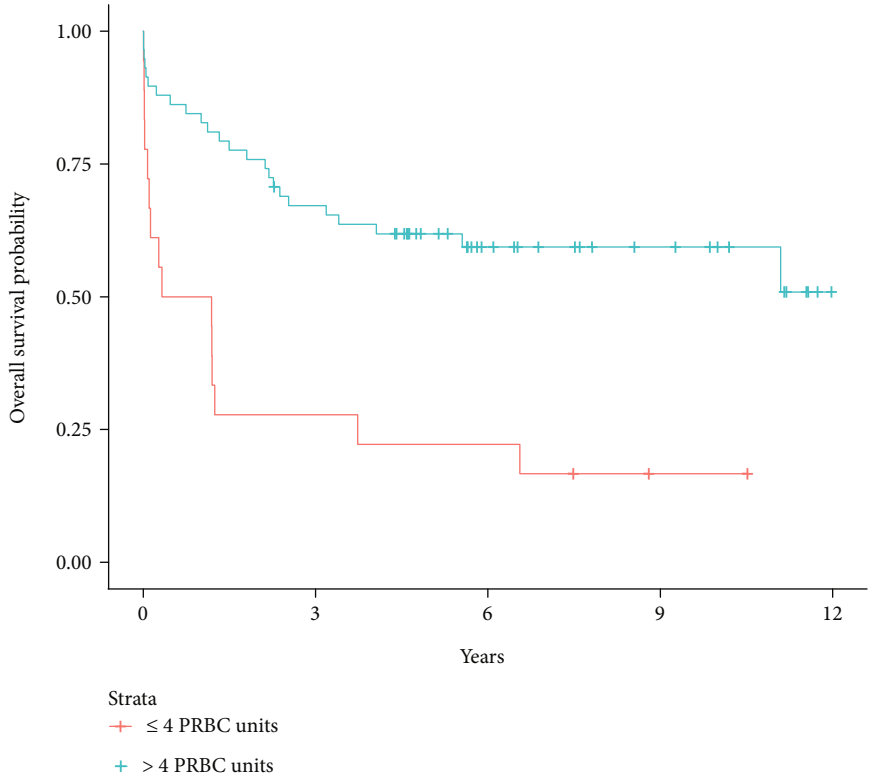

Figure 2: Overall survival according to the number of packed red blood cell units transfused during liver transplantation surgery (HR: 1.15, 95% CI: 1.05 to 1.25, $P = 0.001$).

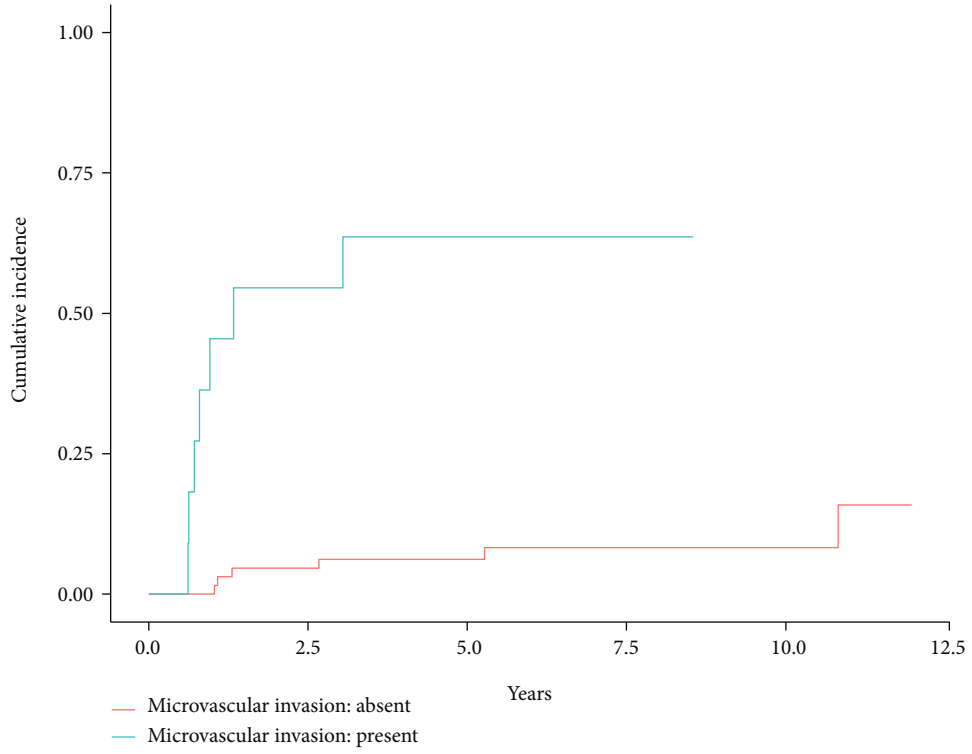

Figure 3: Cumulative incidence of hepatocellular carcinoma recurrence after liver transplantation according to the presence of microvascular invasion in the pathological examination of the explanted liver (HR: 14.86, 95% CI: 4.47 to 49.39, $P < 0.001$).

absent. Microvascular invasion was associated with a 3.8- to 4.9-fold increase in HCC recurrence in prior studies with heterogeneous samples [26]. Our results showed a higher impact of microvascular invasion in terms of risk of HCC recurrence for patients whose underlying liver disease was HCV-cirrhosis, which could possibly be explained by particularities of the molecular mechanisms driving hepatocarcinogenesis in HCV-cirrhosis, such as the methylation of multiple genes and the compromise of the DNA damage response [36–40].

Unfortunately, current practice for the diagnosis of microvascular invasion still relies solely on the pathological examination of surgical specimens. However, recent advances in the field of radiology, radiomics, and radiogenomics have shown promising results concerning the noninvasive diagnosis of microvascular invasion [41–44].

Our study has some potential limitations worth noting. First, our sample was relatively small and our analyses may not have had enough statistical power to detect other predictive variables for overall survival and HCC recurrence. Second, our study was restricted to a single center and our findings may not be generalizable to other settings. Third, we were not able to include in our models several variables related to the histopathological and immunohistochemical profile of the HCC. Nevertheless, our study is valuable for providing data on a subgroup of HCC with a single underlying liver disease in the context of a middle-income country, for which little information is available in the medical literature.

5. Conclusion

In summary, our results provide evidence of a significant mortality and cancer recurrence burden in a population of patients with HCC associated with HCV-cirrhosis that underwent LT. For that population, the number of PRBC units transfused during surgery and the preoperative AFP serum levels were associated with decreased overall survival, whereas the presence of tumor microvascular invasion was the single most important predictor of HCC recurrence.

Data Availability

The anonymized dataset is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the present manuscript.

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Research Article

Four Autophagy-Related lncRNAs Predict the Prognosis of HCC through Coexpression and ceRNA Mechanism

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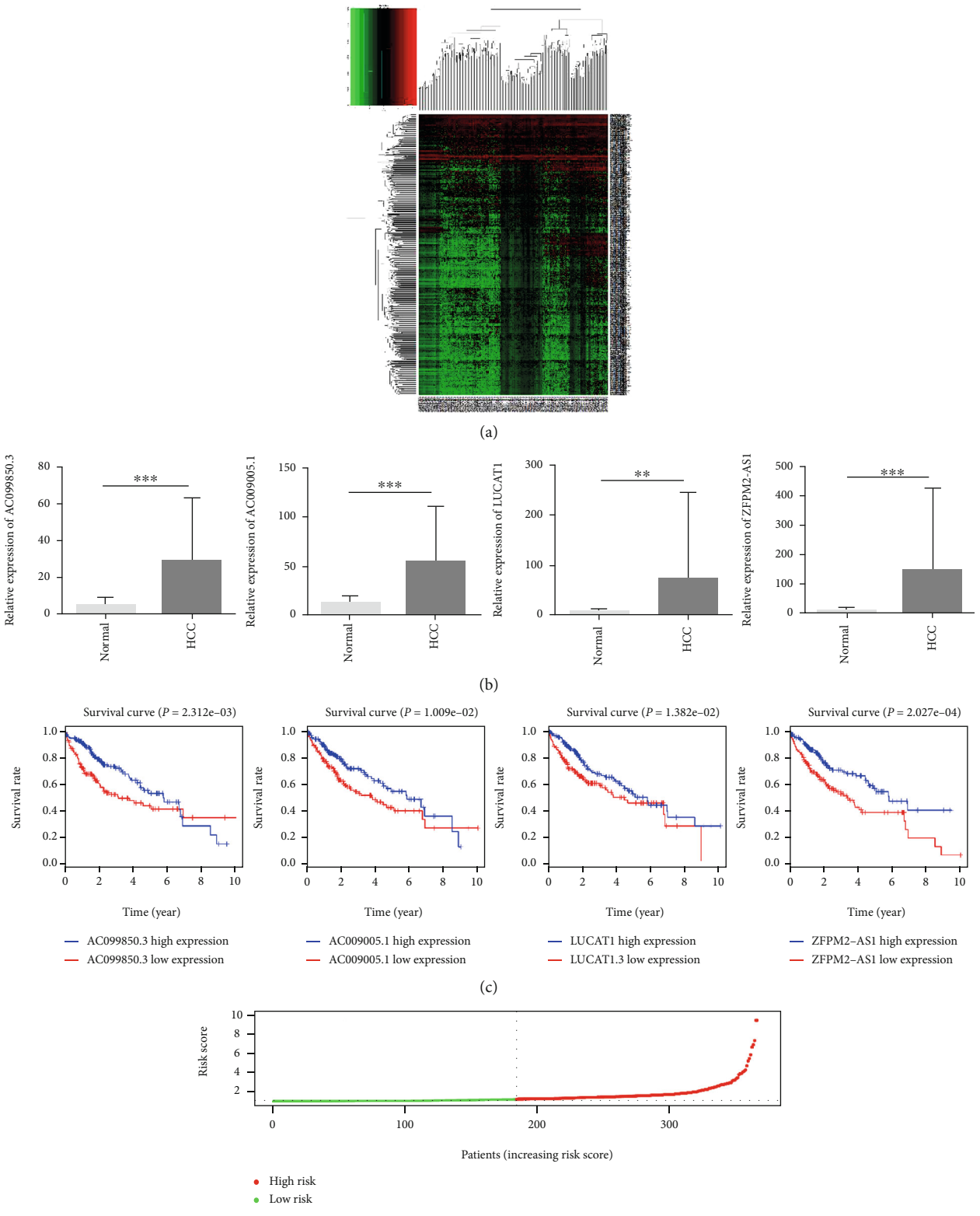
Abnormally expressed long noncoding RNAs (lncRNAs) have been reported to affect the occurrence and progression of hepatocellular carcinoma (HCC) by modulating the autophagy axis. However, none of studies has explored the clinical significance of these autophagy-related lncRNAs in HCC comprehensively. In this study, the RNA-seq, miRNA-seq, and clinical data of normal and HCC patients from the TCGA database and autophagy genes from the Human Autophagy Database were extracted. Subsequently, we screened out 78 differentially expressed autophagy-related lncRNAs, and four prognostic-related lncRNAs (LUCAT1, AC099850.3, ZFPM2-AS1, and AC009005.1) were eventually used to develop the prognostic model. This signature could be regarded as an independent prognostic signature for HCC patients and has the highest prediction efficiency than other clinicopathological factors for the 1-, 3-, and 5-year survival (AUC = 0.764, 0.738, and 0.717, respectively). Additionally, regardless of whether the clinical information is complete for HCC patients, the autophagy-related lncRNA model shows a good predictive power for the overall survival. Importantly, the coexpression network of 4 lncRNAs and 11 autophagy-related genes was constructed. Moreover, based on the bioinformatic analyses, our results found that LUCAT1 and ZFPM2-AS1 may affect the autophagic activity in HCC through the hsa-miR-495-3p/DLC1 and hsa-miR-515-5p/DAPK2 axis, respectively. In conclusion, we establish an effective prognostic model for HCC patients and shed new light on the autophagy-related regulatory mechanisms of the identified lncRNAs.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent liver tumor, accounting for 75–80% of all primary liver cancer cases and arises from chronic liver inflammation and liver fibrosis mostly [1, 2]. Despite the major progress in risk factors, early diagnosis, and treatment techniques for HCC, the poor prognosis of HCC patients remains unsatisfactory (overall mortality to incidence rate, 0.95) [2]. Because of the complex molecular mechanisms and high cellular heterogeneity of HCC patients, traditional clinical parameters including AFP, TNM stage, and vascular invasion have the limited predictive power. Therefore, new and more accurate methods with a better understanding of the underlying HCC development mechanisms are urgently needed to facilitate early detection, help prognostic prediction, and guide individualized treatment.

Autophagy is a key intracellular process for degradation of damaged or unwanted protein and dysfunctional organelles, which is vital to maintain cellular homeostasis, metabolism, and survival [3, 4]. Dysregulation of the autophagic process has been reported to regulate a variety of pathological conditions and cancer development, including HCC [5–7]. The function of autophagy in HCC is a hotspot, and the autophagy process can play either a protective or a detrimental role in the occurrence and development of HCC



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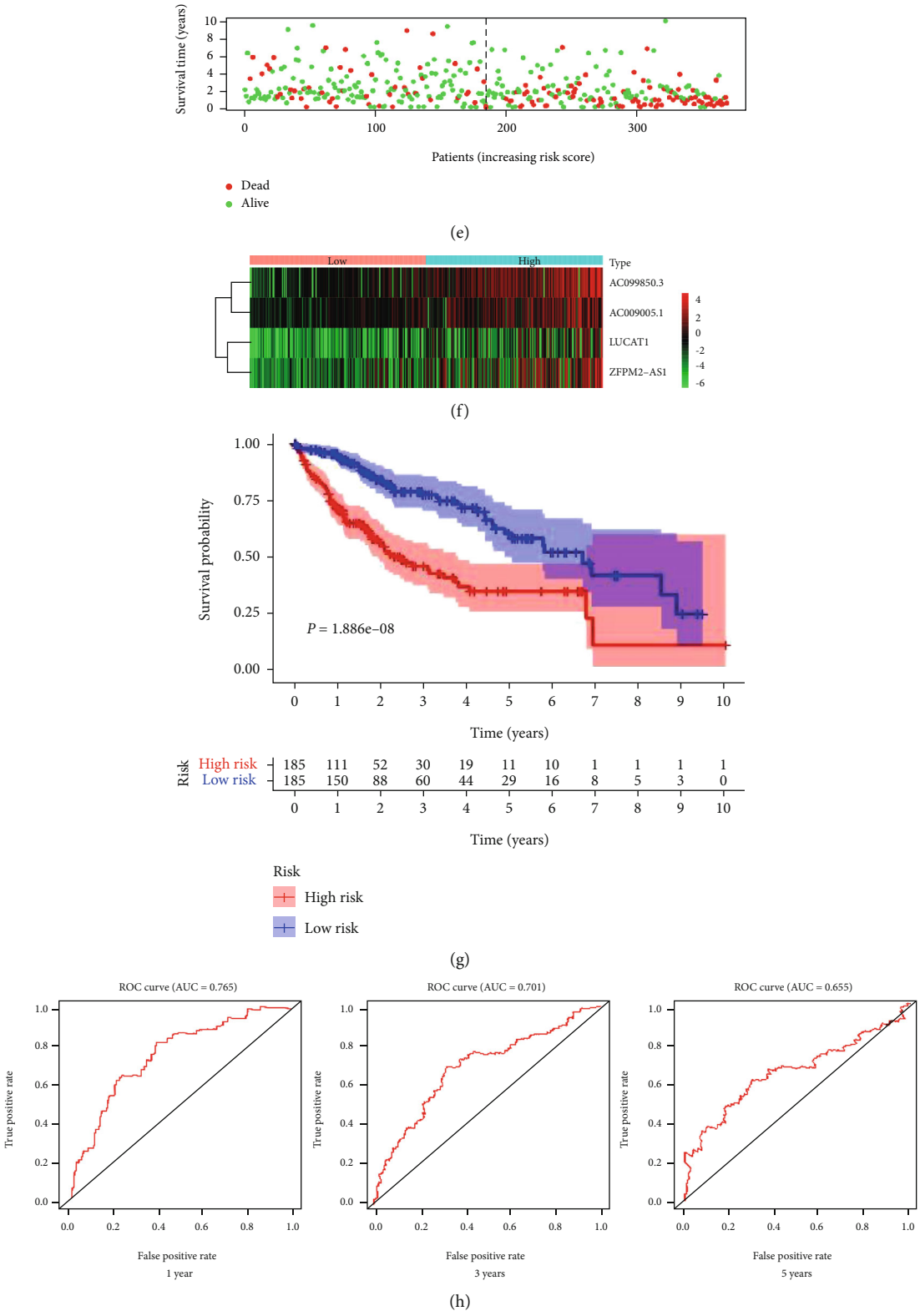


Figure 1: Construction of an autophagy-related lncRNA predictive model in HCC patients. (a) A heat map showing the differential expression of lncRNAs. (b) Differential expression of each lncRNA between normal and HCC liver tissues. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (c) K-M curves of OS of the four lncRNAs in HCC patients. (d) The distribution of the risk score. (e) Survival status of HCC patients in different groups. (f) A heat map showing the differential expression of each lncRNAs between the high-risk group and the low-risk group. (g) K-M curves of the autophagy-related lncRNA model for HCC patients. (h) ROC curves for the 1-, 3-, and 5-year survival prediction.

Table 1: K-M and univariate Cox regression analyses of lncRNAs for OS of HCC patients.

lncRNA	KM	B	SE	HR	95% CI	*P* value
LUCAT1	0.013	0.164	0.038	1.179	1.095-1.269	<0.001
AC092171.2	0.005	0.077	0.032	1.080	1.015-1.149	0.016
MYLK-AS1	0.002	0.216	0.101	1.241	1.018-1.513	0.033
AC009005.1	0.010	0.152	0.042	1.165	1.073-1.264	<0.001
AC099850.3	0.002	0.135	0.024	1.145	1.093-1.199	<0.001
ZFPM2-AS1	<0.001	0.092	0.019	1.096	1.056-1.138	<0.001
AL606489.1	0.007	0.181	0.071	1.199	1.043-1.378	0.011
AC024361.1	0.038	0.308	0.137	1.360	1.040-1.779	0.025
LINC00942	0.014	0.036	0.008	1.037	1.021-1.053	<0.001

depending on its activation status and different cellular conditions [7, 8]. For example, Wu et al. identified that the high expression of autophagic LC3B positively correlated with malignant progression and might be a prognostic biomarker for HCC [9]. In addition, growing research has demonstrated that autophagy-related gene signatures can act as a kind of new emerging biomarkers to robustly predict clinical outcomes in various types of cancers including HCC [10–12]. Therefore, exploring the mechanism of regulating autophagy in the tumorigenesis, metastasis, and treatment of HCC could contribute to the study of new therapeutic strategies and prognostic biomarkers for HCC patients. Studies have shown that autophagy is regulated by various factors. Except for classic energy signal molecules, protooncogenes, and suppressor genes, noncoding RNAs also play an important role [13–15].

Noncoding RNA refers to RNA that does not encode proteins. Among them, those with a length greater than 200 nucleotides are called "long noncoding RNA (lncRNA)," and the length less than 200 nucleotides is called "small noncoding RNA (sncRNA)," such as microRNA (miRNA) [16]. Unlike sncRNAs, the length of lncRNA allows it to regulate gene expression levels in a more complex transcription and translation network. Increasingly, recent studies have shown that lncRNAs can modulate autophagy effector molecules and pathways at different autophagic stages in HCC [15, 17–19]. However, the lncRNAs and their role in the autophagy axis in the prognosis of HCC are still under investigation. And the clinical role, particularly the prognostic role of autophagy-related lncRNAs in HCC, has yet to be determined.

In this study, we established an effective autophagy-related lncRNA signature for predicting the survival of HCC patients. Additionally, we comprehensively explored the molecular mechanism by which these autophagy-related lncRNAs affect the progression of HCC by regulating autophagy. Our study provides a theoretical basis in the potential therapeutic target selection.

2. Materials and Methods

2.1. Data Source. The raw RNA-Seq data, miRNA-Seq data, and the corresponding clinical information of patients with HCC were obtained from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database, which consisted of 374 HCC tumor and 50 normal liver tissue specimens. The 222 autophagy genes were extracted from the Human Autophagy Database (HADb, http://autophagy.lu/clustering/index.html), containing a list of genes directly or indirectly involved in the autophagy process reported in literature.

2.2. Identification of Differentially Expressed lncRNAs and Autophagy-Related lncRNAs. We downloaded the *Homo sapiens* ensemble ID (https://www.ensembl.org/) of RNA to retrieve the required expression information from RNA-Seq data. The differentially expressed lncRNAs were calculated using the package "edgeR" from R by comparing the HCC group and normal liver tissues. Differentially expressed lncRNAs with an absolute log2 fold change (FC) ≥ 2 and an adjusted P value < 0.05 were filtered out for subsequent analysis. Subsequently, we used the Pearson correlation to calculate the correlation between the lncRNAs and these 222 autophagy-related genes. Finally, the differentially expressed lncRNAs with the correlation coefficient > 0.3 and $P < 0.05$ with the autophagy-related genes were filtered out to be the autophagy-related lncRNAs [20].

2.3. Construction of the Autophagy-Related lncRNA Prognostic Signature. Univariate Cox regression and Kaplan-Meier (K-M) analyses were used to screen out autophagy-related lncRNAs that are significantly correlated with the overall survival (OS) of patients with HCC. The autophagy-related lncRNAs with a P value < 0.05 by univariate analysis and K-M analysis were included in the multivariate regression Cox analysis. Subsequently, we used the stepwise selection of variables based on the Akaike information criterion to identify optimal independent prognostic autophagy-related lncRNAs and the most appropriate model. The prognosis signature was constructed based on a linear combination of the regression coefficient derived from the multivariate Cox regression model (β) multiplied with its expression level. The cut-off point for the risk score was identified with the median to stratify HCC patients into the high-risk group and the low-risk group. The survival differences between the high-risk and the low-risk group were compared by the log-rank test. The time-dependent receiver operating characteristic (ROC) curves for predicting OS were drawn, and area under the curve (AUC) values were generated using R with the survival ROC package.

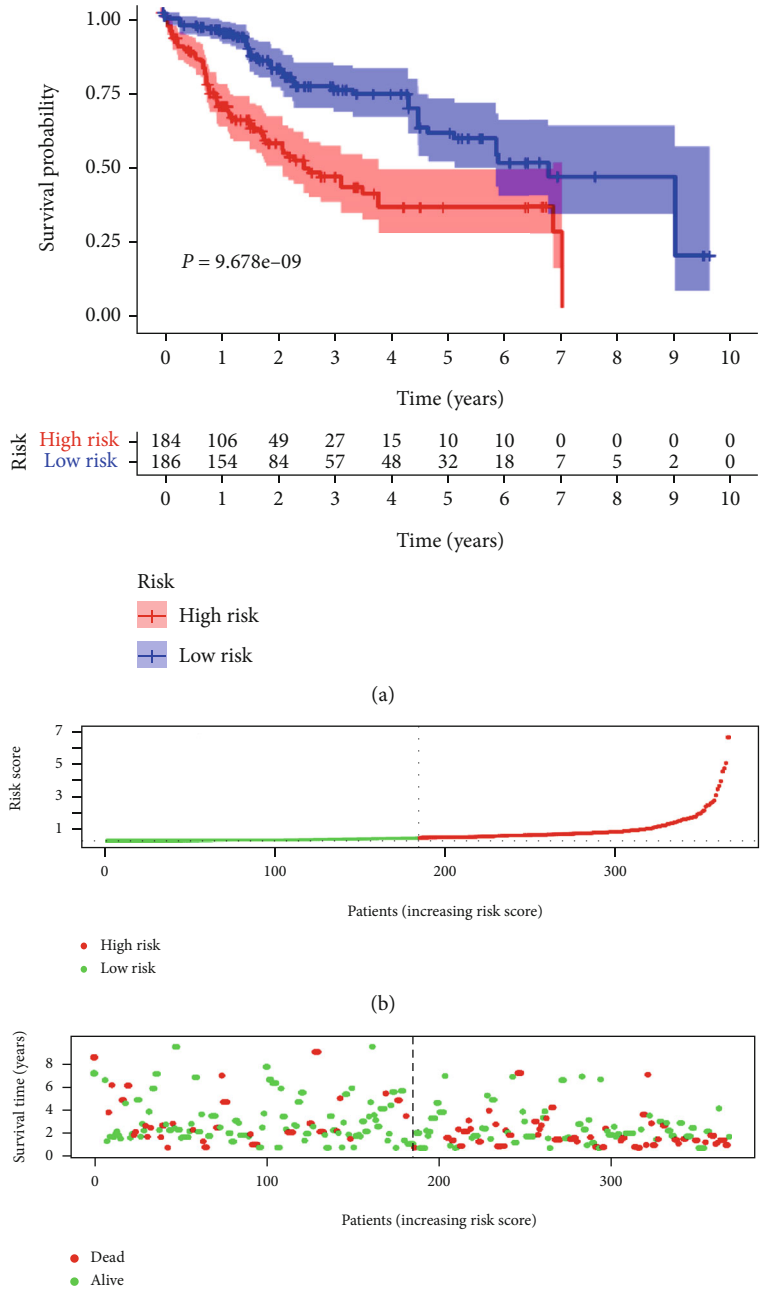
2.4. Internal Validation. An internal validation was performed to validate the predictive performance of the present prognostic model. The validation dataset was constructed by drawing 370 HCC patients with known survival times in the TCGA database using the bootstrap resampling method, which was recommended for internal validation of the prognostic model [21, 22].

2.5. Clinical Samples. We collected thirty-seven tumor tissues from primary HCC patients in Shandong Provincial Hospital, Shandong University, Jinan, Shandong, China, from July 2016 to December 2016. The inclusion criteria were as

Table 2: The information of the 4 lncRNAs in the signature.

lncRNA	Ensemble ID	Chromosome	Coefficient†	HR†	95% CI†	*P* value†
AC099850.3	ENSG00000265415	Chr17q22	0.125	1.133	1.078-1.192	<0.001
LUCAT1	ENSG00000248323	Chr5q14.3	0.109	1.116	1.019-1.222	0.018
ZFPM2-AS1	ENSG00000251003	Chr8q23.1	0.055	1.056	1.010-1.104	0.016
AC009005.1	ENSG00000267751	Chr19p13.3	0.106	1.112	1.018-1.214	0.018

†Statistics derived from multivariate Cox proportional hazards regression analysis.



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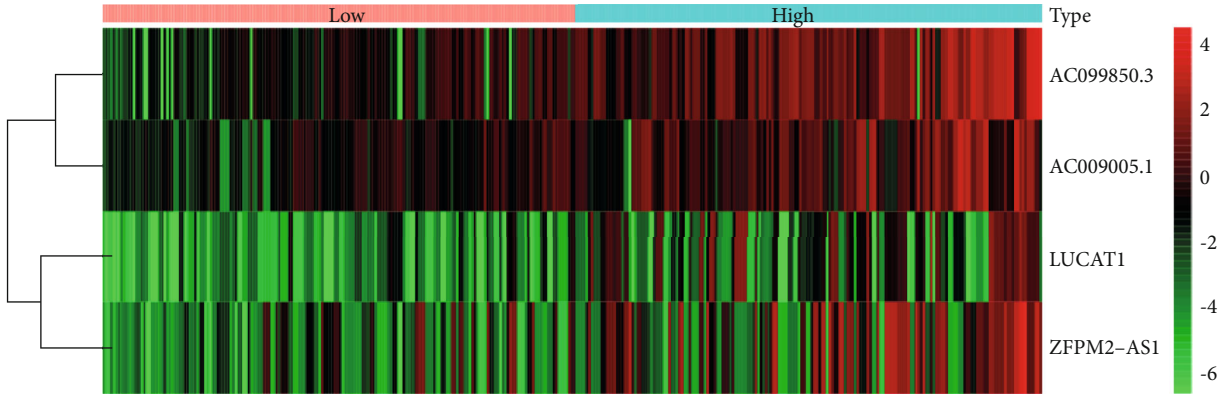

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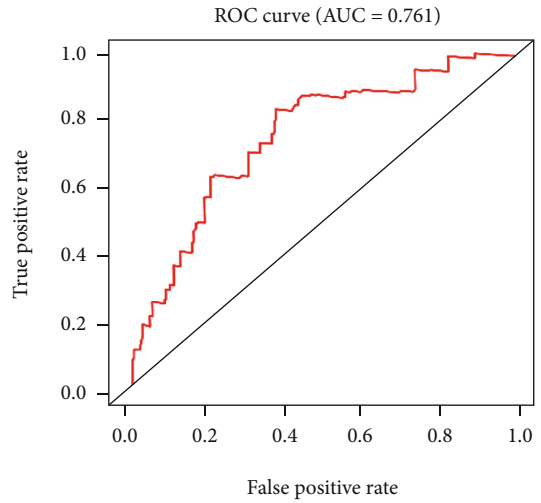


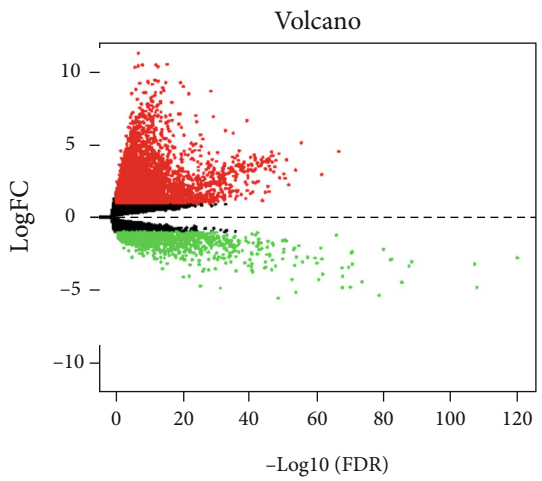
(e)

Figure 2: Validation of the autophagy-related lncRNA prognostic signature. (a) The survival curve of the model for the probability of OS in the validation of HCC cohort. (b) The distribution of the risk score in the validation cohort. (c) Survival status and survival time of HCC patients in the validation cohort. (d) A heat map showing the differential expression of each lncRNA. (e) ROC curve validates the prognostic significance of the signature in the validation cohort.

follows: (1) patients > 18 years old, (2) patients with pathologically confirmed HCC, and (3) patients who underwent curative surgical resection. Patients were excluded if they had other tumors or had recurrent HCC. A total of 11 normal liver tissues were collected from the patients with hepatic trauma undergoing surgical treatment. All tissues were fresh-frozen in liquid nitrogen immediately following surgical resection and stored at -80°C. And all procedures were approved by the Ethics Committee of Shandong Provincial Hospital.

2.6. Cell Culture. HCC cell lines (Huh7, MHCC97-h, LM3, and Bel-7402) and the LO2 cell line, human immortalized normal hepatocyte, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The LO2 cell line and HCC cell lines were cultured using Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Grand Island, NY, USA) with 10% fetal bovine serum (GibcoBRL, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco, Grand Island, NY, and Scotland, UK). The humidified incubator containing 5% CO_2 at 37°C was used to culture cell lines.

2.7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA from human tissues and cultured cells were extracted using the TRIzol reagent (Takara, Shiga, Japan). cDNAs were then generated using a reverse transcription kit (Takara, Shiga, Japan), and the gene expression was determined with real-time-PCR using a SYBR Green PCR kit (Takara, Shiga, Japan) in accordance with the manufacturer's instructions. The PCR primers are listed as follows: GAPDH-F: 5′-ACCCA CTCCT CCACC TTTGAC-3′, GAPDH-R: 5′-TGTTG CTGTA GCCAA ATTCG TT-3′; AC099850.3-F: 5′-TCGCT ATGTT TCCCA GGCTG TATT-3′, AC099850.3-R: 5′-TGCCA AGGAA TCTCT GAAGT CCAT-3′; LUCAT1-F: 5′-GTGTC CAAAT GCTGT CCTCA TCTC-3′, LUCAT1-R: 5′-ATCCT CGGGT TGCCT CTGTT TA-3′; ZFPM2-AS1-F: 5′-TGGTG GTATT TCTGC TGTTC TC-3′, ZFPM2-AS1-R: 5′-GTTCC ATCTT CCTCC TTGTC TAC-3′; and



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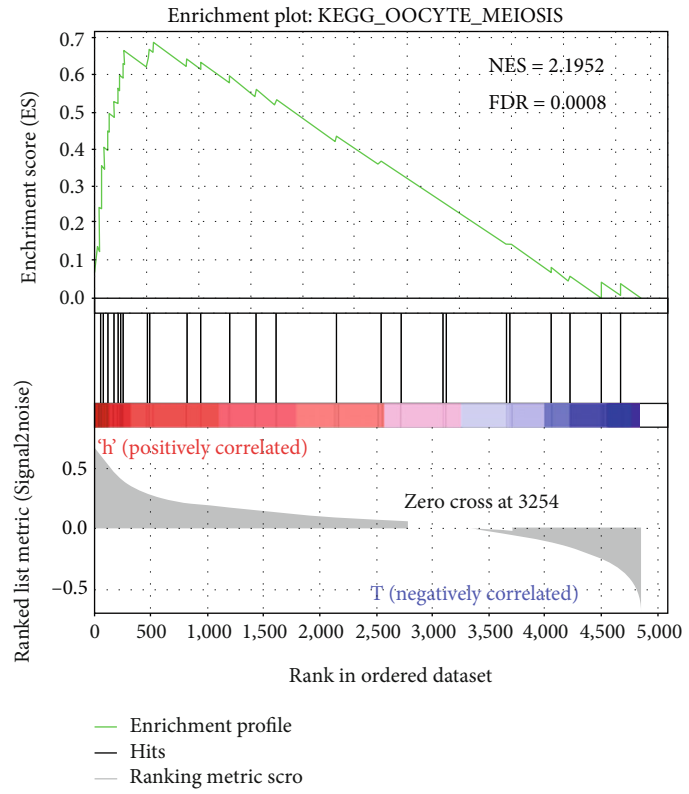

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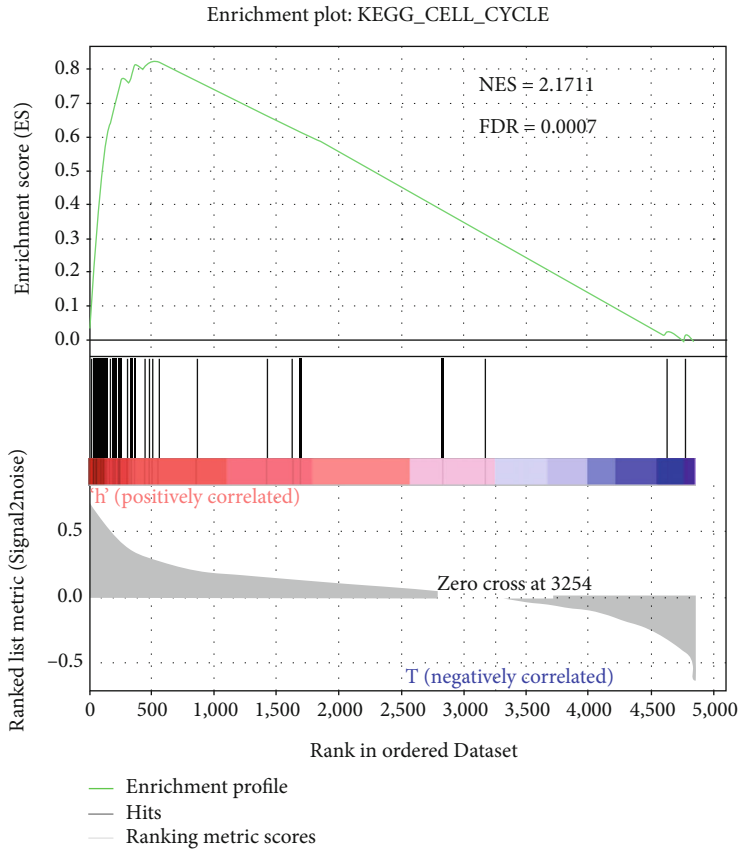

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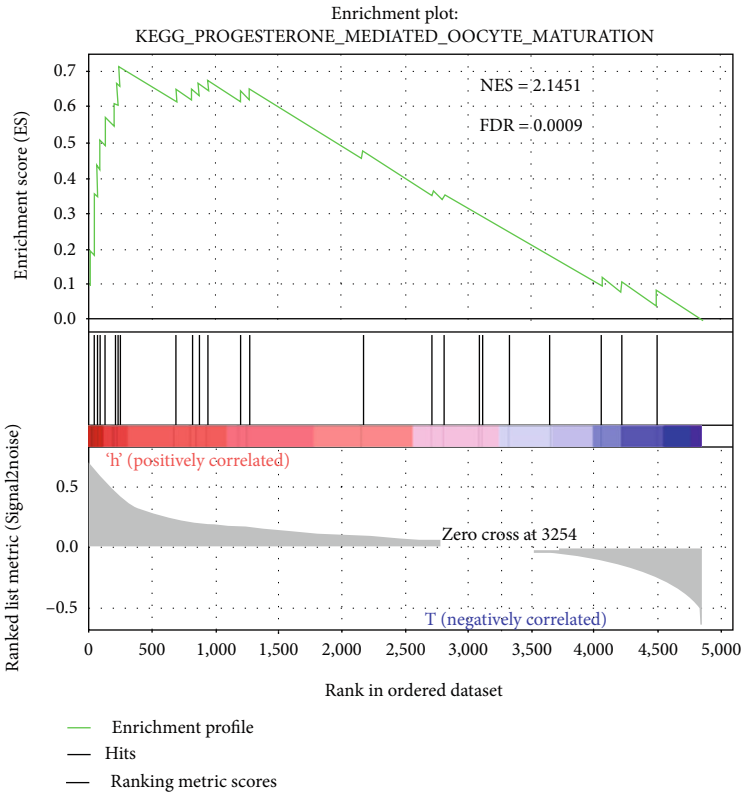

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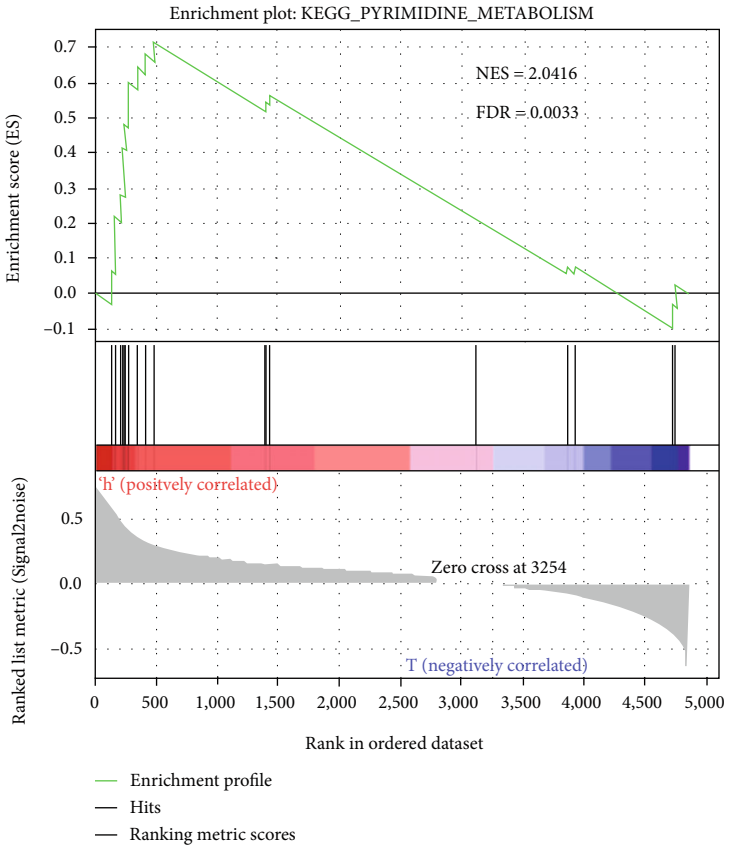

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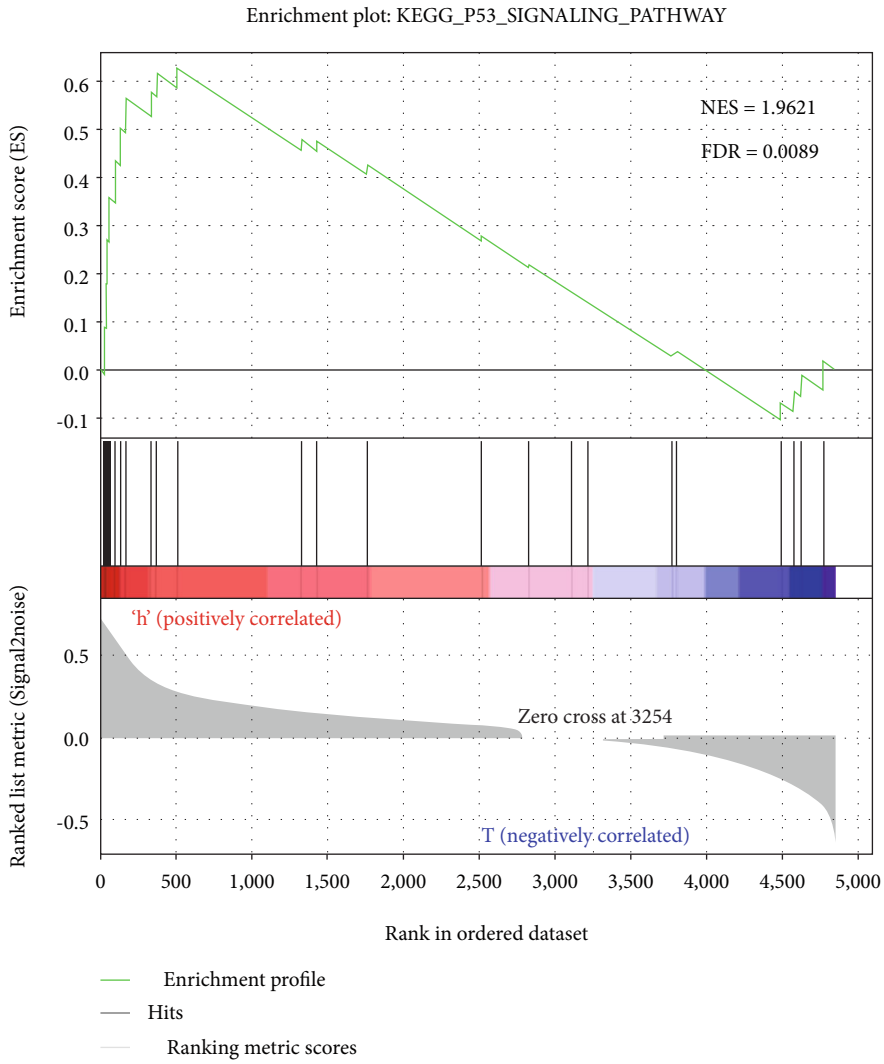

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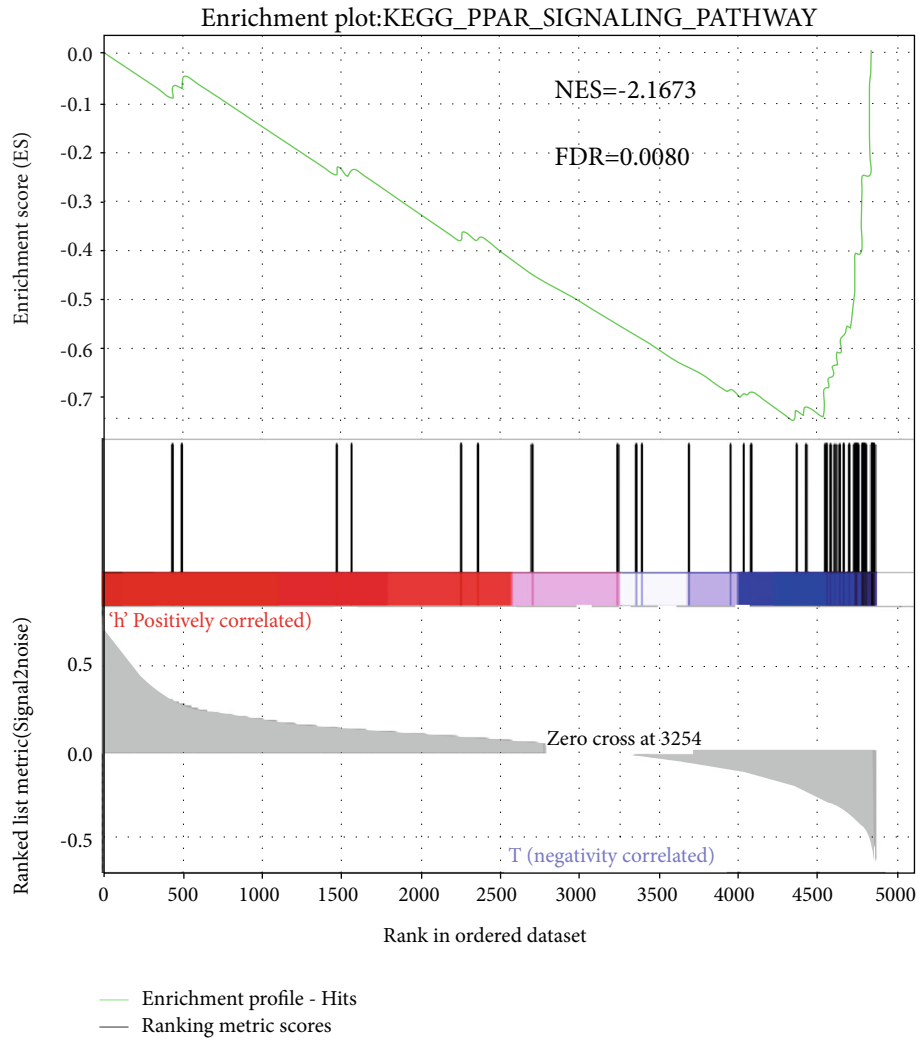


Figure 3: Gene set enrichment analysis. (a) Differentially expressed mRNAs. Red dots represent upregulated RNAs, and green dots represent downregulated RNAs. Gene set enrichment analysis indicated significant enrichment pathways in the high-risk group (b–f) and the low-risk group (g).

AC009005.1-F: 5′-GGCAA ACATC TCTTG TCCAT CCT-3′, AC009005.1-R: 5′-CTCTC CGCAT ATCCC TCCTT CT-3′. The $2^{-\Delta\Delta Ct}$ method was conducted to calculate the lncRNA expression. The Student *t*-test was used to compare the expression level of each lncRNA betwe3en different groups.

2.8. Gene Set Enrichment Analysis. In order to explore the pathways that are affected in the high-risk group and low-risk group, gene set enrichment analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp, version 3.0) was performed. Firstly, differentially expressed mRNAs were filtered out between tumor and adjacent normal liver tissues (absolute logFC ≥ 1.0 and $P < 0.05$). Then, we tested whether the differentially expressed mRNAs were enriched in the high-risk group and low-risk group using GSEA. The hallmarks were calculated using a normalized enrichment score (NES) and false discovery rate (FDR). Pathways with NES > 1 and FDR < 0.01 were considered significant enriched functional pathways.

2.9. Construction of the Coexpression and ceRNA Network. Differently expressed autophagy-related genes with an absolute log2 FC ≥ 1 and an adjusted *P* value < 0.05 were filtered out for subsequent analysis. These genes that highly correlated with autophagy-related lncRNA were used to construct the coexpression network.

The lncRNA-miRNA interactions were predicted by the miRcode database (http://www.mircode.org/) and starBase (http://starbase.sysu.edu.cn/) containing putative miRNA target sites in the long noncoding transcriptome. Differently expressed autophagy-related genes targeted by matched miRNAs were retrieved from miRDB, TargetScan (http://www.targetscan.org/), and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php). Cytoscape software (version 3.7.0) was used to visualize the network.

2.10. Statistical Analysis. Data was presented as the mean ± standard error of mean (SEM). Statistical analyses were performed using R language (version 3.5.), SPSS 25.0 software (SPSS Inc., Chicago, IL), or GraphPad Prism 7 (GraphPad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Differentially Expressed Autophagy-Related lncRNAs in HCC. The RNA-Seq data of 374 HCC tissues and 50 normal liver tissues were obtained from the TCGA database. To retrieve the required lncRNA expression information using Homo sapiens' ensemble ID, the 14370 lncRNA expression profiles were included in the study. After differential expression analysis, 1097 differentially expressed lncRNAs were screened out by comparing HCC and normal liver tissues ($|\log FC| \geq 2.0$, adjusted $P < 0.05$, Figure 1(a)). Furthermore, a total of 222 genes involved directly or indirectly in autophagy were downloaded via the online database HADb. The expression data of these autophagy-related genes were extracted from TCGA, which were used for further identifying their relationship with differentially expressed lncRNAs. Finally, 78 lncRNAs were selected according to correlation coefficient > 0.3 and $P < 0.05$ with autophagy-related genes, and these lncRNAs were regarded as autophagy-related lncRNAs.

3.2. Establishment and Internal Validation of an Autophagy-Related lncRNA Signature for the Prognosis of HCC Patients. Univariate Cox regression and K-M analyses based on 78 autophagy-related lncRNAs were used to screen prognostic biomarkers. A total of 9 autophagy-related lncRNAs which are identified as risk factors (HR > 1) and have prognostic value for HCC patients were screened out (Table 1). Furthermore, 4 independent prognostic autophagy-related lncRNAs (AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1) were selected to develop the prognostic signature according to the multivariate Cox regression analysis based on the Akaike information criterion (Table 2). The expression levels and K-M curves for these lncRNAs were presented in Figures 1(b) and 1(c). Finally, the prognostic model was developed as follows: risk score = $(0.125 * \text{AC099850.3}) + (0.109 * \text{LUCAT1}) + (0.055 * \text{ZFPM2-AS1}) + (0.106 * \text{AC009005.1})$. Based on the risk score, 370 HCC patients with survival times were classified as high-risk and low-risk groups according to the cut-off point. The distribution of the risk score and survival status of HCC patients is shown in Figures 1(d) and 1(e). The heat map of these four signature-related lncRNAs in the high-risk group and the low-risk group of HCC patients is displayed in Figure 1(f). K-M curves confirmed that the survival times of patients in the low-risk group were longer than those of patients in the high-risk group (2.636 ± 0.158 years vs. 1.753 ± 0.126 years, $P < 0.0001$, Figure 1(g)). ROC curves of OS were used to reveal the predictive performance of the four lncRNA risk signatures. The AUC values of the signature for the 1-, 3-, and 5-year survival were 0.765, 0.702, and 0.655, respectively (Figure 1(h)).

Table 3: Distribution of HCC patients' characteristics and the clinical correlation with the lncRNAs signature ($n = 235$).

Clinical parameter	Group	n	Risk score		
			Mean	SD	P value
Age	≤55	95	1.402	1.550	0.30946
	>55	140	1.208	1.241	
Gender	Female	74	1.218	1.348	0.60302
	Male	161	1.318	1.389	
Grade	G1-2	132	1.084	0.940	0.01671
	G3-4	103	1.546	1.754	
Stage	Stages I-II	163	1.200	1.377	0.14686
	Stages III-IV	72	1.482	1.356	
T	T1-2	167	1.193	1.362	0.10477
	T3-4	68	1.517	1.388	
M	M0	231	1.294	1.385	0.00021
	M1	4	0.835	0.12	
N	N0	231	1.293	1.385	0.00728
	N1	4	0.908	0.166	

An internal validation cohort ($n = 370$) was assembled by random drawing with the replacement method from the model cohort ($n = 370$). Risk prediction scores for patients in the validation cohort was calculated. 370 patients in the validation cohort were stratified into the high-risk group ($n = 185$) and low-risk group ($n = 185$) following the median cut-off predicted value. The survival curve analysis indicated that the OS rate in the high-risk group was significantly poorer than that in the low-risk group ($P = 9.672e - 09$, Figure 2(a)). The distribution of the risk prediction score in the validation cohort is presented in Figure 2(b). The survival status and survival time in the validation cohort are presented in Figure 2(c). The heat map of these five signature-related lncRNAs in the high-risk and low-risk groups of HCC patients in the validation cohort is displayed in Figure 2(d). The AUC value of the signature was 0.761 (Figure 2(e)).

3.3. Molecular Pathways Disturbed between the High-Risk Group and the Low-Risk Group. Using $P < 0.05$ and absolute $\log FC \geq 1.0$ as cut-offs, we found that 4851 mRNAs were differentially expressed between tumor and adjacent normal liver tissues (Figure 3(a)). Only these genes were included in the further study. GSEA was computed to pick up the molecular pathways disturbed between the high-risk group and the low-risk group. Only when the FDR < 0.01 were achieved could gene sets be considered significantly enriched. The results revealed that the "oocyte meiosis," "cell cycle," "progesterone-mediated oocyte," "pyrimidine metabolism," and "P53 signaling" pathways were enriched in the high-risk group (Figures 3(b)–3(f)). Genes coexpressed in the low-risk group were significantly enriched in the "PPAR signaling pathway" (Figure 3(g)). Several studies have indicated that these pathways were associated with the development of HCC. Taken together, the GSEA analyses implied that the four-autophagy-related lncRNA signature was associated

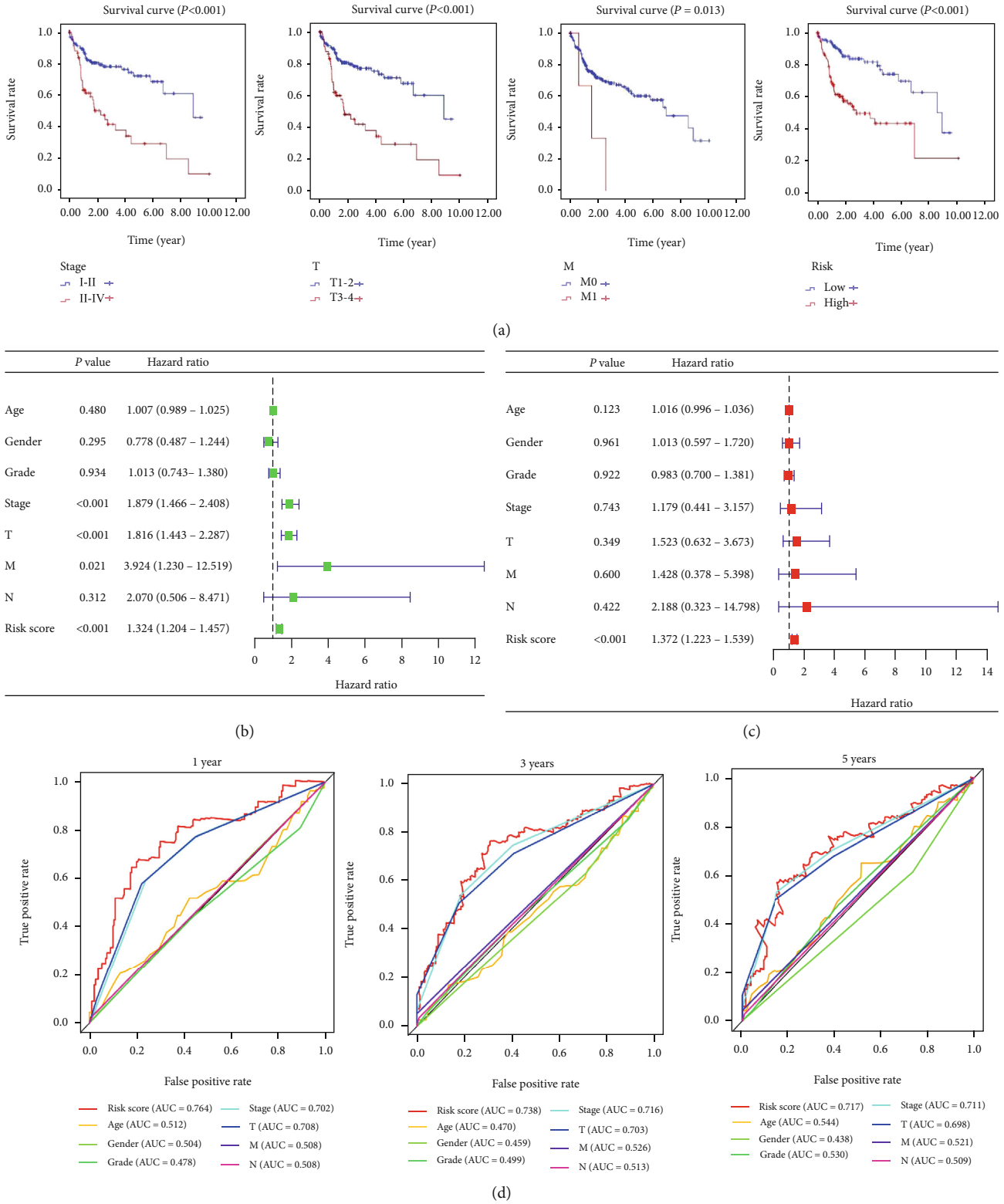


FIGURE 4: Relationship between the autophagy-related lncRNA prognostic signature and clinicopathological features of HCC. (a) K-M curves of stage, T stage, metastasis, and risk scores for the probability of OS in the HCC patients. The forest plot of univariate (b) and multivariate (c) Cox regression analyses in HCC patients. (d) ROC curves validate the prognostic significance of autophagy-related lncRNA prognostic indicators and clinicopathological features.

with the HCC development and progression, which might provide strong evidence for a cancer-targeted treatment.

3.4. Relationship between the Four-Autophagy-Related lncRNA Model and Clinicopathological Features. After filtering out patients with incomplete clinical information, a total of 235 HCC patients were included in the analysis. Firstly, we determined the clinical value of the autophagy-related lncRNA signature regarding the age, gender, grade, and the tumor stage. Results showed that the signature was significantly associated with the grades ($P = 0.017$) and M and N stages ($P < 0.001$ and $P = 0.007$, respectively), suggesting that this lncRNA signature might be associated with the progression of HCC (Table 3).

K-M curves showed that patients with high stage, high T stage, distant metastasis, or high-risk scores have worse prognosis (Figure 4(a)). Subsequently, we performed univariate and multivariate Cox regression analyses to verify the independent predictive value of the four lncRNA signatures for OS. The univariate Cox analysis showed that the tumor stage, T and N stages, and the autophagy-related lncRNA signature were all correlated with the survival of HCC patients (Figure 4(b)). Then, those factors were included in a multivariate Cox analysis, which showed only this signature to be an independent predictive factor (HR = 1.921, 95% CI = 1.013 – 3.644, $P < 0.0001$, Figure 4(c)). Thus, our results confirmed that the autophagy-related lncRNA signature could be used as an independent prognostic factor in clinical practice.

Furthermore, the predictive power value for survival of this signature and clinical factors for survival were compared using ROC curve analysis. The results suggested that the pathological stage and T stage show better prognostic ability for survival than the other factors. The AUCs of the pathological stage were 0.702, 0.716, and 0.711, respectively, for the 1-, 3-, and 5-year survival (Figure 4(d)). In addition, the AUC of the T stage was 0.708, 0.703, and 0.698 at the survival time of 1, 3, and 5 years, respectively. However, the autophagy-related lncRNA model shows the best favorable indicator for survival prediction in value in HCC patients than other clinicopathological factors for the 1-, 3-, and 5-year survival (AUC = 0.764, 0.738, and 0.717, respectively. Figure 4(d)).

3.5. Prognostic Value of Autophagy-Related lncRNA Signature in HCC Patients without Complete Clinical Information. We also included the other 136 patients with incomplete clinical information in the subsequent analysis. K-M curves confirmed that the survival times of patients in the low-risk group were longer than those of patients in the high-risk group (2.072 ± 0.181 years vs. 1.651 ± 0.196 years, $P = 0.004963$, Figure 5(a)). The distribution of the risk prediction score and survival status in this cohort .is presented in Figure 5(b). ROC curves of OS were used to reveal the predictive performance of the four-autophagy-related lncRNA risk model in HCC patients without complete clinical information. The AUC value of the signature for the 1-year survival was 0.756 (Figure 5(c)). Altogether, the results show that this risk score model also has good prediction efficiency in HCC patients with incomplete clinical information.

3.6. Validating the Expression Level of the Four lncRNAs in Clinical HCC Patients and In Vitro. AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1 were highly expressed in tumor tissues than normal liver tissues according to the result of the TCGA database. Subsequently, we determined the expression levels of these four lncRNAs in 37 tumor tissues from primary HCC patients and 11 normal liver tissues using qRT-PCR. Hematoxylin-eosin staining was used to assess whether the tissue is normal or HCC (Figure 6(a)). As the results, all lncRNAs—AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1—displayed high expression patterns in HCC tumor tissues when compared with normal samples (Figure 6(b)), which was consistent with the findings in the TCGA cohort.

Additionally, we detected the expression level of each lncRNA in LO2 and HCC cell lines (Huh7, MHCC97-h, LM3, and Bel-7402). All HCC cell lines indicated higher expression levels of each lncRNA compared to the normal hepatocyte cell line LO2 (Figure 6(c)).

3.7. Mechanism of Regulatory Network for the Four Autophagy-Related lncRNAs. We found that 99 autophagy-related genes are related to the expression of lncRNAs (AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1) according to correlation coefficient > 0.3 and $P < 0.05$ (Table S1). Among these 99 autophagy-related genes, only 11 genes were differentially expressed and selected to construct the coexpression networks (Figure 7(a)). The visualization of coexpression networks of the 4 lncRNAs and mRNAs is shown in Figure 7(b).

For further analysis of the mechanisms of these four prognostic lncRNAs, the ceRNA network was also considered. The target relationships between the four autophagy-related lncRNAs and miRNAs were assessed using the miRcode and starBase. The result showed that 22 miRNAs have the binding domains with LUCAT1, ZFPM2-AS1, and AC009005.1. Furthermore, we predicted the target mRNAs of these miRNAs through miRDB, miRtarBase, and TargetScan. A total of 367 mRNAs were filtered out for subsequent analysis. Lastly, DAPK2 and DLC1 were selected as the differently expressed and autophagy-related overlapping genes (Figure 7(c)). Finally, according to the above results, LUCAT1 and ZFPM2-AS1 can regulate the biological behavior through the ceRNA network. lncRNA LUCAT1 functioned as an autophagy promoter in HCC through sponging hsa-miR-495-3p (Figures 7(d) and 7(e)). What is more, ZFPM2-AS1 affects the autophagic activity in HCC through the hsa-miR-515-5p/DAPK2 axis (Figures 7(d) and 7(e)). In addition, we collect and analyze the expression levels of miR-495-3p/DLC1 and miR-515-5p/DAPK2 and the survival information of HCC patients based on the TCGA database. The univariate Cox regression and K-M analyses were presented to evaluate the prognostic value of miR-495-3p, DLC1, miR-515-5p, and DAPK2. As shown in Table S3, only DLC1 was correlated with the overall survival of patients with HCC.

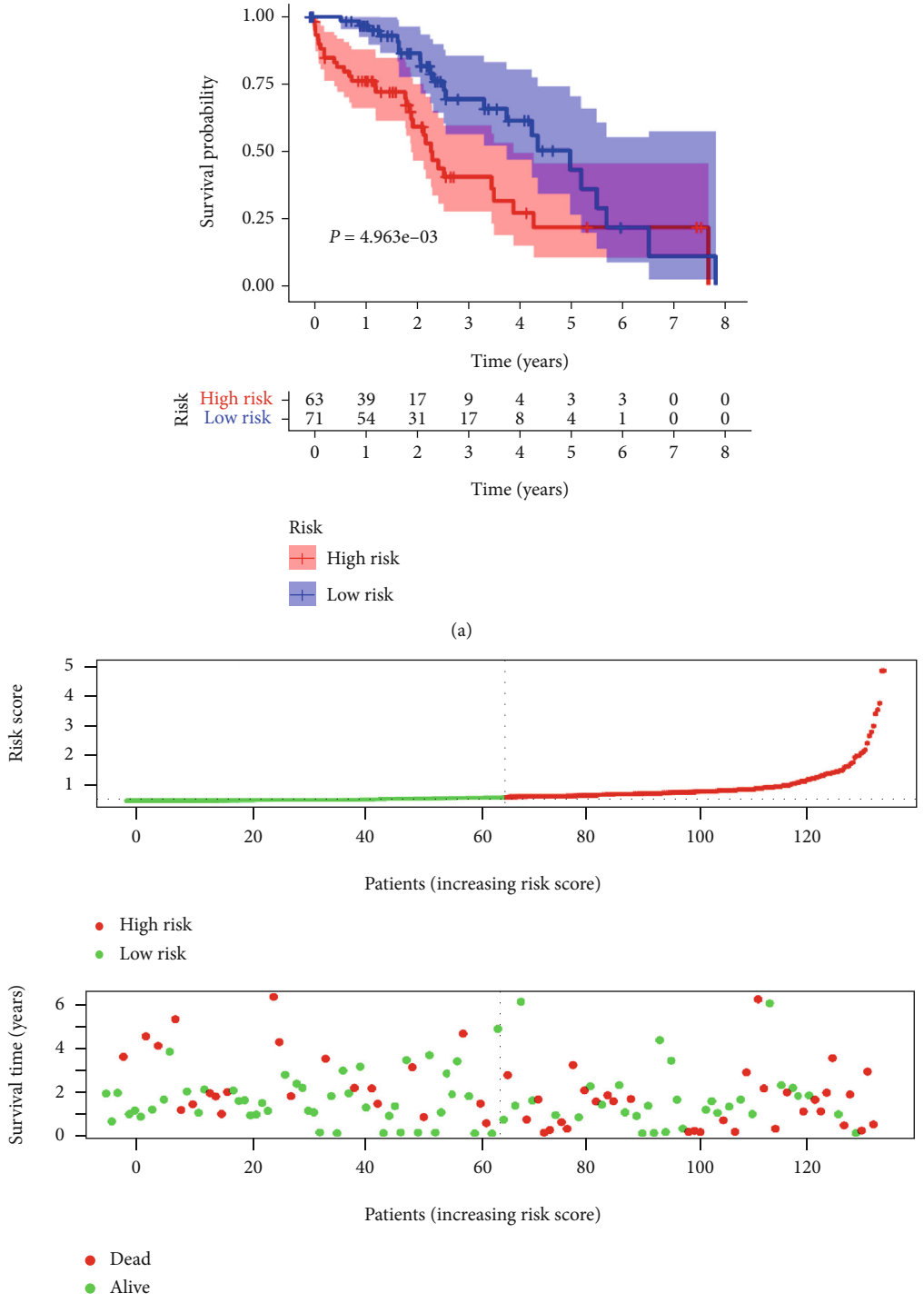
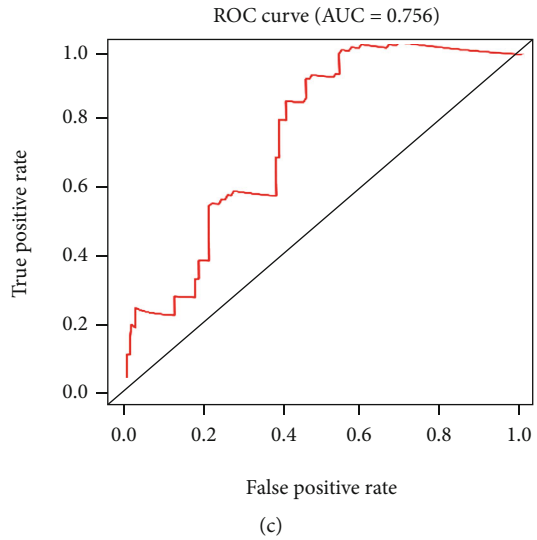


Figure 5: Continued.



(c)

Figure 5: Prognostic value of the autophagy-related lncRNA signature in HCC patients without complete clinical information. (a) K-M curve of OS of the signature. (b) The distribution of the risk score and survival status of HCC patients with incomplete clinical information. (c) ROC curve for survival prediction.

4. Discussion

Autophagy is considered to play a crucial role in the occurrence and treatment of tumors. In recent years, as the understanding of lncRNA has gradually deepened, its role in the regulation of autophagy has also received increasing attention. Several studies have described the role of lncRNAs and autophagy in liver disease and particularly in HCC [23–27]. Therefore, it is important to understand the molecular pathogenesis mechanisms underlying the relationship between lncRNAs and autophagy in the initiation and development of HCC. Moreover, an increasing number of autophagy-related genes signatures serve as valuable prognostic signatures for tumor patients. However, none of the studies has comprehensive analysis of autophagy-related lncRNAs and explores its clinical significance in HCC. Here, we aimed to establish an autophagy-related lncRNA signature in HCC and explore the molecular mechanism of these lncRNAs and their role in the autophagy axis. Our study may lead to a better understanding of potential therapeutic approaches and biomarker assessment for HCC patients.

In this study, four lncRNAs, AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1, were found to be significantly associated with autophagy-related genes and the survival of HCC patients and were selected to develop the prognostic model according to the TCGA and HADb databases. This signature has the highest prediction efficiency in the model cohort (AUC = 0.765) and in the validation cohort (AUC = 0.761) for 1-year OS, respectively. The lncRNA risk prediction score could stratify HCC patients into the low-risk group and high-risk group, and the OS rate of high-risk patients was significantly poorer than that of low-risk patients. Subsequently, we evaluated the clinical value of the autophagy-related lncRNA signature. Results showed that the model was significantly associated with the grade M and N stages, suggesting that this lncRNA signature might be associated with the progression of HCC. Our results also confirmed that the autophagy-related lncRNA risk score could be used as an independent prognostic factor in clinical practice according to the univariate and multivariate Cox regression analyses. Furthermore, ROC curve analysis suggested that the autophagy-related lncRNA model showed better predictive value in HCC patients than other clinicopathological factors. Importantly, we found that this risk score model also has good prediction efficiency in HCC patients with incomplete clinical information.

The Coding Potential Calculator (CPC, http://cpc.cbi.pku.edu.cn/) and Coding Potential Assessment Tool (CPAT, http://lilab.research.bcm.edu/cpat/index.php) were used to evaluate the coding ability of these lncRNAs [28, 29]. Table S2 showed that these lncRNAs (AC099850.3, LUCAT1, ZFPM2-AS1, and AC009005.1) were noncoding RNA. Subsequently, we analyze the regulatory network of the four autophagy-related lncRNAs comprehensively. Among these lncRNAs, LUCAT1 influences the proliferation, migration, and invasion of tumor cells, being involved in the cell cycle of many cancer cells [30–32]. It has been shown as novel players in predicting tumor recurrence and promotes tumorigenesis by inhibiting ANXA2 phosphorylation in HCC [33]. However, the role of LUCAT1 in the prognosis of HCC through autophagy remains unclear. In this study, we confirmed the expression level of LUCAT1 in HCC tissue samples and cell lines. Additionally, according to the coexpression analysis, our study found that LUCAT1 might promote the tumorigenesis of HCC by regulating autophagy via SQSTM1 (cor = 0.526, $P < 0.0001$). Furthermore, it has been reported that lncRNAs are able to regulate miRNAs through binding and separating them from their target mRNAs to affect the autophagic activity [34]. Few studies have reported the LUCAT1-related ceRNA regulatory mechanism. For example, Wang et al. found that LUCAT1

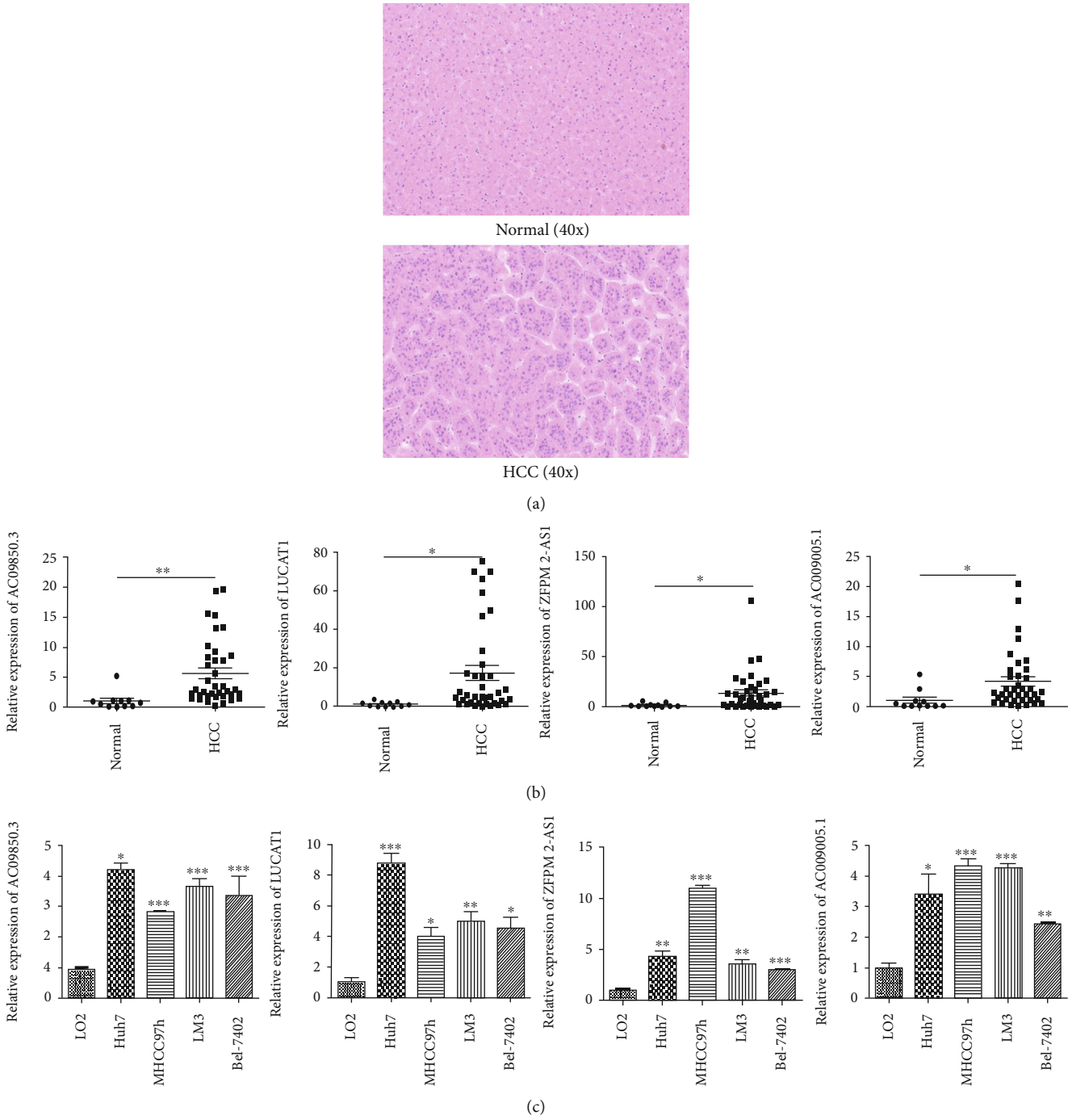


Figure 6: Validating the expression level of the four lncRNAs. (a) Hematoxylin-eosin staining shows that all patients were diagnosed as primary HCC (40x). (b) Differential expression of each lncRNA between normal liver samples and HCC tissues. (c) The expression level of each lncRNA in the normal hepatocyte cell line and HCC cell lines. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

was critical for proliferation and invasion of ccRCC cells by inhibiting the expression of miR-495-3p, which subsequently regulated the expression of SATB1 [35]. However, in this study, we further constructed the LUCAT1-related ceRNA network, in which LUCAT1 regulated miR-495-3p through directly sponging it from the target DLC1 to affect the autophagic activity in HCC.

lncRNA ZFPM2-AS1 has been verified to be upregulated and plays tumor-promoting roles in human cancers [36–39]. For instance, lncRNA ZFPM2-AS1 promotes lung adenocarcinoma progression by interacting with UPF1 to destabilize ZFPM2 [40]. Recently, researchers reported that the cancer-promoting activities of ZFPM2-AS1 were mediated by the MIF–p53 signaling pathway in gastric cancer, by the miR-18b-5p–VMA21 axis in lung adenocarcinoma, by miR-137 in renal cell cancer, and by miRNA-511-3p and consequently increasing the FGFR2 expression in cervical cancer [36–39, 41]. ZFPM2-AS1 was previously identified as a prognostic

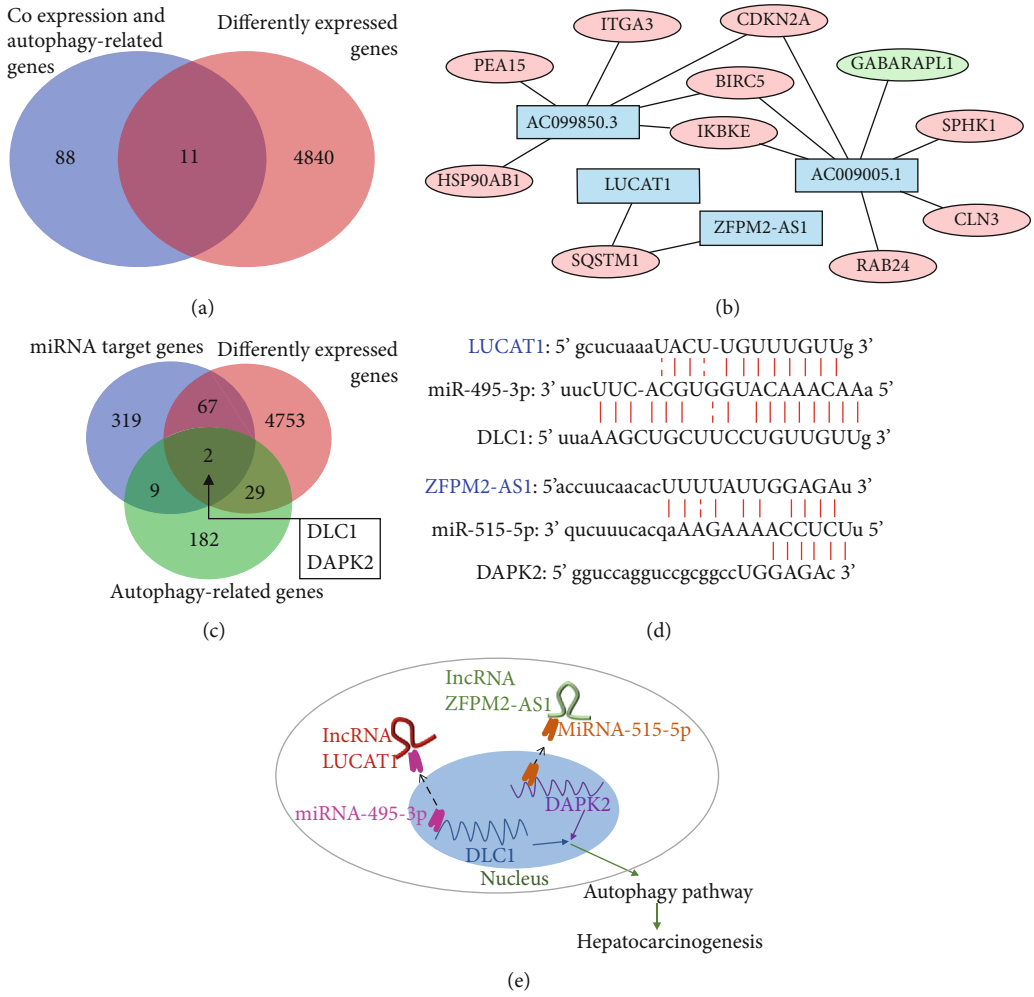


Figure 7: Construction of the regulatory network of the four autophagy-related lncRNAs. (a) Venn diagram analysis showing the genes that were differently expressed autophagy-related and having expression correlation with these four lncRNAs. (b) The coexpression networks of the four autophagy-related lncRNAs. The blue rectangles represent the lncRNAs. Red and green ellipses represent the upregulated and downregulated differentially expressed autophagy-related mRNAs, respectively. (c) Venn diagram analysis of the miRNA target genes. (d) The predictive binding site of the ceRNA network. (e) The schematic illustrates the mechanism by which LUCAT1 and ZFPM2-AS1 affect autophagy through the ceRNA mechanism to regulate HCC.

lncRNA in a TCGA lncRNA-based prognostic signature investigation in HCC patient prognoses [42]. Additionally, Luo et al. identified that the expression levels of lncRNA ZFPM2-AS1 were significantly increased in HCC tissues compared with normal liver tissues, and higher expression levels of ZFPM2-AS1 were significantly associated with a less favorable prognosis of HCC [43], which were consistent with our finding. Nonetheless, none of these studies focus on the relationship between ZFPM2-AS1 and autophagy in cancers. In addition, the expression level and functions of ZFPM2-AS1 in HCC remain poorly understood. In this study, we explore the mechanisms of ZFPM2-AS1 in HCC. On one hand, bioinformatic analysis indicated that ZFPM2-AS1 might promote the tumorigenesis of HCC by regulating autophagy via SQSTM1. On the other hand, the ceRNA network is composed of ZFPM2-AS1, miR-515-5p, and DAPK2. ZFPM2-AS1 harbors a potential binding site for miR-515-5p. And the miR-515-5p has a potential binding site for the autophagy-related gene DAPK2.

Among these four autophagy-related lncRNA model, there is no report about the expression characteristics and related regulatory mechanisms of AC099850.3 and AC009005.1 in tumors. In the study, we used clinical specimens (HCC tissue samples and normal liver tissues) and cell lines (normal and HCC cell lines) for testing to confirm the expression level and stability of lncRNA AC099850.3 and AC009005.1, and the results were consistent with the findings in the TCGA cohort. Additionally, we identified the potential mechanisms of these lncRNAs in HCC using bioinformatic analysis. The results identified that AC099850.3 have the coexpression with the differently expressed autophagy-related genes, PEA15, IKBKE, CDKN2A, BIRC5, ITGA3, and HSP90AB1. In addition, AC009005.1 may be a novel oncogene in hepatocarcinogenesis by interacting with

IKBKE, CDKN2A, BIRC5, SPHK1, RAB24, CLN3, and GABARAPL. Our results show that these two lncRNAs may influence the underlying mechanism of liver cancer development through the regulation of autophagy.

Although this identified risk score model is robust and promising, there are several limitations. We tried to search other databases to find an appropriate cohort for validation of our prediction model. However, we did not find a suitable dataset with expression profiles of all four lncRNAs and corresponding clinical data for survival analysis in the Gene Expression Omnibus (GEO) database and the International Cancer Genome Consortium (ICGC) data portal. In addition, our present study only validated the expression levels of the lncRNAs and conducted the bioinformatic analyses to provide a potential network of these 4 lncRNAs and two specific ceRNA mechanisms. However, comprehensive in vitro experiments need to be investigated to further verify the ceRNA regulation mechanism of LUCAT1/miR-495-3p/DLC1 and ZFPM2-AS1/miR-515-5p/DAPK2 and the coexpression networks of these lncRNAs.

5. Conclusions

In summary, our study has constructed a robust autophagy-related prognostic signature with four lncRNAs (LUCAT1, AC099850.3, ZFPM2-AS1, and AC009005.1) for survival prediction of HCC. Importantly, we have provided comprehensively regulatory mechanism understanding into the four lncRNAs and their role in the autophagy axis, which could be considered as prognostic biomarkers and contribute to the individual therapy research for HCC.

Data Availability

The data that support the findings of the study are available in The Cancer Genome Atlas database at https://cancergenome.nih.gov/ and the Human Autophagy Database at http://autophagy.lu/clustering/index.html.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary 1. Table S1: the relationship between the four lncRNAs and the differentially expressed autophagy-related mRNAs.

Supplementary 2. Table S2: the coding ability of the four lncRNAs according to the Coding Potential Calculator (CPC) and Coding Potential Assessment Tool (CPAT).

Supplementary 3. Table S3: K-M and univariate Cox regression analyses of RNAs for OS of 370 HCC patients.

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Review Article

Recompensation of Decompensated Hepatitis B Cirrhosis: Current Status and Challenges

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Liver-function decompensation or hepatocellular carcinoma (HCC) gradually appears after chronic hepatitis B progresses to cirrhosis. Effective antiviral treatment can significantly improve the long-term prognosis of decompensated patients, and some patients present recompensation of decompensated hepatitis B cirrhosis. At present, there are limited research data on the recompensation of decompensated hepatitis B cirrhosis. There is still controversy regarding the evaluation time, evaluation indicators, influencing factors, and long-term prognosis of recompensation.

1. Introduction

The World Health Organization reported that there are an estimated 240 million people globally with chronic hepatitis B virus (HBV) infection. Approximately 2% to 4% of patients develop compensated cirrhosis each year without effective treatment. Each year, approximately 1.5% to 4% of patients with cirrhosis further develop decompensated cirrhosis (with symptoms such as ascites, hepatic encephalopathy, and gastrointestinal-varix bleeding), leading to repeated hospitalization, severe reduction in quality of life, and even death. As a result of cirrhosis, hepatocellular carcinoma (HCC) occurs in approximately 3% to 6% of patients [1, 2]. Patients with decompensated cirrhosis have a higher rate of liver transplantation, mortality, and HCC, and a worse prognosis [3, 4]. Effective antiviral therapy can inhibit the hepatitis B virus (HBV) replication, improve liver function in patients with decompensated cirrhosis [5], and recompensate liver function in some patients [6], thereby improving their quality of life, prolonging survival time, and reducing the burden of HBV-related diseases [7–9]. In this paper, the current research status, problems, and challenges of the recompensation of decompensated hepatitis B cirrhosis are reviewed.

2. Definition of the Recompensation of Decompensated Hepatitis B Cirrhosis

Liver cirrhosis is usually divided into compensated and decompensated phases on the basis of whether patients with hepatitis B experienced severe complications such as ascites, gastrointestinal-varix bleeding, and hepatic encephalopathy [10]. Untreated patients with decompensated hepatitis B cirrhosis were previously reported to have poor prognosis, with a five-year survival rate of only 14% to 35% [11, 12]. The long-term prognosis of decompensated hepatitis B cirrhosis can be improved with increased levels of symptomatic and supportive therapies, and the active use of antiviral drugs [7].

Clinically, some patients with decompensated hepatitis B cirrhosis demonstrate significant improvements in liver function and a reduction in portal-hypertension-related complications through effective antiviral therapies. Patients are stable for a long time. They do not develop syndromes similar to compensated cirrhosis, such as ascites, gastrointestinal-varix bleeding, and hepatic encephalopathy, which are considered to be "recompensation" for the development of decompensated hepatitis B cirrhosis.

According to the Chinese Society of Hepatology Guidelines for the Diagnosis and Treatment of Cirrhosis, cirrhosis

patients may no longer have decompensated cirrhosis events (such as ascites, gastrointestinal bleeding, and hepatic encephalopathy) for a long period of time (at least one year) due to effective etiology control and the effective treatment or prevention of complications. Moreover, there may still be clinical and laboratory characteristics of compensated cirrhosis. This situation can be considered recompensation of decompensated hepatitis B cirrhosis.

Recompensation of decompensated hepatitis B cirrhosis is a state in which, after a period of active treatment, the liver's reserved function can meet the patient's daily activities, and no complications related to cirrhosis decompensation occur. In this state, the liver disease of the patient has no obvious progress or improvement, and it is not clear whether it can be maintained for a long period of time. From a pathological point of view, there is no sufficient clinical evidence to support that liver fibrosis in patients with decompensated hepatitis B cirrhosis can be reversed.

3. Current Status of Recompensation of Hepatitis B Cirrhosis Decompensation

At present, there are few studies on the recompensation of decompensated hepatitis B cirrhosis. After combining the literature on the clinical efficacy of antiviral therapies for decompensated hepatitis B cirrhosis, the research status of the recompensation of decompensated hepatitis B cirrhosis was summarized, as shown in Table 1. We list several studies related to oral antiviral therapy in HBV-related decompensated cirrhosis. In these studies, the number of cases was more than 50, and the follow-up time was more than 1 year. These studies included different patient populations with different severity in terms of Child–Turcotte–Pugh (CTP) or model for end-stage liver disease (MELD) scores, while the trials had different aims/designs. Most of these studies lacked a specific description of the occurrence of complications in decompensated liver cirrhosis. The occurrence of complications is very important for evaluating the therapeutic effect of decompensated hepatitis B liver cirrhosis.

Shim et al. [13] enrolled 70 HBV-infected patients with decompensated cirrhosis who were primarily treated with 0.5 mg of entecavir (ETV) daily, and they evaluated the clinical outcomes using intention-to-treat analyses. Cumulative transplantation-free survival was 87.1% at one year. ETV treatment for 12 months resulted in improved Child–Turcotte–Pugh (CTP) and model for end-stage liver disease (MELD) scores. In total, 66% (36/55) of patients achieved CTP Class A, and 49% (27/55) showed an improvement in CTP score of two points after 12 months of ETV.

A randomized, open-label comparative study of ETV versus adefovir therapy was performed by Liaw et al. [14], involving subjects with chronic hepatitis B who had hepatic decompensation (CTP score ≥ 7). Adult subjects were randomized and treated ($n = 191$) with 1.0 mg of ETV or 10 mg of adefovir daily for up to 96 weeks from the date of the last subject randomization. Approximately two-thirds of the subjects in both groups showed improvement and stabilization in CTP status. MELD score changes at week 48 were −2.6 for ETV and −1.7 for adefovir. Among those with baseline hepatic encephalopathy, clinical improvement was observed in 17/22 (77.3%) ETV-treated and 10/23 (43.5%) adefovir dipivoxil- (ADV-) treated patients. Similarly, in patients with baseline ascites, reversal was seen in 26/63 (41.3%) ETV-treated and 23/61 (37.7%) ADV-treated patients. Cumulative death rates were 23% for ETV and 33% for adefovir. Week 24 mortality rates were 12% for both groups.

Singal and Fontana [15] performed a meta-analysis of one-year efficacy and safety outcomes in 22 studies conducted between 1995 and 2010 on oral nucleotide analogs in patients with decompensated HBV cirrhosis. Pooled one-year data showed a favorable benefit of ETV (lamivudine; LAM) vs. untreated controls. CTP score was improved by ≥ 2 (odds ratio (OR): 117 (15, 921), $p \leq 0.0001$). Transplant-free survival was also improved (OR: 3.2 (1.2, 9), $p = 0.022$). Overall, one-year transplant-free survival rates ranged from 78% with LAM to 95% and 94% with tenofovir (TDF) and telbivudine (LdT), respectively. All oral antiviral agents were associated with improved virological, biochemical, and clinical parameters at one year. However, the efficacy of ETV and LdT was compromised by drug resistance. In addition, adefovir had low potency and a slower onset of action.

Srivastava et al. [16] evaluated the usefulness of various prognostic indicators in predicting the 24-month survival of patients with HBV-related decompensated cirrhosis after tenofovir (TDF) therapy, as well as the posttreatment outcome. The 24-month survival and mortality of 96 HBV-related decompensated patients were studied after TDF therapy. Overall survival was 0.947 at 12 months and 0.833 at 24 months. Multivariate analysis showed that an MELD score > 20 was the most robust predictor of mortality. Reversal of decompensation was observed in 48.6% of cases at the end of 24 months (i.e., without ascites or any other feature of liver failure). Posttreatment response with 24 months of TDF therapy was significantly improved in terms of hepatic function, with reversed decompensation. It showed incredible efficacy in the improvement of hepatic functional status with reduced viremia in a great majority of decompensated cirrhosis subjects who had high MELD and HBV DNA levels.

Yue-Meng et al. [17] retrospectively evaluated 130 treatment-naïve patients with HBV-related decompensated cirrhosis who had started treatment with telbivudine (LdT; $n = 31$), lamivudine (LAM; $n = 45$), or entecavir ($n = 54$). After 24 months of treatment, CTP and MELD scores were significantly decreased in all groups from 12 months onward in comparison to the baseline. Cumulative survival rates at 24 months were 80%, 93.3%, and 86.8% in the LdT, LAM, and ETV groups, respectively ($p = 0.222$, log-rank test). During the study, 16 patients died of the following causes: variceal bleeding ($n = 6$), liver failure ($n = 6$), pneumonia ($n = 1$), spontaneous bacterial peritonitis ($n = 1$), and HCC metastasis to the lungs ($n = 2$). Nineteen patients developed HCC. The cumulative rates of HCC development at 24 months were 15.0%, 14.0%, and 13.5% in the LdT, LAM, and ETV groups, respectively.

Jang et al. [18] performed a 10-year observation analysis using data from the Epidemiology and Natural History of Liver Cirrhosis study of patients with decompensated liver cirrhosis in Korea. Of the entire cohort (1595 patients

Table 1: Oral antiviral therapy in HBV-related decompensated cirrhosis.

Year, (ref.)	Country	Drugs used	Number of patients	Follow-up period	ALT normalization (%)	HBV DNA undetectable (%)	MELD score	CTP score	HCC (%)	Liver transplantation (%)	Death (%)
2018, Jang JW	Korea	ETV/LAM	179/116	10 years	n.r.	<20 IU/ml 39.3%	Change from baseline LAM −3.28 ETV −5.00	Change from baseline LAM −3.09 ETV −2.40	20.0%	0-0.5y 10.5% 0.5-10 y	0-0.5y 6.8% 0.5-10y 25.8%
2017, Wan YM	China	LdT/LAM/ETV	31/45/54	2 years	LdT 83.3% LAM 64.3% ETV 85.4%	<500 copies/ml LdT 83.7% LAM 65.3% ETV 89.1%	Significantly improved compared to baseline.	Significantly improved compared to baseline.	LdT 15.0% LAM 15.6% ETV 14.8%	n.r.	LdT 20.0% LAM 6.7% ETV 12.7%
2017, Lee SK	Korea	TDF	57	1 year	77.2%	<116 copies/ml 70.2%	Change from baseline −2.9	Change from baseline −1.7	n.r.	n.r.	0%
2012, Chan HL	Global	LdT/LAM	116/116	2 years	LdT 61.4% LAM 52.4%	<300 copies/ml LdT 49.1% LAM 39.5%	n.r.	Decrease ≥ 2 point LdT 38.6% LAM 40.4%	LdT 15% LAM 16%	LdT 4.35% LAM 3.48%	LdT 16% LAM 22%
2011, Liaw YF	Taiwan	ETV (1 mg)/ADV	100/91	1 year	ETV 63% ADV 46%	<300 copies/ml ETV 57% ADV 20%	Change from baseline ETV −2.6 ADV −1.7	Improvement ETV 38% ADV 36%	ETV 12% ADV 20%	ETV 11% ADV 3%	ETV 23% ADV 33%
2011, Liaw YF	Global	TDF/FTC +TDF/ETV	45/45/22	1 year	TDF 56.8% FTC+TDF 75.6% ETV 54.5%	<400 copies/ml TDF 70.5% FTC+TDF 87.8% ETV 72.7%	Change from baseline TDF −2.0 FTC+TDF −2.0 ETV −2.0	Decrease ≥ 2 point TDF 25.9% FTC+TDF 48.0% ETV 41.7%	n.r.	5.6%	TDF 4.4% FTC +TDF 4.4% ETV 9.1%
2010, Shim JH	Korea	ETV	70	1 year	76.4%	<51 copies/ml 89.1%	Change from baseline −2.3	Change from baseline −1.5	6.9%	4.3%	12.9%
2007, Schiff E	Global	ADV	176	1 year	77%	<1000 copies/ml 59%	Change from baseline −2.0	n.r.	n.r.	n.r.	14.0%
2003, Hann HW	USA	LAM	75	1 year	55%	<0.7 MEq/ml 69%	n.r.	Decrease ≥ 2 point 31%	n.r.	n.r.	13.3%

ALT: alanine aminotransferase; HBV: hepatitis B virus; MELD: model for end-stage liver disease; CTP: Child–Turcotte–Pugh; HCC: hepatocellular carcinoma; ETV: entecavir; LAM: lamivudine; n.r.: not reported; LdT: telbivudine; ADV: adefovir; TDF: tenofovir disoproxil fumarate; FTC: emtricitabine.

enrolled at the onset of decompensation since 2005), their analysis comprised 295 patients. In total, 60.1% of patients survived for five years and 45.7% survived for 10 years without liver transplantation. Maintained virologic response (MVR, defined as persistent undetectable HBV DNA during therapy) was observed in 116 patients (39.3%); these patients had significantly longer transplant-free survival than those of patients without an MVR. Baseline MELD score > 20 and multiple complications were associated with short-term mortality. MVR was the factor that had the strongest association with long-term transplant-free survival. Patients with an MVR had significant improvement in hepatic function over time. However, no significant reduction in the risk of HCC or HCC-related mortality was observed in these patients.

As can be seen from the above, currently published studies on the efficacy of antiviral therapy for decompensated hepatitis B cirrhosis mainly focused on comparisons of the efficacy of different antiviral agents during an observation time of one to two years. The only study with a 10-year follow-up cohort was the Korean study. Results in this study suggested that a virologic response was achieved in most patients after active antiviral therapy. Treated patients demonstrated an improvement in liver-function-related measures [13, 14, 17, 19–23]. The long-term efficacy of patients was generally assessed on the basis of a reduction in MELD and CTP scores [17–19] or the incidence of HCC, liver transplantation, and liver-disease-related death. Study results showed that MELD and CTP scores in patients with decompensated hepatitis B cirrhosis were decreased after effective antiviral therapy, suggesting that some patients may be recompensated for cirrhosis. However, only a few studies mentioned the complications related to decompensated cirrhosis. Therefore, at present, there are not many data on the recompensation of decompensated hepatitis B cirrhosis, and whether it can reduce the occurrence of HCC is controversial. The long-term prognosis of these patients is not clear.

4. Issues and Challenges

4.1. Complexity in Mechanisms of Recompensation of Decompensated Hepatitis B Cirrhosis. Hepatic-function decline and portal hypertension are the most important pathophysiological changes observed in decompensated cirrhosis. Several studies showed that effective antiviral therapies can improve liver function in patients with decompensated hepatitis B cirrhosis and help to recompensate cirrhosis. Severe portal hypertension can cause uncontrolled or recurring complications of decompensated liver cirrhosis, causing a significant reduction in survival rate without liver transplantation. The hepatic venous pressure gradient (HVPG) indirectly reflects portal-vein resistance. Studies showed that, in patients with portal hypertension, HVPG is reduced by at least 20% or to below 12 mmHg from the baseline using medication/nondrug treatment, which significantly reduces the risk of bleeding and the incidence of decompensation or progressive decompensation; risk of death is also significantly reduced. Effective antiviral therapy can reduce portal pressure and the risk of bleeding in some patients [24].

The pathogenesis of complications of decompensated hepatitis B cirrhosis is very complicated. Under portal hypertension, the formation of portal collateral circulation and the occurrence of a portosystemic shunt are promoted. The formation of portal hypertension increases the risk of ascites and esophagogastric varices. The formation of a portal shunt increases the risk of hepatic encephalopathy. Research by Nagaoki et al. [25] found that, even in patients with hepatitis B cirrhosis who responded well to antiviral therapy, baseline portal-vein collateral circulation and the extrahepatic portal shunt still had a higher incidence of esophagogastric-varix exacerbation and a risk of portal-venous systemic shunt-associated hepatic encephalopathy. Patients with liver cirrhosis have decreased resistance and are more easily infected. Studies showed that infection increases the mortality of patients with cirrhosis fourfold, resulting in patient death within one month of infection in 30% of cases [26]. Hepatorenal syndrome is a serious complication of liver cirrhosis. Patients with liver cirrhosis show a sevenfold increase in mortality, with 50% of patients dying within one month [27]. Therefore, patients with repeated complications often have poor prognosis.

In fact, the clinical manifestations of patients with decompensated hepatitis B cirrhosis are different. Some patients may present with massive ascites, while others may present with variceal bleeding or recurrent hepatic encephalopathy, and a few may present with hepatorenal syndrome and hepatopulmonary syndrome. The nutritional problems of patients with chronic liver disease are also receiving increased attention [28]. Sarcopenia may be considered one of the most common and significant complications of liver cirrhosis, and it is associated with adverse outcomes and increased morbidity and mortality [29].

Comprehensive treatment of complications can also affect the incidence of recompensation, such as the use of diuretics, portal-vein pressure-lowering drugs, endoscopic treatment of esophagogastric varices, shunt or devascularization of the portal-vein system, splenectomy, and nutritional support. These treatments affect the occurrence and duration of complications and change the long-term prognosis of patients with cirrhosis. However, it is unclear whether different types of complications need to be separately investigated.

4.2. Lack of Objective Evaluation Indicators for Recompensation of Decompensated Hepatitis B Cirrhosis. Previous studies showed that partially decompensated patients with hepatitis B cirrhosis can achieve cirrhosis recompensation through effective antiviral treatment. However, not all patients can achieve cirrhosis recompensation by inhibiting HBV replication. Some patients still have bad prognosis [30, 31]. Jang et al. [18] reported that, among 295 patients with decompensated hepatitis B cirrhosis who had started antiviral therapy at the time of first decompensation, 20 patients (6.8%) died of cirrhosis-related complications within six months of antiviral therapy. Fontana et al. [32] prospectively enrolled 154 patients with decompensated hepatitis B cirrhosis. After treatment with LAM, patients had a median follow-up of 16 months (0.5–37 months). Most deaths (78%) occurred in the first six months after initiation of antiviral therapy, with

cirrhosis-related complications as the primary cause of death. The three-year survival rate is 88% in patients treated with antiviral therapy for more than six months. Thus, patients with severely decompensated cirrhosis might not be recompensated, and they may even die before a virologic response. It is essential to promptly identify high-risk patients and implement effective treatment strategies.

It is reported in the literature that antiviral therapy for one year can reduce the score of patients with decompensated hepatitis B cirrhosis who have a baseline CTP score of ≥7 points. The treatment can also decrease the score by ≥2 points or by 49% to 72% [15]. A MELD score of >20 is considered to be the most effective predictor of death in patients with decompensated hepatitis B cirrhosis treated with TDF (the two-year mortality rate of patients with MELD score > 20 and <20 points is 60% and 1.4%, respectively) [16]. Similarly, the baseline CTP score and the MELD score after three months of antiviral treatment can predict a patient's six-month mortality rate. In liver transplantation, although the CTP score at three months is not statistically different between the death and survival groups, the survival group had a higher score than that of the death group. CTP score decreases in the first six months after treatment, but the decrease is not significant afterward [31]. Other prediction methods with important potential include the end-stage liver-disease-model dynamic score (ΔMELD) and MELD combined with serum sodium, APRI, and FIB-4.

Therefore, a comprehensive evaluation index of liver function may be helpful for the early identification of patients with "recompensation advantage." The CTP score integrates the two aspects of liver function and complications, and it can be dynamically monitored. We speculated that a dynamic change in CTP score may be a good early evaluation indicator, but it cannot reflect the dynamic changes in complications such as gastrointestinal-varix bleeding, hepatorenal syndrome, hepatopulmonary syndrome, and sarcopenia.

4.3. Lack of Liver-Pathology Research to Support Recompensation of Decompensated Hepatitis B Cirrhosis. Liver histology remains the gold standard for the diagnosis of cirrhosis. Histological evaluation of liver cirrhosis can be divided into active and quiescent periods. In the Laennec cirrhosis scoring system that is commonly recommended, the pathological diagnosis of liver cirrhosis can be further divided into Laennec 4A, 4B, and 4C substages according to the width of fibrous septa and the size of sclerosing nodules [33, 34]. The width of the fiber interval and the size of the nodules are independent predictors of portal hypertension.

The reversal of cirrhosis has become a research hotspot in recent years. Increasing clinical evidence shows that effective etiological treatments can reverse liver fibrosis/cirrhosis [35–39]. Bedossa [40] believes that the fibrous tissue in liver tissue degrades. Then, liver cells replace the disappearing fibrosis, resulting in the liver lobular structure returning to normal in order to consider cirrhosis reversal. According to pathophysiological mechanisms, the probability of cirrhosis reversal is higher if the occurrence of cirrhosis is recent, if etiology is controlled, if patients are young, or if nodular cirrhosis and avascular thrombosis are large.

The main clinical problem with the reversal of cirrhosis is the lack of reliable methods for measuring long-term changes in liver fibrosis. The Ishak fibrosis stage and Laennec cirrhosis scoring system, although commonly used, struggle to accurately assess dynamic changes in liver pathology. P-I-R classification can reflect dynamic changes in liver pathology [41]. The quantitative analysis and dynamic monitoring of liver fibrosis are more suitable for evaluating pathological changes related to decompensated cirrhosis.

According to China's Guidelines for the Diagnosis and Treatment of Cirrhosis, clinical cirrhosis can be divided into four critical periods, namely, the compensatory, decompensated, and recompensated periods, and cirrhosis reversal. In these guidelines, the criteria for the reversal of fibrotic cirrhosis include (1) a decrease in Ishak fibrosis stage by ≥1 or (2) a P-I-R classification decline after treatment [41]. Previous studies on liver pathology related to hepatitis B cirrhosis focused more on patients with chronic hepatitis B and compensated cirrhosis. Results suggested that effective antiviral therapy can improve liver histology and even end cirrhosis in some patients.

However, patients with decompensated cirrhosis often suffer from, for example, thrombocytopenia, abnormal coagulation function, and ascites. This significantly increases the risk of percutaneous liver biopsies. Although a transjugular liver biopsy can reduce the abovementioned risks, the necessary conditions are limited, and it is not widely applied in clinics. We hope that there will be relevant pathological data to support the recompensation of decompensated hepatitis B cirrhosis.

4.4. Limitations of Antiviral Therapy for Treatment of Decompensated Hepatitis B Cirrhosis. Current clinical studies showed that continuing viral suppression can recompensate partially decompensated HBV cirrhosis, and this recompensation is limited to some patients. After HBV replication is controlled, patients with HBV-related cirrhosis still have a risk of HCC. It is unclear whether patients who develop HCC after HBV replication is controlled still have cirrhosis or whether HCC occurrence is independent of cirrhosis reversal.

In addition to antiviral therapy, cell transplantation, antihepatic fibrosis therapy, and immunomodulatory therapy are hot research topics in the treatment of decompensated cirrhosis [42, 43]. Stem cells were proposed as an alternative to hepatocytes for cell transplantation. They are very attractive to the scientific community because of their high availability, good cell quality, and the possibility of using them in autologous cell transplantation [43]. Antihepatic fibrosis treatment is also a focus of cirrhosis treatment. Hepatic stellate cells are the central link of liver fibrosis. Peroxisome proliferator-activated receptor agonists and farnesate X receptor antagonists can inhibit hepatic-stellate-cell activation through related signaling pathways, thereby delaying the progression of fibrosis. There are also studies showing that statins can reduce portal hypertension in cirrhosis and even reduce the incidence and mortality of decompensation and HCC [44].

5. Summary and Outlook

There are many articles on the treatment and prognostic evaluation of decompensated hepatitis B cirrhosis; however, most articles do not provide original data, and some test indicators are different, making it difficult to compare the status of recompensation in hepatitis B cirrhosis.

Effective antiviral therapy can improve the liver biochemical indices of patients with decompensated hepatitis B cirrhosis. About 30% to 70% of patients have significantly improved CTP scores, suggesting that decompensated hepatitis B cirrhosis can be recompensated. However, the mechanism underlying liver cirrhosis and its related complications are not clear. At present, there are few studies comprehensively evaluating the long-term treatment effects of hepatitis B liver-cirrhosis-related complications, and on the recompensation of decompensated hepatitis B cirrhosis. Therefore, the evaluation time, evaluation indicators, influencing factors, and long-term prognosis of recompensated patients are still unclear. There is no parameter describing all recompensation characteristics. To explore the pathogenesis of recompensation of decompensated hepatitis B cirrhosis, further cohort studies and pathological research are needed. The identification of high-risk populations who struggle to achieve cirrhosis recompensation at an early stage, and the exploration of effective treatment strategies is hotspots in the field of liver disease at home and abroad. In short, how to clinically evaluate and achieve the recompensation of decompensated hepatitis B cirrhosis is still a contentious topic.

Data Availability

Not applicable.

Conflicts of Interest

All authors declare that they have no any conflict of interests.

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Research Article

Intestinal *Clostridioides difficile* Can Cause Liver Injury through the Occurrence of Inflammation and Damage to Hepatocytes

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This study investigated if intestinal *Clostridioides difficile* (CD) causes liver injury. Four-week-old male C3H/HeN mice were treated with phosphate-buffered solution (control), CD, diethylnitrosamine (DEN) to induce liver injury with PBS (DEN+PBS), and DEN with CD (DEN+CD) for nine weeks. After sacrifice, livers and mesenteric lymph nodes (MLNs) were removed and bacterial translocation, transcriptomes, and proteins were analysed. CD was found in 20% of MLNs from the control and DEN+PBS groups, in 30% of MLNs from the CD group, and in 75% of MLNs from the DEN+CD groups, which had injured livers. Also, CD was detected in 50% of the livers in the DEN+CD group with CD-positive MLNs. Elevated *IL-1β*, *HB-EGF*, *EGFR*, *TGF-α*, *PCNA*, *DES*, *HMGB1*, and *CRP* expressions were observed in the CD and DEN+CD groups as compared to the control and DEN+PBS groups. Protein levels of IL-6 and HMGB1 were higher in the CD and DEN+CD groups than in the control and DEN+PBS groups. These results indicate that intestinal CD can initiate and aggravate liver injury, and the mechanism of pathogenesis for liver injury should be investigated in further studies.

1. Introduction

An enormous number of microorganisms, including bacteria, viruses, and archaea, inhabit the human body. The community of microorganisms that coexists peacefully has been called the microbiota, normal flora, or microflora [1]. Microorganisms that comprise the microbiota can colonise every surface of the body. The gastrointestinal tract is the most extensively colonised organ, housing approximately 70% of all microorganisms in the human body [2].

A balanced gut microbiota is critical to host health. However, overgrowth of pathogenic bacteria results in various diseases [3]. Changes in the gut microbiota can greatly impact the liver, because gut bacteria and their byproducts can enter the liver through the portal vein [4]. There are several reports on changes in gut microbiota associated with liver diseases such as nonalcoholic fatty liver disease, cirrhosis, alcoholic liver cirrhosis, and cirrhosis with encephalopathy [5–9].

An imbalance in the gut microbiota can be induced by exposure to a broad range of antibiotics. Several studies have shown the adverse effects of various antibiotics on the host gut microbiota in human subjects [10, 11] and animal models [12, 13]. Over the past few decades, both the incidence and severity of *Clostridioides difficile* infection (CDI) have increased dramatically worldwide [14]. In addition, several studies have shown an increase in CDI in patients with liver cirrhosis and liver transplant recipients [15, 16]. Patients with CDI and liver disease risk prolonged hospitalisation,

immunosuppression, multiple comorbidities, chemotherapy, and the need for treatment with proton pump inhibitors [16]. In addition, the extent of microbiota perturbation in patients has been linked to the likelihood of developing recurrent CDI [17]. Thus, studies are needed to investigate whether *C. difficile* (CD) causes liver disease and aggravates liver disease when already present, in addition to its role in liver disease. To investigate the effect of *C. difficile* in intestine on liver injury, a mouse model is needed for preclinical studies.

Therefore, the objective of this study was to investigate the relationship of CD as an intestinal bacterium with the initiation and aggravation of liver injury using a mouse model.

2. Materials and Methods

2.1. Bacterial Inoculum Preparation. Toxigenic CD ATCC43594 was used in this study. CD was cultured in brain-heart infusion (BHI) medium with 10% fetal bovine serum (FBS), 0.2% glucose, and 1% sodium thioglycolate (ST) (BHI+FBS+G+ST) at 37°C for 48 h under anaerobic conditions established with Oxoid AnaeroGen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After incubation, the bacterial cells were harvested by centrifugation at 1,912 × g and 4°C for 15 min, washed twice, and resuspended in phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4{\cdot}7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water).

2.2. Animal Procedures and DEN-Induced Liver Injury. All animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University, and the ethical approval number was KUIACUC-2017-62. The animal facility was a biosafety level 2 laboratory with individually ventilated cages under 12 : 12 light/dark cycles, and the bedding material was beta-chips. The animals were allocated to different experimental groups randomly, and three to four animals were placed in a cage. The experimental design is shown in Figure 1. Three-week-old male C3H/HeNCrljOri mice (Orient Bio, Inc., Seongnam, Gyeonggi, Korea) were given free access to chow diet and water, with a combination of clindamycin (100 mg/L) and streptomycin (5 g/L) supplied for five days in their drinking water to decrease commensal bacteria in their intestines and to enhance CD colonisation [18, 19]. The mice were then orally gavaged with (1) 200 μL PBS (control; $n = 5$), (2) 200–300 μL CD (at 4 log CFU/mL) three times a week for nine weeks (CD; $n = 9$), (3) weekly intraperitoneal injection (i.p.) of DEN (diethylnitrosamine; 40–120 mg/kg body weight) to induce liver injury and 200 μL PBS oral gavage three times a week (DEN+PBS; $n = 5$), and (4) weekly DEN and CD treatment (DEN+CD; $n = 8$). The order in which the mice in the different experimental groups was treated was changed once a week to avoid bias effect.

During treatment, the activity and appearance of mice were monitored at 2–3-day intervals. After the mice were anesthetized by ether inhalation, they were euthanized by exsanguinating from vena cava, followed by removing livers and mesenteric lymph nodes (MLNs). To confirm the effect of DEN treatment for inducing liver injury, histological analysis was performed as follows. The mouse liver tissues were fixed in the 10% neutral buffered formalin and paraffin embedded, followed by staining with hematoxylin and eosin (H&E).

2.3. Bacterial Translocation Analysis. Detection of CD in an MLN culture was an indication of bacterial translocation from the gastrointestinal tract [20]. Hence, all MLNs were removed, and all were homogenised with 5 mm stainless beads (Qiagen) and TissueLyser LT (Qiagen) after resuspending MLNs in 1 mL 0.1% buffered peptone water (Difco, Becton, Dickinson and Company, Sparks, MD, USA). Each suspension was plated on *Clostridium difficile* moxalactam norfloxacin (CDMN) agar (Thermo Fisher Scientific, Inc.) to isolate CD, followed by anaerobic incubation at 37°C for 48 h. To detect CD in livers, a portion of each liver was homogenised using the same protocol as MLNs and plated on CDMN agar. After anaerobic incubation at 37°C for 48 h, colonies on plates were confirmed to be CD by PCR analysis. To identify CD, a primer set for *tpi* (CD species-specific gene) was used, and *tcdA* and *tcdB* (Table 1) were used for determining the toxin type of CD by multiplex PCR analysis using a Qiagen Multiplex PCR Kit (Qiagen) on a Rotor-Gene Q (Qiagen) with the following touchdown procedures: 95°C for 15 min; 94°C for 30 sec, decreased from 65°C to 55°C for an initial 11 cycles of 90 sec each; and then 40 cycles of 72°C for 30 sec, followed by a final extension at 72°C for 10 min. The PCR products were separated on 2% agarose gels, and the bands of PCR products were visualised in a LAS-3000 Imager (Fujifilm, Tokyo, Japan).

2.4. Transcriptome Analysis. Total RNA was extracted from livers and small intestines using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and the RNA was quantified with a Take3 system in an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

For the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis, complementary DNA was synthesized from extracted total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Primers used for qRT-PCR are listed in Table 1; primers from the QuantiTect Primer Assay (Qiagen) were used for tumour necrosis factor alpha (*TNF-α*) and interleukin-6 (*IL-6*). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for normalisation of expression levels. qRT-PCR was performed on a Rotor-Gene Q instrument (Qiagen) using a Rotor-Gene SYBR Green PCR Kit (Qiagen) following the manufacturer's instructions. Relative fold changes were analysed using the $-2^{\Delta\Delta C_T}$ method. Relative expression levels are expressed as follows. One sample in the control group was designated the reference, and relative gene expression levels in other samples were calculated, followed by a calculation of the mean value and standard error of the control group. In treatment groups, the gene expression levels were measured against the level of the reference sample in the control group, and fold-changes in expression levels were expressed as means for the groups. According to a study by Sambanthamoorthy et al. [21], more

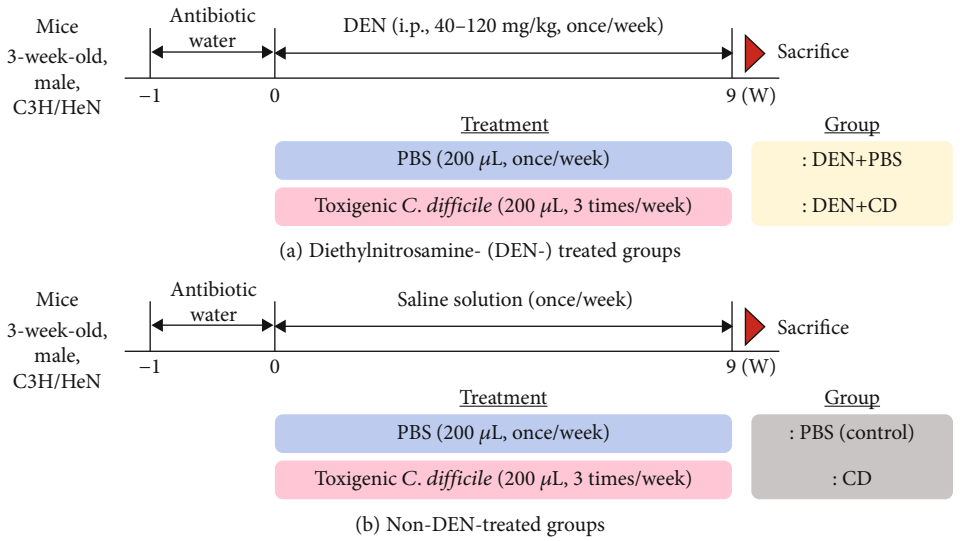

Figure 1: Experimental design in this study.

Table 1: Primers for detection of *Clostridioides difficile* and quantitative real-time reverse transcription polymerase chain reaction.

Gene	Sequence (5′ to 3′)	Tm (°C)	Reference
tpi	F: AAAGAAGCTACTAAGGGTACAAA R: CATAATATTGGGTCTATTCCTAC	55-65; touch-down	[49]
tcdA	F: AGATTCCTATATTTACATGACAATAT R: GTATCAGGCATAAAGTAATATACTTT		
tcdB	F: GGAAAAGAGAATGGTTTTATTAA R: ATCTTTAGTTATAACTTTGACATCTTT		
23S	F: GGGAGCTTCCCATACGGGTTG R: TTGACTGCCTCAATGCTTGGGC	60	[44]
GAPDH	F: TCCTGCACCACCAACTGCTTAG R: TGCTTCACCACCTTCTTGATGTC	55	[50]
IL-1β	F: CTCCATGAGCTTTGTACAAGG R: TGCTGATGTACCAGTTGGGG	55	[51]
HB-EGF	F: GAAAGCAGGATCGAGTGAGC R: CTTGCGGCTACTTGAACACA	60	[52]
EGFR	F: GGCGTTGGAGGAAAAGAAAG R: TTCCCAAGGACCACTTCACA	60	[52]
DES	F: AGCTCAAGTCATCGCCCTTC R: GCAGATCCCAACACCCTCTC	60	[52]
TGF-α	F: CAGGGAGCAACACAAATGGA R: AGCCTCCAGCAGACCAGAAA	60	[52]
PCNA	F: TTTGAGGCACGCCTGATCC R: GGAGACGTGAGACGAGTCCAT	55	[53]
ICAM-1	F: TCGGAAGGGAGCCAAGTAACT R: GATCCTCCGAGCTGGCATT	60	[54]
CRP	F: ATG GAG AAG CTA CTC TGG TGC R: ACA CAC AGT AAA GGT GTT CAG TG	60	[55]
HMGB1	F: CTTCGGCCTTCTTCTTGTTCT R: GGCAGCTTTCTTCTCATAGGG	60	[27]

than two-fold changes in gene expression were considered significant.

2.5. Immunoblotting. To investigate the expression level of protein related to cytokines in liver, at least three liver samples, randomly selected, per group were used for immunoblot analysis. To extract total proteins from livers and small intestines, frozen liver and small intestine tissues were prepared and homogenised in a PRO-PREP protein extraction solution (iNtRON Biotechnology, Inc.) for 25 min on ice, followed by centrifugation (13,000 rpm, 4°C, and 30 min). Proteins extracted from livers, and small intestines were quantified using a DC Protein Assay Kit I (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's manuals. Forty micrograms of total protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Marlborough, MA, USA). These membranes were blocked with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Immunoblots were performed with primary antibodies specific for IL-6 (sc-57315, 1 : 500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), HMGB1 (ab18256, 1 : 2500; Abcam, Cambridge, UK), PCNA (ab29, 1 : 2500; Abcam), and β-actin (sc-81178, 1 : 1000; Santa Cruz Biotechnology, Inc.). β-actin was used as a loading control. Horseradish peroxidase-conjugated anti-mouse IgG (sc-2005, 1 : 5000; Santa Cruz Biotechnology, Inc.) was used as a secondary antibody. To visualise reactive bands, membranes were developed with ECL Select Western blotting Detection Reagent (GE Healthcare Life Sciences), followed by chemiluminescence detection with an LAS-3000 Imager (Fujifilm). The intensity of immunoreactive bands was quantified using GelQuant software v. 2.7 (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

2.6. Investigation of Intestinal Inflammation Effect on CD Translocation. An additional experiment (approval number: KUIACUC-2018-0043) was approved by the Institutional Animal Care and Use Committee of Korea University to prove if CD was translocated to MLNs and livers due to intestinal inflammation, which may be caused by DEN; DEN treatment was performed to induce liver injury. To induce intestinal inflammation in mice, five-week-old male C3H/HeN mice (Orient Bio, Inc.) were treated with 2% dextran sulfate sodium salt (DSS; MP Biomedicals, CA, USA) dissolved in drinking water for 7 days, followed by drinking water without DSS for the next 7 days. The mice were then orally injected with (1) 200 μL PBS (control group; $n = 8$), (2) 200 μL PBS and water with DSS (DSS+control group; $n = 3$), (3) 200 μL *C. difficile* (a mixture of *C. difficile* strains ATCC43594 and ATCC BAA-1803) at 4 log CFU/mL (*C. difficile* group; $n = 8$), and (4) 200 μL of the *C. difficile* mixture at 4 log CFU/mL and water with DSS (DSS+*C. difficile* group; $n = 4$) every day for 2 weeks. During treatment, the activity and appearance of mice were monitored at 2–3-day intervals. After the mice were anesthetized by ether inhalation, they were euthanized by exsanguinating from vena cava, followed by removing MLNs and livers. The MLNs and livers were homogenised with 5 mm stainless beads (Qiagen) and TissueLyser LT (Qiagen).

Table 2: Positive cultures of *Clostridioides difficile* in mesenteric lymph nodes (MLNs).

Experimental group	n	CD-positive MLN culture	CD-positive percentage (%)
PBS	5	1	20
DEN+PBS	5	1	20
CD	9	3	33
DEN+CD	8	6	75

From each tissue, DNA was extracted by a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. DNAs were used to detect CD by qRT-PCR, using the primer sets for 23S rRNA gene sequence (Table 1). qRT-PCR was performed in a Rotor-Gene Q instrument (Qiagen) using a Rotor-Gene SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. To convert C_T values to log CFU/g, a standard curve was prepared. To produce the standard curve, CD culture was diluted to 1-7 log CFU/mL, the cell counts were enumerated on CDMN agar (37°C of incubation for 48 h), and qRT-PCR was also performed using the respective cultures. A linear equation was then applied to get the relationship between CD cell counts and C_T values.

2.7. Statistical Analysis. The statistical analysis was performed using SAS (version 9.2; SAS Institute, Inc., Cary, NC, USA). All data were analysed by the general linear model procedure, and the test of significance of least squares mean was performed using a pairwise t-test at α=0.05.

3. Results

3.1. Bacterial Translocation through MLN. In our experiment, CD was detected in the MLN in three (33%) of nine mice inoculated with CD, whereas CD was detected in the MLN of only one (20%) of five mice inoculated with PBS (Table 2; Suppl. Figure 1). CD was detected in the MLN of one (20%) of five mice in the control and DEN+PBS groups, whereas CD was detected in three (33%) of nine mice in the CD group and in six (75%) of eight mice in the DEN+CD group (Table 2; Suppl. Figure 1). In addition, three liver samples from the DEN+CD group were CD-positive (Figure 2). The mice with these livers were identical with those of the mice with CD-positive MLNs. In both MLNs and livers, CD-positive rates were calculated based on the presence of *tpi* (species-specific DNA). After discontinuing antibiotic administration, the inhibited CD in intestine was recovered slowly; thus, both toxigenic and nontoxigenic CDs could be found. Even nontoxigenic CDs were found in more CD-treatment groups (CD and DEN+CD) than in non-CD treatment groups (control and DEN+PBS). It can be inferred that the injected CD weakened the gut barrier, causing more transmission of intestinal CDs (whether or not toxins exist), or toxigenic CDs administered may have been difficult for toxin genes to detect due to genetic variation or problems with DNA purification through the intestinal environment. To evaluate if CD is translocated from the intestine to the liver due to the intestinal inflammation, which may be induced by DEN

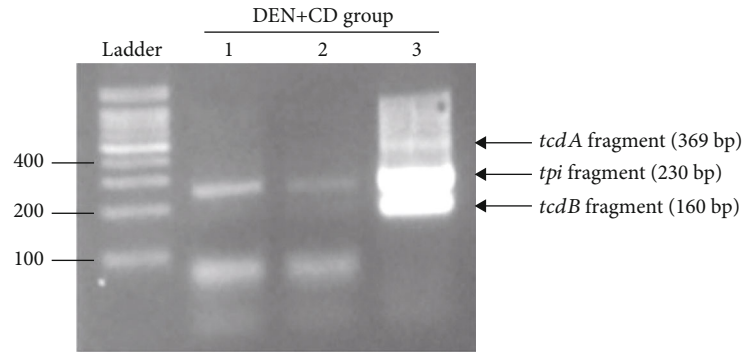

Figure 2: Multiplex PCR to detect *Clostridioides difficile* (CD) in livers of mice in the diethylnitrosamine (DEN)+CD group. CD colonies were confirmed from liver cultures in three of eight mice. Lane 1: *tpi*: +; *tcdA*: −; *tcdB*: −. Lane 2: *tpi*: +; *tcdA*: −; *tcdB*: −. Lane 3: *tpi*: +; *tcdA*: +; *tcdB*: +.

treatment, we used DSS to induce intestinal inflammation without any damage to the liver. The result showed that no CD was detected in all liver tissues (data not shown). The additional experiment result showed that CD cell counts in the pure culture obtained by plating (x axis) and the corresponding C_T values obtained by qRT-PCR (y axis) showed a good correlation ($R^2 > 0.997$) (Figure 3).

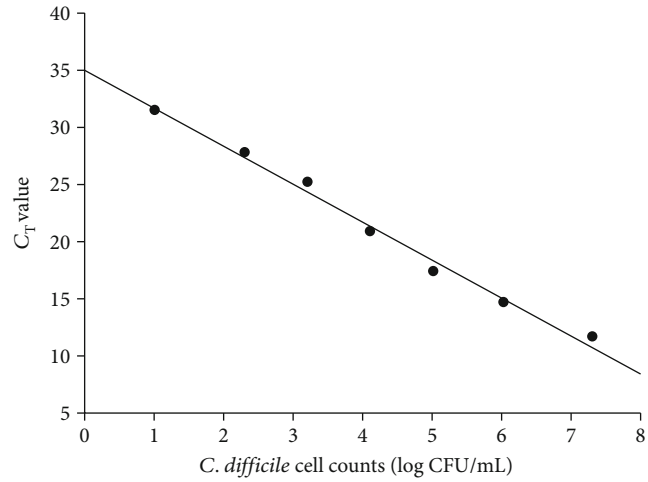

Figure 3: Standard curve between *Clostridioides difficile* cell counts (log CFU/g) and C_T values measured by qRT-PCR.

3.2. Change of Transcripts in Liver. To evaluate the relative expression levels of genes associated with liver inflammation and injury, a qRT-PCR analysis was performed. The indicators of a proinflammatory response, liver injury, and hepatocarcinoma were investigated regarding TNF-α, IL-6, IL-1β, heparin-binding epidermal growth factor (HB-EGF), epidermal growth factor receptor (EGFR), transforming growth factor alpha (TGF-α), proliferative cell nuclear antigen (PCNA), desmin, intracellular adhesion molecule (ICAM-1), high mobility group box-1 (HMGB1), and C-reactive protein (CRP). Levels of *TNF-α* and *IL-6*, proinflammatory cytokines, were under the cut-off cycle threshold for this study. In non-DEN-treated groups, the relative gene expression of IL-1β was 35.85-fold higher in the CD group than in the control groups, indicating that a proinflammatory immune response occurred in livers with CD overgrowth in the intestinal tract. The *HMGB1* expression level was 11.38-fold higher in the CD group than in the control groups. In this study, in the non-DEN-treated groups, *CRP* was 12.37-fold higher ($p < 0.05$) in the CD group than in the control group (Figure 4). Gene expression of ICAM-1 in the CD group was 0.40-fold lower than that in the control group (Figure 4), indicating that inflammation may have occurred in the liver. In our study, *PCNA* levels were elevated 24.60-fold in the CD group relative to that in the control group (Figure 4), indicating that regeneration of hepatocytes might occur in the CD group. In Figure 4, *EGFR* levels in the CD group were 29.60-fold higher than those in the control group, indicating that cell regeneration and transformation might occur in the liver. *HB-EGF* expression levels were 3.54-fold higher in the CD group than in the control group (Figure 4). *DES* expression levels in the CD group were 4.07-fold higher than those in the control group (Figure 4). *TGF-α* expression in the CD group was 14.62-fold higher than that in the control group (Figure 4). These results indicate that intestinal CD may cause inflammatory responses in the liver and contribute to a liver cancer microenvironment.

In the DEN-treated groups of mice, among genes (*IL-1β*, *HMGB1*, and *CRP*) related to inflammation, *IL-1β* (6.40-fold), *HMGB1* (7.37-fold), and *CRP* (18.31-fold) expression levels were significantly higher ($p < 0.05$) in the DEN+CD group than in the DEN+PBS group (Figure 5), but among genes related to liver damage (*ICAM-1* and *DES*), only expression levels of *ICAM-1* were 0.25-fold lower in the DEN+CD group than in the DEN+PBS group (Figure 5). *Desmin* gene expression levels were 9.76-fold higher in the DEN+CD group than in the DEN+PBS group. The expression levels of hepatocyte regeneration-related genes (*PCNA*, *EGFR*, and *HB-EGF*) were 9.42-, 1.98-, and 3.20-fold higher in the DEN+CD group than in the DEN+PBS group (Figure 5). *TGF-α* was 14.88-fold higher in the DEN+CD group than in the DEN+PBS group (Figure 5).

3.3. Protein Level Related to Inflammation and Injury in Liver. Relative protein levels were expressed as the levels of target protein to normalised protein (β-actin). In Figure 6, IL-6 protein levels, normalised to β-actin, were higher ($p = 0.07$) in the CD group (0.19 ± 0.05) than in the control group (0.04 ± 0.02) (Suppl. Figure 2A). The immunoreactivity

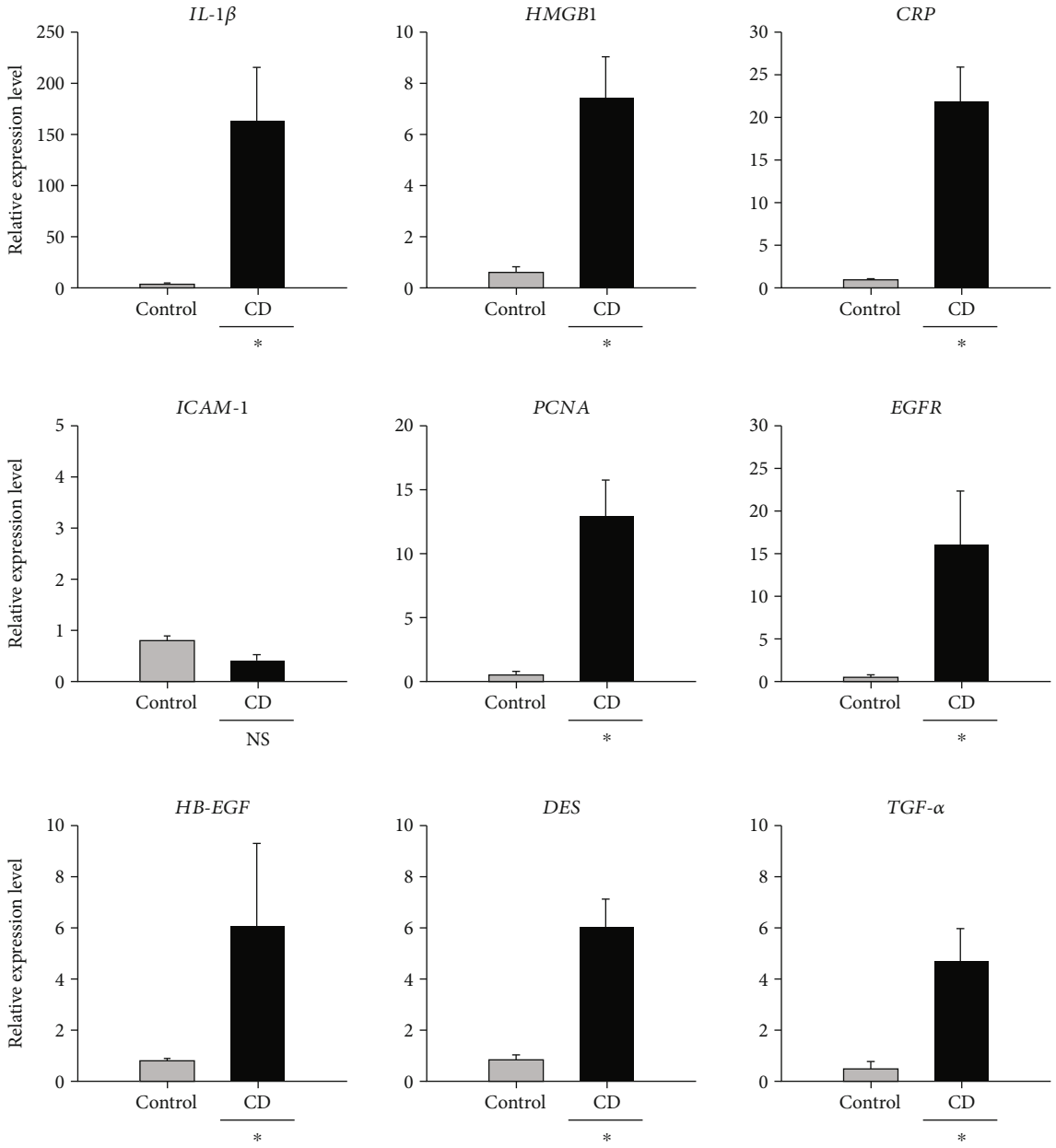

Figure 4: Relative gene expression levels in liver tissues from mice in the nondiethylnitrosamine- (DEN-) treated groups, the phosphate-buffered solution- (PBS-) treated group, and the *Clostridioides difficile*-only-treated (CD) group. All data are presented as the mean and standard error. *>2-fold changes were considered significant.

of PCNA has been used to assess proliferative activity in normal, regenerative, and tumoral livers in humans and rodents [22]. PCNA protein levels were significantly elevated in the CD group (0.26 ± 0.02) ($p < 0.05$) relative to those in the control group (0.19 ± 0.00) (Figure 6; Suppl. Figure 2A). The relative protein levels of HMGB1, which plays a pivotal role in liver injury, were assessed in the control and CD-treated groups. The levels of HMGB1 protein in the PBS and CD groups were 0.08 ± 0.00 and 0.14 ± 0.02 ($p = 0.10$), respectively (Figure 6; Suppl. Figure 2A).

In the DEN-treated groups, significantly increased IL-6 was observed only in the DEN+CD group (1.53 ± 0.08) ($p < 0.05$), and not in the DEN+PBS group (0.89 ± 0.15) (Figure 6; Suppl. Figure 2B). The levels of PCNA in the DEN+CD (0.80 ± 0.25) and DEN+PBS (0.82 ± 0.12) groups were not significantly different ($p > 0.05$), but they were higher than those in the non-DEN-treated group (Figure 6; Suppl. Figure 2B). Even though the levels of HMGB1 in the DEN+CD (0.44 ± 0.06) and DEN+PBS (0.26 ± 0.02) groups were not significantly different ($p = 0.09$) (Figure 6; Suppl. Figure 2B), these levels were reasonably different.

4. Discussion

To investigate the impact of CD on liver damage, DEN was used to induce liver injury. Histological changes in DEN-treatment groups, compared to control, were observed

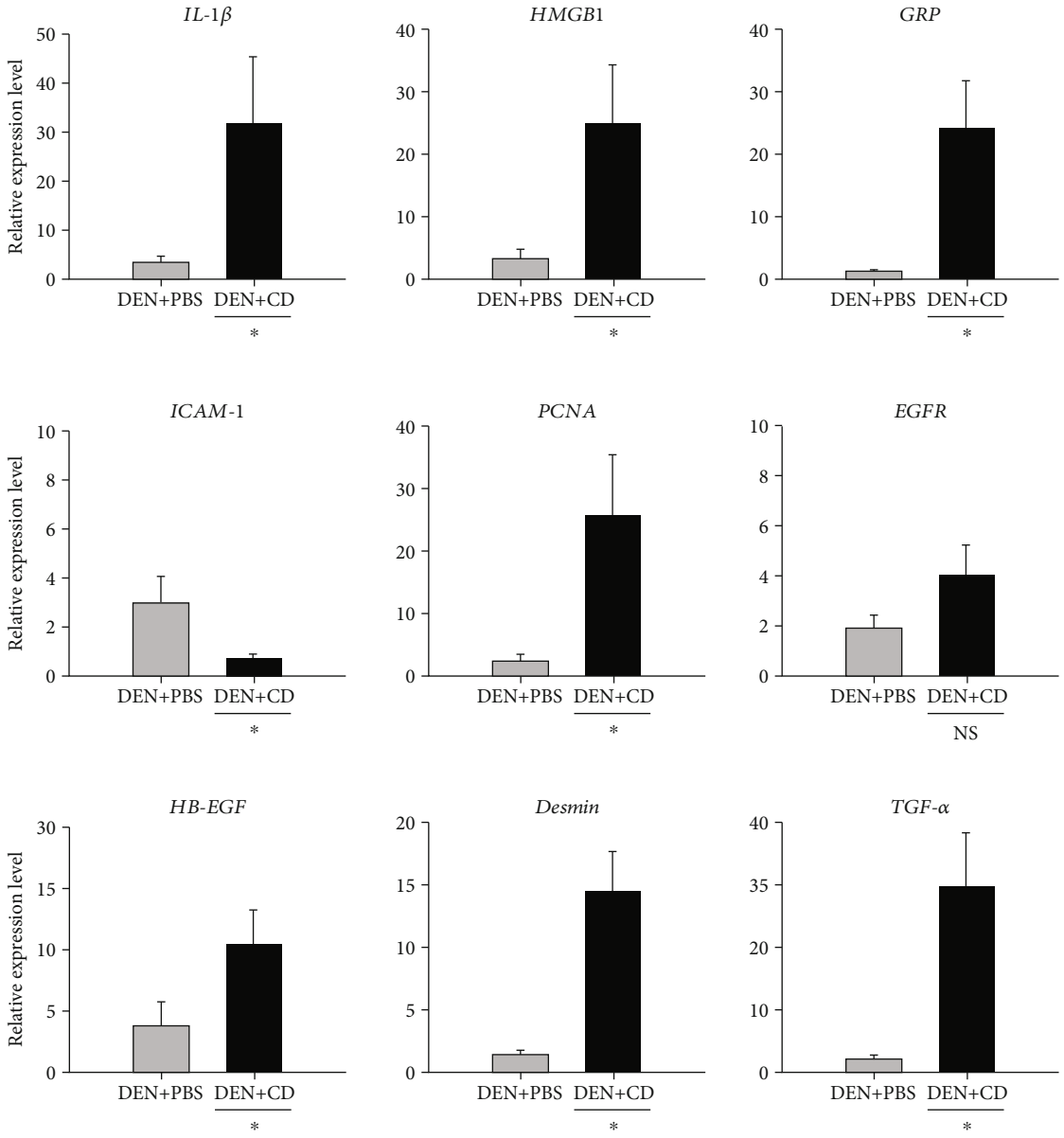

Figure 5: Relative gene expression levels in liver tissues from mice in the diethylnitrosamine- (DEN-) treated groups. DEN+phosphate-buffered solution (PBS) ($n = 3$): control group, intraperitoneally injected (i.p.) with 40–120 mg/kg DEN with PBS by oral gavage. DEN +*Clostridioides difficile* (CD) ($n = 5$): i.p., 40–120 mg/kg DEN with CD by oral gavage. All data are presented as the mean and standard error. *>2-fold changes were considered significant. NS: not significant.

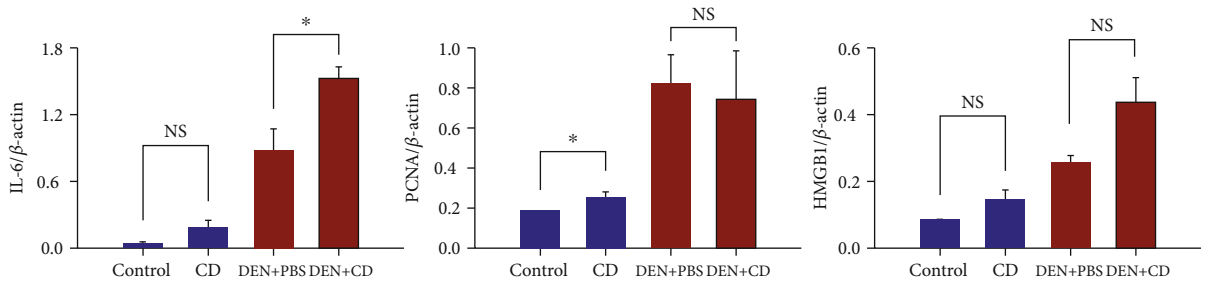

Figure 6: The immunoreactive intensity of liver proteins from mice in the phosphate-buffered solution- (PBS-) treated (control) group, the *Clostridioides difficile*-treated (CD) group, the diethylnitrosamine (DEN) and PBS-treated (DEN+PBS) group, and the DEN and CD-treated (DEN+CD) group. All data are presented as the mean and standard error. NS: not significant.

(Suppl. Figure 3). Bacterial translocation occurs when bacteria colonising the gastrointestinal tract cross the mucous membrane and migrate to the mesenteric lymph nodes (MLNs), spleen, liver, and blood [20]. According to Garcia-Tsao et al. [20], CD-positive MLNs are an indication of bacterial translocation from intestines. In our study, we treated mice with DEN to induce liver injury, and the result showed that the rates of CD-positive MLNs were higher in the CD and DEN+CD groups than in the control and DEN+PBS groups, and the rates of CD-positive liver were higher in the DEN+CD group than in the control, DEN+PBS, and CD groups. However, DEN treatment may cause intestinal inflammation as Shirakami et al. [23] suggested, and it may help in the translocation of CD from the intestine to the liver. Thus, we induced intestinal inflammation in the mice with DSS, but no CD was detected in all liver samples. These results indicate that bacterial translocation of CD can be accelerated through MLNs if CD colonises the intestinal lumen and that this translocation can be accelerated when the liver is injured.

In analysis of transcripts in livers, the gene expression levels of IL-1β, HMGB1, and CRP were analysed to investigate immune reactions. IL-1β is a proinflammatory cytokine mainly produced by macrophages [24]. It is also a potent myofibroblastic activator of hepatic stellate cells [25]. HMGB1 is a nuclear protein released from immune cells or injured nonimmune cells [26] and a critical mediator of various inflammatory responses to injury, infection, and inflammation [27]. CRP, secreted by the liver, is an acute-phase protein that can bind to a microbial capsular polysaccharide and that is involved in innate immune reactions against bacteria [28]. This protein is mainly regulated by IL-6 or IL-1β from hepatocytes [22], and in humans, CRP is the most widely studied marker of systemic inflammation. An association between CRP expression levels and liver disease, including nonalcoholic fatty liver disease (NAFLD), fibrosis, and hepatitis has been reported [29]. Indicators related to liver injury and damage including ICAM-1 and desmin were investigated. ICAM-1 is a member of the immunoglobulin superfamily, and it is expressed on various cell types, including epithelial cells, endothelial cells, and fibroblasts. ICAM-1 is overexpressed in response to proinflammatory cytokines such as TNF-α and IL-1 [30]. In addition, this molecule has been reported to be involved in leukocyte-mediated tissue injury [31, 32] and to bind to leukocytes after partial hepatectomy, inducing hepatocyte proliferation in response to the release of TNF-α and IL-6 [33]. Desmin is a smooth muscle protein composed of intermediate filaments, and it is regarded as a representative marker of hepatic stellate cells [34]. To determine the level of liver regeneration, *PCNA*, *EGFR*, and *HB-EGF* were used. PCNA has been found in the nuclei of cells of organisms from yeasts to animals. It regulates cell division, the cell cycle, and/or DNA replication [35]. Several studies [36, 37] have shown that expression of this protein is linked to proliferation or neoplastic transformation. Hepatocytes are quiescent in the normal adult liver, and the cells are renewed quite slowly [38]. However, cell regeneration can occur following injury, and cell proliferation is a critical component to a regenerative reaction. This reaction is also regarded as essential for the initiation of carcinoma [39]. Hepatocyte proliferation was found to be elevated in human cirrhotic livers, and patients with elevated cell proliferation in cirrhotic livers are at increased risk of developing hepatocellular carcinoma (HCC) [40]. EGF and its tyrosine kinase receptor, EGFR, have been suggested to play a critical role in liver regeneration and transformation [41, 42]. EGFR is highly increased in human cirrhosis cases [43]. HB-EGF is a member of the EGF family, and it is produced in various tissues, including the lung, brain, heart, and skeletal muscle [44]. It is associated with various physiological and pathological processes such as wound healing, development, atherosclerosis, and blastocyst and tumour formation [44]. TGF-α plays a vital role in hepatocarcinogenesis in humans and animals, and *TGF-α* expression is increased in hepatocellular carcinoma (HCC) tissues; furthermore, TGF-α has been reported to be linked to the differentiation of HCC cells [45]. The results of this study showed that the relative gene expressions of IL-1β, HMGB1, CRP, PCNA, EGFR, HB-EGF, desmin, and TGF-α were significantly higher in the CD-treated groups (CD and DEN+CD groups) than in the PBS groups (control and DEN+PBS groups), while the *ICAM-1* level in the CD group was not significantly different from that in control. In addition, these results became more obvious when mice were treated with DEN. Thus, CD overgrowth in intestines appears to promote inflammation in the liver, and this effect can be more deleterious to injured livers. Although histological changes were not observed within nine weeks, these transcriptional changes indicate the potential for liver damage in the long term.

In protein expression analysis using western blot assay, IL-6, a proinflammatory cytokine, and PCNA and HMGB1 related to liver injury, were examined. IL-6 and TNF-α are considered critical drivers of inflammation [46]. They are regarded as pathogenic markers, and their expression is associated with liver inflammation and fibrosis [47]. In addition, according to research by Streetz et al. [48], IL-6 induces the production of acute phase proteins in the liver and accelerates liver generation. In both the CD and DEN+CD groups, IL-6 expression levels in the liver were increased compared to the control and DEN+PBS groups, although the increase in IL-6 of the CD group was not significant (Figure 6). However, we observed the tendency of the proinflammatory cytokine level in liver when CD is overgrown in the intestines. The increase in PCNA protein expression was observed only in the CD group compared to the control. The levels between the DEN+CD and DEN+PBS groups were similar, whereas transcriptional expression of PCNA was elevated in the DEN+CD group than in the DEN+PBS group. Also, HMGB1 protein levels were not significantly different, but they were elevated in CD-treated groups. These results indicate that colonisation of CD in intestines can cause transcriptional changes, and consequently it may promote to produce proteins affecting inflammation and damage to hepatocytes.

5. Conclusions

In summary, the positive rates of CD in MLNs, an indicator of bacterial translocation, were higher for the CD-treated groups compared to the control groups. In transcriptome

analysis, the expression of genes related to proinflammatory cytokine, liver injury, and hepatocellular carcinoma was elevated in the CD and DEN+CD groups compared to the control groups. Also, the protein levels in the liver related to proinflammatory cytokine or liver injury were increased in the CD and DEN+CD groups compared to the PBS and DEN+PBS groups. Although this study has limitations as to whether the effect of CD in the intestines of mice with liver injury can be relevant to humans due to the different intestinal environment between mice and humans, these results have implicated CD in the intestines as a cause of liver disease through inflammation, and liver injury can be aggravated by CD from the intestines.

Data Availability

The data in this study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Soomin Lee participated in the design and coordination of the study, performed the experiments, analysed the data, and wrote the manuscript. Heeyoung Lee participated in the design of the study, performed the experiments, and analysed the data. Sejeong Kim, Jeeyeon Lee, Jimyeong Ha, Yukyung Choi, Hyemin Oh, Yewon Lee, and Yujin Kim performed the experiments and helped in drafting the manuscript. Kyoung-Hee Choi and Yohan Yoon participated in the design of the study, oversaw the data collection in the study, and contributed to the manuscript revision process. All authors read and approved the final manuscript.

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Supplementary Materials

Supplementary Figure 1: gel bands for *Clostridioides difficile* (CD) in mesenteric lymph nodes (MLNs) of mice in the diethylnitrosamine (DEN)+CD and CD groups. Presumed CD colonies on *Clostridium difficile* moxalactam norfloxacin agar were confirmed with tpi (species-specific) bands. White numbers on the gels indicate the MLN sample of mice. Supplementary Figure 2: protein expressions of IL-6, PCNA, and HMGB1 in liver tissues of non-DEN treatment groups (A) and DEN-treatment groups (B). DEN: diethylnitrosamine. Supplementary Figure 3: histopathological changes of mice liver in the control and diethylnitrosamine (DEN) treatment groups (200). Hematoxylin and eosin- (H&E-) stained liver from control, DEN+PBS, and DEN+CD. Liver from control presented a healthy state. In DEN+PBS, degenerated hepatocyte (black arrow), hepatocellular necrosis (black triangle), karyomegaly (star), oval cell hyperplasia (white arrow), and cholestasis (white triangle) were found. In DEN +CD, degenerated hepatocyte (black arrow), hepatocellular necrosis (black triangle), oval cell hyperplasia (white arrow), and cholestasis (white triangle) were found. Each scale bar is indicated in the lower right corner with a black line and represents 50 μm. *(Supplementary Materials)*

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Clinical Study

The Prognosis Analysis of Liver Cirrhosis with Acute Variceal Bleeding and Validation of Current Prognostic Models: A Large Scale Retrospective Cohort Study

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Background. Acute variceal bleeding is a major cause of death in liver cirrhosis. This large scale retrospective cohort study aims to analyze the prognosis of patients with cirrhosis and acute variceal bleeding and to validate the current prognostic models. *Methods*. Patients with cirrhosis and acute variceal bleeding were enrolled from Jan 2019 to March 2020. The independent prognostic factors for in-hospital death were identified by logistic regression analyses. Area under curves (AUCs) was compared among Child-Pugh, cirrhosis acute gastrointestinal bleeding (CAGIB) score, and model for end-stage liver disease (MELD) and neutrophil-lymphocyte ratio (NLR) scores. *Results*. Overall, 379 patients with liver cirrhosis and acute variceal bleeding were consecutively evaluated. The majority of the patients were males (59.1%) and the mean age of all patients were 53.7 ± 1.3 years (range 14-89). Hepatitis B virus (HBV) was the most common underlying cause of liver cirrhosis (54.1%). 72 (19%) patients had hepatocellular carcinoma. Multivariate logistic regression analyses showed that age, HCC, WBC, total serum bilirubin, serum creatinine, and ALT were independently associated with in-hospital death. And the odds ratios (ORs) for in-hospital death were 1.066 (95% CI 1.017-1.118, $P = 0.008$), 7.19 (95% CI 2.077-24.893, $P = 0.001$), 1.123 (95% CI 1.051-1.201, $P = 0.001$), 1.014 (95% CI 1.005-1.023, $P = 0.003$), 1.012 (95% CI 1.004-1.021, $P = 0.006$), and 1.005 (95% CI 1.000-1.009, $P = 0.036$), respectively. In the whole cohort with HCC patients, the AUCs of Child-Pugh, CAGIB, MELD and NLR scores were 0.842 (95% CI 0.801-0.878), 0.840 (95% CI 0.799-0.876), 0.798 (95% CI 0.754-0.838), and 0.688 (95% CI 0.639-0.735), respectively. The differences were statistically significant between Child-Pugh and NLR scores ($P = 0.0118$), and between CAGIB and NLR scores ($P = 0.0354$). *Conclusion*. Child-Pugh and CAGIB scores showed better predictive performance for prognosis of patients with cirrhosis and acute variceal bleeding than NLR scores.

1. Introduction

Acute variceal bleeding is a frequent medical emergency with the 6-week mortality of 15-20% in patients with liver cirrhosis [1, 2]. Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide with approximately 850,000 new cases each year [3]. The HCC patients with cirrhosis may suffer from both the tumor burden and variceal bleeding associated with liver cirrhosis. Combined treatment with prophylactic antibiotics, vasoactive drugs, endoscopic techniques, and interventional treatments are the recommended therapy methods for patients with acute variceal bleeding. However, treatment failure remains as high as 20% [4].

The consensus suggested the importance of early use of risk stratification scores in patients with acute upper gastrointestinal bleeding, which could help reduce the costs and

resources without influencing the outcomes of patients [5]. Conventional scoring systems with acute upper gastrointestinal bleeding included Glasgow-Blatchford score (GBS), Rockall score, and AIMS65 score [6–8]. However, these systems were not designed for patients with cirrhosis. As we know, variceal bleeding is the most frequent reasons of acute upper gastrointestinal bleeding in patients with liver cirrhosis. Recent studies have shown that these scoring systems were successful for predicting mortality risk in patients with nonvariceal upper gastrointestinal bleedings [9, 10]. Oakland et al. developed a new scoring system based on the data from Canada, the United Kingdom, and Australia (CANUKA), which was used to identify low-risk patients with 30-day rebleeding or death [11]. Tammaro et al. developed T-score to predict high-risk endoscopic stigmata and the need for early intervention [12]. Robertson et al. validated the AIMS65 score and found that AMIS65 score was equivalent to other liver disease severity risk stratification scores in predicting short term mortality [13]. However, these scoring systems were designed for acute upper gastrointestinal bleeding rather than for liver cirrhosis patients with acute variceal bleeding. Although multiple scoring systems have been proposed about liver diseases or acute upper gastrointestinal bleeding, very limited data are available for the prognostic value of current scoring systems in patients with acute variceal bleeding.

Child-Pugh, model for end-stage liver disease (MELD), and neutrophil-lymphocyte ratio (NLR) scores have been widely used in clinical practice considering they were used for prognostic assessment in patients with liver cirrhosis. Child-Pugh score was proposed to predict the risk of surgery for patients with variceal bleeding. MELD score was designed to predict the prognosis of patients who received transjugular intrahepatic portosystemic shunts (TIPS) therapy. Currently, it has been widely used to rank the priority of liver transplantation candidates. NLR is a scoring system through evaluating the degree of inflammation reaction and has been considered as a marker for the severity of liver fibrosis and cirrhosis. Lately, cirrhosis acute gastrointestinal bleeding (CAGIB) was proposed by Bai et al. They use a large scale of patients with cirrhosis and acute gastrointestinal bleeding to propose and validate the performance of CAGIB score. And their results showed that CAGIB score performed better than Child-Pugh, MELD, and NLR [14]. Although several previous studies have compared the discriminative abilities of the staging systems, it still remains controversial which could reflect the prognosis more accurately.

Therefore, we conducted this large cohort retrospective study to evaluate the prognostic factors for the liver cirrhotic patients with acute variceal bleeding and further to compare the discriminate ability of these current stage systems.

2. Methods

We screened all consecutive patients with acute gastrointestinal bleeding who were admitted to our hospital between January 2019 and March 2020. The inclusion criteria were acute variceal bleeding because of liver cirrhosis. The time frame for the acute bleeding episode should be 120 h (5 days) according to the Baveno V criteria [15]. The exclusion criteria were ulcer diseases, acute gastric mucosa hemorrhage, Mallory-Weiss syndrome, tumor diseases related bleeding, inflammatory bowel diseases, obscure gastrointestinal bleeding, and other reasons-caused bleeding. All consecutive patients who met these criteria were included. Because of the nature of this study, the informed written consent was waived. The following data were collected: age, gender, etiology, α-fetoprotein (AFP), history of GIB, hepatic encephalopathy (HE), ascites, and the laboratory tests at admission including white blood cell (WBC), platelet (PLT), hemoglobin (Hb), red blood cell (RBC), total bilirubin (TBIL), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ-GGT), prothrombin time (PT), international normalized ratio (INR), serum creatinine (Scr), and in-hospital death. Child-Pugh, CAGIB, MELD, and NLR scores were calculated for every patient, respectively [16–18].

$\text{MELD} = 0.957 \times \log_e(\text{creatinine mg/dL}) + 0.378 \times \log_e(\text{bilirubin mg/dL}) + 1.120 \times \log_e(\text{INR}) + 0.643 \times (\text{cause of cirrhosis})$. For cause of cirrhosis, use 0 for alcohol-related liver disease or for cholestatic liver disease; 1 for all other causes.

$$\begin{aligned}\text{CAGIB} = {} & \text{Diabetes (yes = 1, no = 0)} \times 1.040 \\ & + \text{HCC (yes = 1, no = 0)} \times 0.974 + \text{TBIL}(\mu\text{mol/L}) \\ & \times 0.005 - \text{ALB (g/L)} \times 0.091 + \text{ALT(U/L)} \times 0.001 \\ & + \text{Scr } (\mu\text{mol/L}) \times 0.012 - 3.964.\end{aligned} \tag{1}$$

The NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. NLR ≥5 was considered raised [19].

The Child-Pugh scores were consisted of encephalopathy, ascites, bilirubin, albumin, prothrombin time, and INR. The patients whose score 5 or 6 were good operative risks (grade A); 7, 8, or 9 moderate (grade B); and patients with 10-15 poor operative risks (grade C) [20].

2.1. Statistical Analysis. Continuous variables were summarized as the means and standard deviation. Categorical variables were expressed as frequencies and percentages. Logistic regression analyses were used to assess the prognostic values of the variables associated with in-hospital death. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Then, receiver operating characteristic curve (ROC) analysis was performed to evaluate the predictive performance of Child-Pugh score, CAGIB score, MELD score, and NLR score. The area under curve (AUC) was calculated. The predictive performance of each scoring system was compared. All statistical analyses were performed using SPSS software version 17.0 (IBM Corp, Armonk, NY, USA) and MedCalc software version 19.0.4 (MedCalc Software, Mariakerke, Belgium). $P < 0.05$ was considered statistically significant.

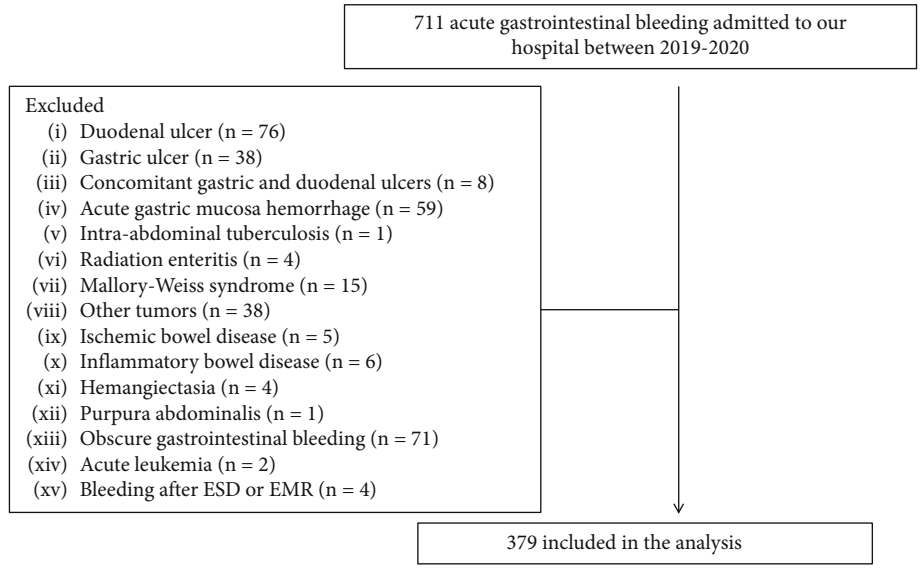

Figure 1: Patients flow chart.

3. Results

3.1. Patients. Overall, we followed 711 consecutive patients with acute upper gastrointestinal bleeding who admitted to our hospital from Jan 2019 to March 2020. Of these patients, 379 with liver cirrhosis and acute variceal bleeding were consecutively evaluated (Figure 1). Detailed baseline clinical characteristics of these enrolled patients were provided in Table 1. The majority of the patients were males (59.1%). The mean age of all patients was 53.7 ± 1.3 years (range 14-89). Hepatitis B virus (HBV) was the most common underlying cause of liver cirrhosis (54.1%). 72 (19%) patients had hepatocellular carcinoma. Nine patients had undergone liver transplantation. 96 (25.3%) underwent TIPS treatment. 157 (41.4%) patients received endoscopic variceal ligation treatment, and 144 (38%) patients received gastric variceal obturation treatment.

3.2. Univariate and Multivariate Analyses. Univariate logistic regression analyses demonstrated that age, hepatitis infection, HCC, WBC, RBC, albumin, total serum bilirubin, serum creatinine, ALT, AST, alkaline phosphatase, Child-Pugh score, MELD score, NLR score, and CAGIB score were significantly associated with in-hospital death. Multivariate logistic regression analyses showed that age, HCC, WBC, total serum bilirubin, serum creatinine, and ALT were independently associated with in-hospital death. And the odds ratios (ORs) for in-hospital death were 1.066 (95% CI 1.017-1.118, $P = 0.008$), 7.19 (95% CI 2.077-24.893, $P = 0.001$), 1.123 (95% CI 1.051-1.201, $P = 0.001$), 1.014 (95% CI 1.005-1.023, $P = 0.003$), 1.012 (95% CI 1.004-1.021, $P = 0.006$), and 1.005 (95% CI 1.000-1.009, $P = 0.036$), respectively (Table 2). Child-Pugh score, MELD score, and NLR score are complex variables composed of many clinically significant variables, and therefore, they were not included in the multivariate analysis.

In the whole cohort including HCC patients, the AUCs of Child-Pugh, CAGIB, MELD, and NLR scores were 0.842 (95% CI 0.801-0.878), 0.840 (95% CI 0.799-0.876), 0.798 (95% CI 0.754-0.838), and 0.688 (95% CI 0.639-0.735), respectively (Figure 2). The differences were statistically significant between Child-Pugh and NLR scores ($P = 0.0118$), and between CAGIB and NLR scores ($P = 0.0354$).

In the cohort without HCC patients, the AUCs of Child-Pugh, CAGIB, MELD, and NLR scores were 0.864 (95% CI 0.820-0.900), 0.780 (95% CI 0.729-0.826), 0.800 (95% CI 0.750-0.844), and 0.747 (95% CI 0.694-0.795), respectively (Figure 3). The differences between CAGIB, Child-Pugh, MELD, and NLR scores were not statistically significant.

4. Discussion

Acute variceal bleeding is a lethal complication of liver cirrhosis. Although some scoring models were used to predict the prognosis and mortality in liver cirrhosis and acute upper gastrointestinal bleeding, the prognostic scoring system for the mortality of patients with acute variceal bleeding was relatively rarely. The present work evaluated the prognosis of patients with liver cirrhosis and acute variceal bleeding and further validated the prognostic ability of current models. The strengths of this study were as follows: (1) the data was obtained from the large sample size and the patients with cirrhotic liver and acute variceal bleeding were consecutively enrolled; (2) we evaluated the prognosis of HCC patients with acute variceal bleeding; (3) most of the cases in our cohort were caused by HBV infection which differed from the patients in western countries; (4) this is the first study as an external validation of CAGIB score in patients with cirrhosis and acute variceal bleeding.

Currently, Child-Pugh, MELD, and NLR scores are the most widely known staging scores. Firstly, Child-Pugh is one of the oldest and useful tools utilized in clinical practice to estimate the prognosis of liver cirrhosis. Although Child-Pugh score has some limitations considering that it includes some subjective factors, such as ascites and hepatic encephalopathy which would be affected by therapy, it is still the most widely used prognostic scoring system for liver cirrhosis

Table 1: Demographic, clinical, and biochemical characteristics in patients with acute gastrointestinal bleeding ($n = 379$).

	N
Age (y) (mean ± SD)	53.7 ± 1.3 (range 14-89)
Sex (male)	224 (59.1%)
Cause of cirrhosis	
Hepatic B virus	205 (54.1%)
Hepatic C virus	30 (7.9%)
Both hepatic B and C virus	2 (0.5%)
Autoimmune liver disease	42 (11.1%)
Alcoholic	9 (2.4%)
Other	91 (24%)
History of GIB	86 (22.7%)
Ascites	229 (60.4%)
Hepatic encephalopathy	9 (2.4%)
Hepatocellular carcinoma	72 (19%)
Baseline laboratory values, mean ± SD (range)	
White blood cell ($\times 10^9$/L)	5.3 ± 5.3 (0.93-64.86)
Platelet ($\times 10^9$/L)	78.2 ± 5.8 (1-511)
Red blood cell ($\times 10^9$/L)	2.8 ± 0.7 (1.2-5.4)
Hemoglobin (g/L)	82 ± 2.4 (23-105)
Albumin (g/dL)	31.2 ± 5.9 (14.5-55)
Total serum bilirubin (μmol/L)	36.7 ± 5.3 (2.3-662.4)
Serum creatinine (μmol/L)	63.3 ± 4.3 (11-513)
International normalized ratio	2.2 ± 1.1 (0.97-1.74)
Alanine aminotransferase (U/L)	39.1 ± 6.5 (2.4-734)
Aspartate aminotransferase (U/L)	57.2 ± 1.2 (8-1244)
Alkaline phosphatase (U/L)	110.4 ± 1.1 (10-1425)
Gamma-glutamyl transpeptidase (U/L)	76.8 ± 2.1 (4.4-3331)
Prothrombin time (s)	17.2 ± 4.6 (1.2-80)
α-Fetoprotein (ng/mL)	1324.6 ± 1677.3 (0.19-287000)
Absolute neutrophil count ($\times 10^9$/L)	4.2 ± 5.5 (0.5-58.8)
Absolute lymphocyte count ($\times 10^9$/L)	4.2 ± 0.8 (-10.4)
Child-Pugh	
A	112 (29.6%)
B	205 (54.1%)
C	62 (16.4%)
Child-Pugh score	7.6 ± 1.7 (5-13)
MELD score	6.5 ± 0.7 (5.06-11.9)
NLR score	6.9 ± 9.3 (0.3-101.4)
CAGIB score	−5.6 ± 1.1 (-7.8-1.6)
In-hospital death	25 (6.6%)

MELD: model for end-stage liver disease; NLR: neutrophil to lymphocyte ratio; CAGIB: cirrhosis acute gastrointestinal bleeding.

patients worldwide [21]. In our study, Child-Pugh was shown to be a reliable scoring system with the highest AUCs, which was higher than MELD, CAGIB, and NLR.

Secondly, considering all the patients with score more than 10 were classified as Class C in Child-Pugh system, it was suggested that Child-Pugh classification could not discriminate the patients with serious damaged liver function [17]. Under this background, MELD was created to predict the survival of patients after TIPS treatment and now has been used to evaluate the priority of liver transplantation [17]. A previous study by Salerno et al. demonstrated that MELD score performed better than Child-Pugh model in predicting short-term (3 months) outcome [22]. Then, the study by Schepke et al. suggested that there was only a slight difference in the predictive accuracy of 1-year survival between these two models [23]. However, some studies have shown that MELD correlated well with Child-Pugh score [24]. Moreover, the study by Serste et al. demonstrated that MELD score failed to predict the mortality in patients with refractory ascites [25]. The limitation of MELD is that it originated from advanced liver disease. And the calculation of MELD score is more complex compared with others. Thus, it still remains controversial about the advantage of MELD in clinical practice.

Thirdly, NLR is a scoring system that reflects the degree of inflammatory reaction with integrating two immune pathways. On one hand, neutrophils indicate the continuous inflammation; on the other hand, lymphocytes indicate the regulatory pathway [19]. NLR has been considered as a prognostic marker for patients with various tumors including HCC, gastric cancer, and lung cancer [26, 27]. The advantage of NLR is that the value of neutrophils and lymphocytes could be easily obtained in clinical practice. And as we know, the inflammatory reaction process plays an important role in the progression of liver fibrosis and cirrhosis. Thus, it was suggested that NLR could be used as a marker for the severity of liver fibrosis and cirrhosis. The systematic review by Peng et al. pointed that NLR was particularly associated with the degree of liver fibrosis in patients with nonalcoholic fatty liver disease (NAFLD) [28]. This is associated with the fact that inflammatory reaction is evolved in the progression of NAFLD. However, NLR failed to reflect other factors that may reflect the severity of liver damages.

CAGIB was recently proposed to predict the prognosis of patients with cirrhosis and acute gastrointestinal bleeding. It includes TBIL, ALB, Scr, ALT, diabetes, and HCC as variables predicting prognosis. In our study, we identified age, HCC, WBC, TBIL, Scr, and ALT as prognostic factors, which was similar with CAGIB score. In real-world practice, the rapid increase in Scr level indicated decreased kidney function. The importance of Scr level as a critical prognostic factor for patients with liver disease has been proved in previous studies [29, 30]. It was suggested that patients with renal failure and liver cirrhosis would had worse prognosis compared to patients with similar severity of liver disease [31]. In addition, in the training cohort and internal validation cohort in CAGIB score, there were around 14%-18% HCC patients, which was similar with the percentage of HCC patients in our cohort [14]. And HCC with over a 7-fold increased risk of in-hospital death played a crucial role in the prognosis.

Table 2: Predictors for overall survival in 379 patients with liver cirrhosis and GIB.

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Age	1.043	1.010-1.078	0.011	1.066	1.017-1.118	0.008
Gender (male/female)	1.041	0.455-2.381	0.925			
Etiology (hepatitis/other)	0.378	0.165-0.865	0.021			
AFP	1	1.000-1.000	0.898			
HCC (yes/no)	5.417	2.355-12.458	<0.001	7.19	2.077-24.893	0.001
Diabetes (yes/no)	0.846	0.191-3.748	0.826			
History of GIB (yes/no)	1.354	0.546-3.357	0.513			
White blood cell	1.166	1.079-1.259	<0.001	1.123	1.051-1.201	0.001
Platelet	1.002	0.996-1.008	0.502			
Red blood cell	0.26	0.125-0.541	<0.001	0.375	1.131-1.068	0.066
Hemoglobin	0.987	0.968-1.006	0.175			
Albumin	0.902	0.837-0.972	0.007	1.164	1.031-0.913	0.623
Total serum bilirubin	1.013	1.005-1.020	0.001	1.014	1.005-1.023	0.003
Serum creatinine	1.017	1.007-1.026	<0.001	1.012	1.004-1.021	0.006
International normalized ratio	1.009	0.962-1.059	0.712			
Alanine aminotransferase	1.008	1.003-1.012	0.001	1.005	1.000-1.009	0.036
Aspartate aminotransferase	1.004	1.002-1.006	<0.001			
Alkaline phosphatase	1.002	1.000-1.004	0.036	0.794	0.995-1.006	0.794
Gamma-glutamyl transpeptidase	1	0.999-1.002	0.524			
Child-Pugh score	2.219	1.695-2.905	<0.001			
MELD score	2.789	1.743-4.462	<0.001			
NLR score	1.046	1.017-1.075	0.002			
CAGIB score	3.408	2.214-5.244	<0.001			

HCC: hepatocellular carcinoma; OR: odds ratio; CI: confidence interval; AFP: α-fetoprotein; GIB: acute gastrointestinal bleeding; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Child-Pugh score, MELD score, and NLR score are complex variables composed of many clinically significant variables, and therefore, they were not included in the multivariate analysis. ALT and AST had a potential collinearity for assessing liver function, and therefore, AST was excluded in the multivariate analysis.

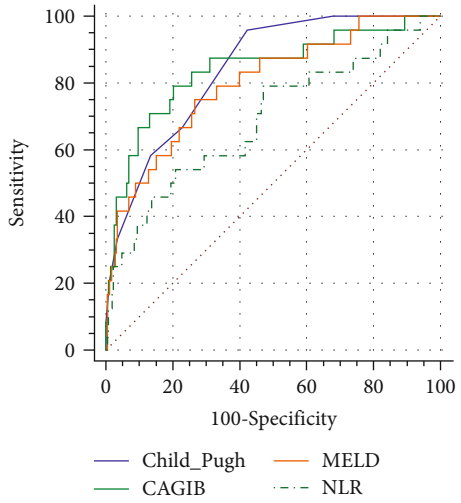

Figure 2: Comparisons of predictive performance of CAGIB score with Child–Pugh, MELD, and NLR score in liver cirrhotic patients including HCC. Blue line refers to the Child–Pugh score, green line refers to the CAGIB score, orange line refers to the MELD score, and black dotted line refers to the NLR score.

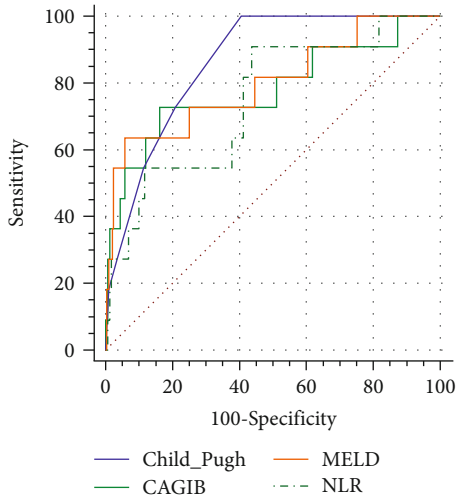

Figure 3: Comparisons of predictive performance of CAGIB score with Child–Pugh, MELD, and NLR score in liver cirrhotic patients without HCC. Green line refers to the Child–Pugh score, blue line refers to the CAGIB score, orange line refers to the MELD score, and black dotted line refers to the NLR score.

All in all, a previous systematic review by Peng et al. compared the Child-Pugh and MELD scores in the evaluation of prognosis in patients with liver cirrhosis and found that both of them had similar prognostic value. However, these two scoring systems performed differently depending on specific conditions. They pointed that studies should illustrate clearly the candidates who should use Child-Pugh or MELD [16]. Our study showed that in the whole cohort including HCC, the AUCs of Child-Pugh and CAGIB were higher than that of MELD and NLR scores. And the differences reached statistically significant between Child-Pugh and NLR scores, and between CAGIB and NLR scores. These results implied that Child-Pugh and CAGIB had better performance than NLR in the evaluation of prognosis for patients with cirrhosis and acute variceal bleeding. We considered that the possible reason was the etiology of our patient mainly consisted of hepatitis infection rather than NAFLD. And 19% patients had tumor burden which was an important prognostic factor. NLR, as an index including inflammation markers, could not reflect accurately both the degree of liver function damage and the effect of tumor burden.

There are a few limitations to our study that need to be acknowledged. Firstly, this is a single-center study; the lack of data from multicenter may cause potential bias. Secondly, this is a retrospective study and patients received different treatments to stop bleeding. 25.3% patients received TIPS treatment, 41.4% patients received endoscopic variceal ligation treatments, and 38% patients received gastric variceal obturation treatments. The various treatment methods may possibly affect the prognostic. However, there was no treatment-related death in this study.

5. Conclusions

In conclusion, our study demonstrated that in the current models, Child-Pugh, CAGIB, and MELD had good prognostic ability in predicting the prognosis of liver cirrhotic patients with acute variceal bleeding. Child-Pugh and CAGIB performed better than NLR in the cohort including HCC patients.

Data Availability

The data of this study would be available on request through the corresponding author. The corresponding author is Shuixiang He with the E-mail address hesx123@126.com. The contacted address is 277 West Yanta Road, Xi'an, Shaanxi 710061, China. Fax: +86-85323112. Tel: +86-85323112.

Conflicts of Interest

The authors have declared no conflicts of interest.

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Research Article

Value of Liver Regeneration in Predicting Short-Term Prognosis for Patients with Hepatitis B-Related Acute-on-Chronic Liver Failure

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Background and Aims. The value of hepatocyte regeneration in predicting the outcomes of hepatitis B-related acute-on-chronic liver failure (HBV-ACLF) is not fully assessed. The present study was aimed at establishing a novel scoring system to predict patients' outcomes within 3 months by applying serological indicators of hepatic regeneration and liver injury. *Methods*. Patients with chronic hepatitis B who had a rapid deterioration were investigated. Patients were observed for 90 days, and the endpoint of follow-up was death or liver transplantation. Serum parameters were estimated on the diagnosis of acute-on-chronic liver failure (ACLF). Cox proportional hazard regression was used to identify independent prognostic factors and create a novel prognostic scoring system, and a receiver operating characteristic (ROC) curve was used to analyze the performance of the model. *Results*. A total of 308 patients with HBV-ACLF were incorporated and divided into the training cohort ($n = 206$) and testing cohort ($n = 102$) randomly. Creatine (Cre), age, total bilirubin (TBil), alpha-fetoprotein (AFP), and international normalized ratio (INR) were found to be independent prognostic factors. According to the results of Cox regression analysis, a new prognostic model (we named it the TACIA score) was calculated. The areas under ROC (AUROC) for the new model were 0.861 and 0.763 in the training and testing cohorts, respectively, and patients with lower TACIA scores (<4.34) would survive longer ($P < 0.001$). *Conclusions*. A pertinent prognostic scoring system for patients with HBV-ACLF was established in our study, and the novel model could predict patients' short-term survival effectively.

1. Introduction

Acute-on-chronic liver failure is a life-threatening clinical syndrome with a rapid progress of hepatic injury on the basis of chronic liver diseases. The likely causes of acute decompensation could be either hepatic or nonhepatic. In China, hepatitis B virus (HBV) infection is a significant health concern, and the reaction of HBV becomes the most common pathogeny of ACLF. Thus, hepatitis B-related acute-on-chronic liver failure becomes a weighty problem [1]. In addition, the rapidly worsening liver dysfunction may finally result in multiple organ failure and a high short-term mortality.

Recently, liver transplantation is the most efficient method for ACLF treatment. Unfortunately, patients seldom have chance to get liver transplantation because of the severe donor liver shortage. As a result, intensive care and supportive therapy have become alternatives to manage ACLF. Except liver transplantation, currently applied therapeutic

methods are aimed at helping to clear cytotoxic items and create a proper circulation for liver regeneration; for that, liver regeneration is a vital procedure to the recovery of severe hepatic injury [2]. In the past few years, the mortality of ACLF has shown a decreasing trend due to the early diagnosis and management of organ failure, but the survival rate is still not as expected [3]. Thus, prognostic models could play an essential role in ACLF management, including the Child-Pugh score (CTP) [4], MELD score [5], AARC score [6], CLIF-SOFA score [7], CLIF-C OF score, and CLIF-C ACLF score [8]. Those models mainly evaluate the severity of liver injury and the occurrence of multiple organ failure. Rarely did they focus on the capability of liver tissue repairing and liver regeneration. Functional liver tissue repairing is the key to the improvement of injured hepatic function. As a maker of liver regeneration, alpha-fetoprotein (AFP) was found to be a parameter correlated with the outcome of acute liver failure [9]. But seldom has its predictive value been assessed in ACLF. In our previous study, we found that an elevated AFP level could predict a better outcome for HBV-ACLF patients [10].

The outcome of liver failure should be assessed from the perspective of both damaged liver function and the ability of liver regeneration. But researchers mostly concentrate on the former one. Thus, we aimed to perform a timely assessment of patients' outcomes upon the diagnosis of ACLF by integrating clinical parameters of both organ damage and liver regeneration and to create and validate a new prognostic model for HBV-ACLF centering on the value of hepatic regeneration.

2. Patients and Methods

2.1. Study Cohort and Data Collection. We retrospectively studied patients with chronic hepatitis B who have an acute progression of liver dysfunction from 2012-2-27 to 2017-9-27 in our hospital. The diagnosis of chronic hepatitis B was based on the existing guidelines. Liver cirrhosis was diagnosed by referring to liver biopsy, ultrasound, fibroscan, laboratory examination, and clinical manifestations. ACLF was diagnosed according to Asian Pacific Association for the Study of the Liver (APASL) [11]: a presentation of jaundice (serum total bilirubin $\geq 85\,\mu$mol/L), coagulopathy (INR ≥ 1.5 or prothrombin activity $\leq 40\%$), and any degree of encephalopathy and/or clinical ascites within 4 weeks on the basis of ongoing chronic liver diseases. Patients with HBsAg positive who were aged between 18 and 80 and had a manifestation of liver dysfunction within 4 weeks were included. After preliminary screening, 903 patients with chronic hepatitis B who had an acute progress of liver dysfunction were studied. Five hundred and ninety-five patients were excluded for the following: (1) coinfection with HAV, HCV, HEV, and HIV; (2) those who do not meet the APASL criteria; (3) any evidence to hepatocellular carcinoma (HCC); (4) combination with reproductive system tumors and other malignancies; (5) pregnancy; (6) a lack of biochemical or imageology examination; and (7) those treated with liver transplantation (LT) or artificial liver support (ALS) previously. Finally, there were 308 patients incorporated into this study. The population was randomly separated into two subgroups at a proportion of 2 : 1 to establish and validate a new prognostic model (Figure 1). Clinical data was collected upon the diagnosis of ACLF. Prognostic models including CTP, MELD, AARC, CLIF-SOFA, CLIF-C OF, and CLIF-C ACLF were recorded as tools of condition assessment. Patients were followed up for 90 days since the date of ACLF diagnosis. The endpoint of follow-up is death or liver transplantation.

2.2. Patient Management. Standard medical treatment was obtained including bed rest, intravenous antibiotics, liver-protective treatment, and energy supplements. Patients also received plasma and albumin infusion, water-electrolyte maintenance, and complication-preventing treatment. Antiviral therapies were administered individually according to the virus replication levels and patients' conditions by using lamivudine, telbivudine, adefovir dipivoxil, or entecavir.

2.3. Statistical Analyses. Statistical analyses were performed by referring to SPSS software (version 16.0; IBM Corporation, Somers, NY, USA). Continuous data were expressed as means ± SD or medians with interquartile range appropriately. Those variables were compared by using Student's t -test or the nonparametric Mann-Whitney U test. Percentages were used to present categorical data, which were compared by the chi-squared test or Fisher's exact test. The independent prognostic factors were identified by multivariate Cox regression analysis, and a new prognosis scoring system was established on the basis of Cox proportional hazard regression. The area under the receiver operating characteristic curve was used for model discrimination and calibration. The comparison of cumulative survival rates was conducted with the Kaplan-Meier method. It was considered of statistical significance when $P \leq 0.05$.

3. Results

3.1. Characteristics and Outcomes of HBV-ACLF Patients. There are 308 patients incorporated in our study. Table 1 reveals the baseline characteristics of HBV-ACLF patients. During a 90-day follow-up, eighty-eight cases (42.72%) were deceased or got liver transplant in the training cohort, and the liver transplant-free survival rate was 53.92% (55/102) in the testing cohort. The rates of liver transplantation were 0.97% (2/206) and 4.90% (5/102) within 90 days in the training and testing cohorts, respectively.

3.2. Independent Prognostic Factors and Development of a New Predictive Model. In the training cohort, age (43.92 ± 11.69 years versus 52.80 ± 12.04 years, $P < 0.001$), total bilirubin (233.91 (88.50, 634.60) μmol/L versus 310.60 (86.50, 795.70) μmol/L, $P = 0.003$), AFP (82.19 (1.80, 3858.00) ng/mL versus 17.50 (1.04, 1155.65) ng/mL, $P < 0.001$), INR (1.81 (1.50, 4.44) versus 2.24 (1.52, 7.26), $P < 0.001$), Cre (69.20 (31.00, 207.70) μmol/L versus 84.55 (39.00, 505.00) μmol/L, $P < 0.001$), leukocyte count ($5.82\,(2.01, 25.51) \times 10^9$/L versus $7.37\ (1.80, 37.50) \times 10^9$/L, $P < 0.001$), and albumin (31.71 (18.60, 43.90) g/L versus 30.48 (13.60, 40.60) g/L, $P = 0.003$) are of statistical significance in survivors and patients with poor outcomes.

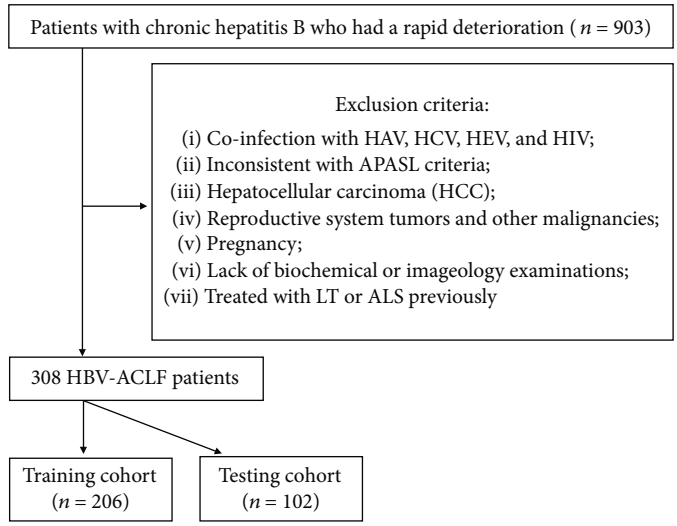

Figure 1: Inclusion and exclusion criteria of this research.

Table 1: Clinical characteristics and outcomes of HBV-ACLF patients.

	Training cohort ($n = 206$)	Testing cohort ($n = 102$)	P
Age (years)	47.71 ± 12.60	47.38 ± 11.77	0.825
Gender (male, %)	174 (84.47%)	82 (80.39%)	0.369
TBil (μmol/L)	252.55 (86.50, 795.70)	281.86 (86.61, 1004.50)	0.528
Cre (μmol/L)	73.70 (31.00, 505.00)	74.30 (43.00, 371.00)	0.793
Alb (g/L)	30.90 ± 4.97	31.39 ± 5.33	0.428
Leukocyte count ($\times 10^9$/L)	6.60 (1.80, 37.50)	6.42 (2.16, 21.60)	0.997
Neutrophil count ($\times 10^9$/L)	4.31 (0.64, 33.38)	4.54 (0.86, 16.83)	0.778
ALT (IU/L)	365.85 (21.80, 5124.20)	302.00 (16.20, 6189.40)	0.988
AST (IU/L)	302.25 (40.50, 7025.20)	318.35 (49.90, 3562.70)	0.883
INR	1.95 (1.50, 7.26)	1.91 (1.50, 6.96)	0.440
AFP (ng/mL)	52.75 (1.04, 3858.00)	35.87 (0.83, 1495.82)	0.740
HBeAg positive (%)	47 (22.82%)	29 (28.43%)	0.282
HBV DNA ($\log_{10}$ IU/mL)	5.07 (2.01, 9.76)	5.49 (2.30, 9.81)	0.298
HE (%)	109 (52.91%)	55 (53.92%)	0.867
Ascites (%)	142 (68.93%)	79 (77.45%)	0.118
28-day mortality (%)	67 (32.52%)	32 (31.37%)	0.839
90-day mortality (%)	86 (41.75%)	42 (41.18%)	0.924

AFP: alpha-fetoprotein; Alb: albumin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Cre: creatine; HE: hepatic encephalopathy; INR: international normalized ratio; TBil: total bilirubin.

After univariate Cox regression, clinically significant parameters were verified by multivariate analysis. Finally, total bilirubin, creatine, age, INR, and AFP were found to be independent factors of patients' outcomes (Table 2). Then, a new prognostic model (we named it the TACIA score) for HBV-ACLF patients was established as the following mathematical formula: TACIA score = $0.003 \times$ TBil (μmol/L) + $0.036 \times$ age + $0.009 \times$ Cre (μmol/L) + $0.525 \times$ INR − $0.003 \times$ AFP (ng/mL).

3.3. Performance of the New Model. Firstly, the performance of the TACIA score was estimated internally in the training cohort (Figure 2(a)), and its area under the ROC curve was 0.861. In addition, we compared the efficiency of the TACIA score and other formulas (including CTP, MELD, CLIF-SOFA, CLIF-C OF, and CLIF-C ACLF scores) in predicting short-term prognosis. The results illustrated that the TACIA score was superior to those models mentioned above. Furthermore, we externally examined the performance of the novel predictive model in the testing cohort (Figure 2(b)), and it showed its validity as well (AUROC = 0.763). The areas under the ROC curve of each model were compared with TACIA by the z test in both the training and testing cohorts. Table 3 demonstrates the differences between TACIA and other models.

Table 2: Univariate and multivariate Cox regression analyses of 90-day mortality in the training cohort.

	Univariate analyses			Multivariate analyses		
	β	HR (95% CI)	P	β	HR (95% CI)	P
Age (years)	0.042	1.043 (1.026, 1.061)	<0.001	0.036	1.037 (1.017, 1.056)	<0.001
Gender (male)	-0.045	0.956 (0.540, 1.693)	0.878			
TBil (μmol/L)	0.003	1.003 (1.001, 1.004)	<0.001	0.003	1.003 (1.001, 1.005)	<0.001
Cre (μmol/L)	0.010	1.010 (1.008, 1.013)	<0.001	0.008	1.008 (1.004, 1.011)	<0.001
Alb (g/L)	-0.074	0.929 (0.891, 0.968)	0.001	-0.010	0.990 (0.939, 1.045)	0.728
Leukocyte count ($\times 10^9$/L)	0.110	1.116 (1.076, 1.157)	<0.001	-0.114	0.892 (0.742, 1.074)	0.227
Neutrophil count ($\times 10^9$/L)	0.127	1.136 (1.095, 1.178)	<0.001	0.169	1.184 (0.965, 1.454)	0.105
ALT (IU/L)	0.000	1.000 (0.999, 1.000)	0.053			
AST (IU/L)	0.000	1.000 (1.000, 1.000)	0.672			
HBeAg positive (%)	0.115	1.122 (0.675, 1.864)	0.657			
HBV DNA ($\log_{10}$ IU/mL)	-0.045	0.956 (0.860, 1.064)	0.956			
INR	0.775	2.170 (1.802, 2.612)	<0.001	0.525	1.691 (1.333, 2.146)	<0.001
AFP (ng/mL)	-0.004	0.996 (0.994, 0.998)	0.001	-0.003	0.997 (0.995, 0.999)	0.021
HE	-0.163	0.849 (0.559, 1.291)	0.445			
Ascites	0.786	2.194 (1.291, 3.727)	0.004	0.307	1.360 (0.739, 2.502)	0.323

AFP: alpha-fetoprotein; Alb: albumin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Cre: creatine; HE: hepatic encephalopathy; INR: international normalized ratio; TBil: total bilirubin; HR: hazard ratio; CI: confidence interval.

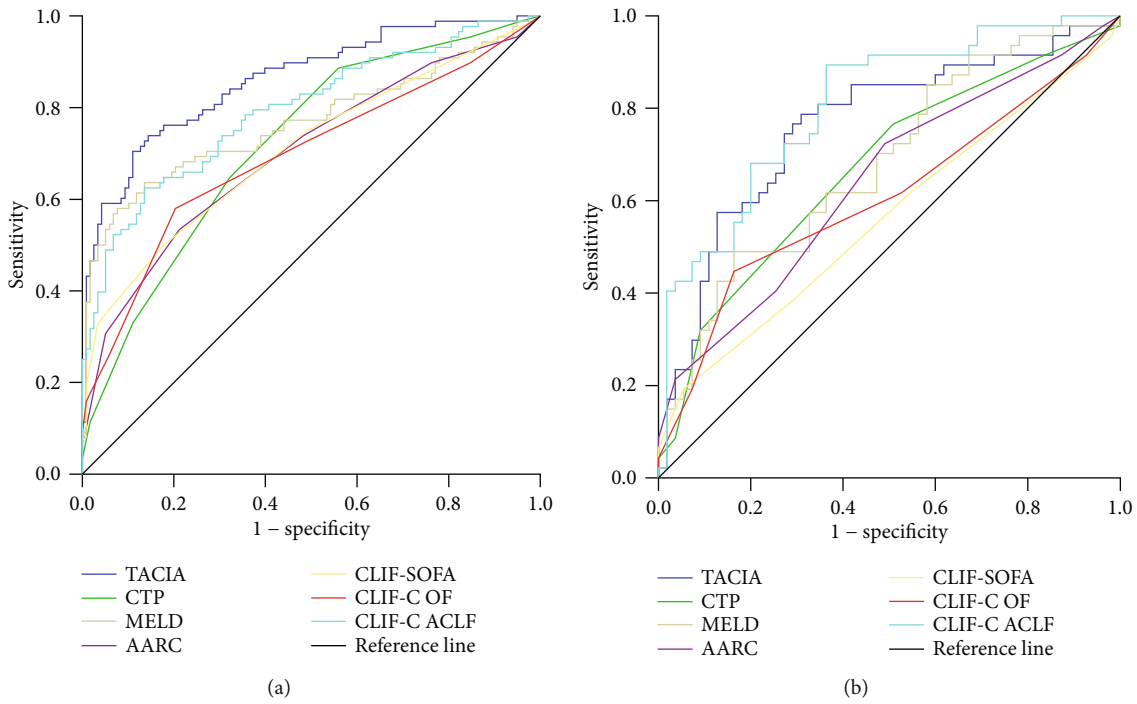

Figure 2: The performance of the novel scoring system compared with that of other models: (a) training cohort; (b) testing cohort.

The newly founded TACIA score showed its applicability in predicting a poor prognosis within 90 days in the training cohort. A cut-off point of the TACIA score ≥ 4.34 was suggested to indicate a poor outcome with 72.73% sensitivity and 86.44% specificity. The results demonstrated that patients with a higher TACIA score (≥4.34) would have increased risk for poor outcomes. Thus, we further analyzed patients' survival according to their TACIA scores (Figure 3). In the training cohort, the transplant-free survival rate at 28 and 90 days were 32.10% (26/81) versus 88.80% (111/125) ($P < 0.001$) and 20.99% (17/81) versus 80.80% (101/125) ($P < 0.001$) in groups of patients with TACIA score ≥ 4.34 and <4.34. In the testing cohort, the transplant-free survival rates at 28 and 90 days were 44.24% (16/37) versus 80.00% (52/65) ($P < 0.001$) and 27.03% (10/37) versus 69.23% (45/65) ($P < 0.001$), respectively.

Table 3: Performance of those prognostic models in the training and testing cohorts.

	Training cohort			Testing cohort		
	AUROC	95% CI	*P*	AUROC	95% CI	*P*
TACIA	0.861	(0.806, 0.905)		0.763	(0.669, 0.842)	
CTP	0.722	(0.655, 0.782)	<0.001	0.670	(0.570, 0.760)	0.176
MELD	0.768	(0.704, 0.824)	0.001	0.680	(0.580, 0.769)	0.076
AARC	0.701	(0.633, 0.762)	<0.001	0.641	(0.540, 0.733)	0.020
CLIF SOFA	0.707	(0.640, 0.768)	<0.001	0.562	(0.460, 0.660)	0.001
CLIF-C OF	0.695	(0.627, 0.759)	<0.001	0.607	(0.505, 0.702)	0.012
CLIF-C ACLF	0.793	(0.731, 0.846)	0.002	0.808	(0.718, 0.879)	0.308

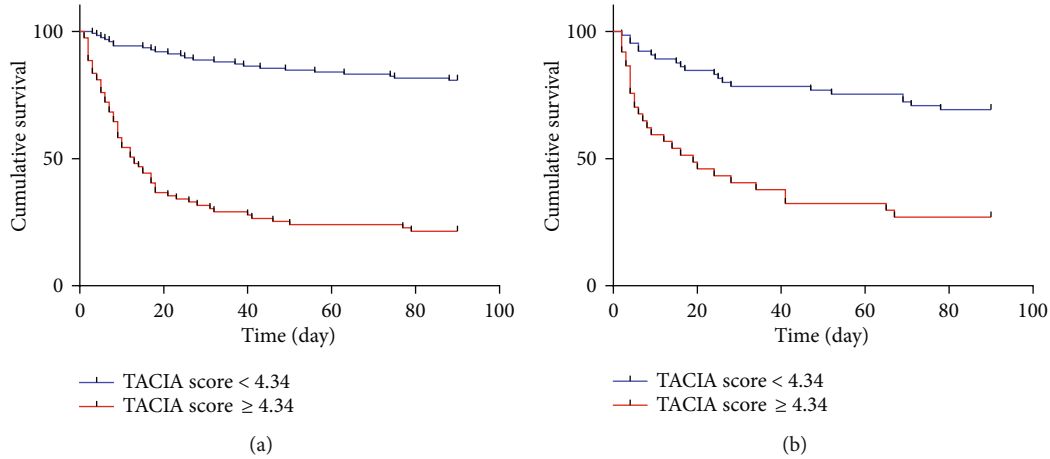

Figure 3: Survival curve of HBV-ACLF patients: (a) training cohort; (b) testing cohort.

4. Discussion

Acute-on-chronic liver failure is a serious clinical syndrome that exhibits a high short-term mortality. Effective prognostic models could be of great value in the management of ACLF and predicting patients' outcomes, including CTP, MELD, and other prognostic formulas. Previous studies have illustrated that these formulas could be efficient tools to estimate the prognosis of cirrhosis and end-stage liver diseases, but their efficiencies might vary from territories and etiologies. In addition, these models mainly assess the condition of organ failure, so that they may not be inadequate enough to evaluate the prognosis of HBV-ACLF. Except for the severity of organ failure, the capability of hepatic regeneration could also be an essential item to the prognosis of ACLF. It is acknowledged that the liver shows its tissue repairing potential after hepatic resection or obvious hepatocyte necrosis. So, liver regeneration could be a significant procedure to the reversal of impaired hepatic function.

Liver transplantation is the most effective therapy for ACLF patients at present, while the lack of a donor liver has made it difficult and even impossible for clinical dissemination. Alternative methods including intensive care and antiviral therapy could help control the progression of liver dysfunction and promote hepatic repairing. The secretion of AFP is minimal in an adult liver, and it could usually be detected just in pathophysiological situations including hepatocyte proliferation and canceration [12]. As a marker of liver regeneration, AFP could be a prognostic item for patients with liver damage [13]. Previous researches have expounded that elevated AFP levels could predict a better prognosis for acute liver failure [14, 15]. Yet its prognostic value in ACLF has not been fully clarified. Considering the predictive value of liver regeneration, a formula combining AFP with other indices of liver function to estimate the prognosis of HBV-ACLF should be proposed.

The present study illustrated that total bilirubin, age, creatine, INR, and AFP were independent factors of patients' outcomes. The level of bilirubin would be elevated when massive necrosis of hepatocytes occurred or under the conditions of biliary obstruction and hemolysis. Patients with severe liver damage exhibit diminished liver function which may lead to multiple organ dysfunction and high short-term mortality. High serum bilirubin concentrations in ACLF patients could indicate an apparent injury of hepatocytes, which was always associated with poor outcomes [16]. Besides, there is a growing risk of poor outcomes for HBV-ACLF patients along with the increase in serum creatine. Patients with a higher creatine level could carry a kidney dysfunction and even to the extent hepatorenal syndrome, which may finally result in unexpected outcomes [17, 18]. Consistent with Cordoba et al.'s research, we found that INR was an independent risk factor of short-term mortality for ACLF patients [19]. The liver would have a weakened

synthesis of coagulation factors when got severely injured, which may lead to coagulopathy and even multiorgan failure. Except for the severity of liver damage, the ability of hepatic regeneration is also a key to the prognosis of liver failure. AFP is considered a marker of hepatocyte regeneration in liver injury and could predict patients' outcomes [20]. Besides, elderly patients with chronic hepatitis B are at risk of developing HBV-ACLF, and age is of prognostic significance. Studies illustrated that elderly HBV-ACLF patients tend to have a higher 3-month mortality [21] [22]. Those patients would exhibit a declined systemic health condition, and the capability of liver regeneration may be diminished, so that the prognosis sometimes trends to be unexpected [23, 24].

The novel scoring system showed its prognostic value for HBV-ACLF by calculating age, creatine, INR, and AFP. The new model could predict the 90-day survival effectively and has an advantage over CTP, MELD, CLIF-SOFA, CLIF-C OF, and CLIF-C ACLF in the training cohort. The novel scoring system also showed its applicability in the testing cohort though no significantly statistical difference was found between TACIA and CTP, MELD, and CLIF-C ACLF, for which the limited sample size might be a potential reason. Patients who have lower TACIA scores (<4.34) might survive longer than those who have a higher TACIA score (≥4.34). The results indicate that patients with high TACIA scores might have a serious liver dysfunction and even incorporated with multiorgan failure, and the capability of hepatic regeneration would be diminished.

Acute-on-chronic liver failure is a syndrome featured as having poor short-term prognosis. This study highlighted a timely assessment of organ dysfunction and liver regeneration at the development of ACLF, which could help in patients' management. However, there exist some limitations in this study. Firstly, the model was constructed by the baseline clinical characteristics; for that, a dynamic observation of serological indicators was lacking. Besides, there is a shortage of multicentre comparative analysis in this research. Hence, further large-scale multicentre prospective studies assessing the availability of this novel prognostic model should be recommended.

5. Conclusion

In summary, the novel model could predict the prognosis of HBV-ACLF effectively. Lower levels of this new model could indicate a better outcome. The results of our research might be helpful in the management of HBV-ACLF for clinicians.

Data Availability

All data arising from this study are contained within the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Shanhong Tang designed the study and carried it out. Mengying Sun, Hongyong Li, Jiani Li, Sen Qin, Dehui Yang, Dejiang Zhou, Min Weng, and Xiaolei Zhou helped to collect data. Xianjun Yang conducted the statistical analysis. Xiaoping Wang drafted the manuscript. Liucun Gao, Xiaoling Wu, and Weizheng Zeng helped to finalize the manuscript. All the authors read and approved the manuscript.

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Clinical Study

Validation of the Combined Model Based on Platelet Count and Albumin to Rule out High-Risk Varices in Liver Cirrhosis

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Background. The Baveno VI criteria based on platelet count and liver stiffness, measured by transient elastography (TE), have been proposed to rule out high-risk varices (HRV) defined as medium or large-sized varices or the presence of high-risk stigmata (cherry red spots and red wale marks). However, TE is not available in all hospitals. Recently, the Rete Sicilia Selezione Terapia hepatitis C virus (RESIST-HCV) criteria recommended that cirrhotic patients with a platelet count > 120000/μL and serum albumin > 36 g/L could avoid esophagogastroduodenoscopy (EGD) screening for HRV. *Aim*. We aimed to validate the performance of the RESIST-HCV criteria in two cohorts predominantly characterized with hepatitis B infection. *Methods*. Patients with compensated cirrhosis who had blood tests within three months of performing EGD and TE were enrolled retrospectively from two centers. RESIST-HCV criteria were applied to identify patients who did not require EGD screening. *Results*. This study included 188 patients from the Xingtai cohort (28 (14.9%) with HRV) and 104 patients from the Beijing cohort (19 (18.3%) with HRV). Of the patients who met the RESIST-HCV criteria (83 in the Xingtai cohort and 26 in the Beijing cohort), 0 and 1 had HRV, respectively, accounting for 44.1% (Xingtai cohort) and 25% (Beijing cohort) of endoscopies that were unnecessary. In the combined cohort, 109 (37.3%) patients met the RESIST-HCV criteria, only 1 (0.9%) HRV was missed, and the negative predictive value was 99.1%. Baveno VI and Expanded Baveno VI criteria spared 15.6% and 23.3% of EGDs, respectively, while missing 0% and 4.8% of HRV, respectively. *Conclusions*. In our population, the combined criteria based on platelet count and serum albumin performed well, saving 30-40% of EGDs and correctly identifying 99.1% of patients who could safely avoid screening endoscopies for high-risk varices in compensated cirrhotic patients.

1. Introduction

Portal hypertension (PH) is a common complication of liver cirrhosis, and it promotes the transition from the preclinical to the clinical phase of liver cirrhosis. Gastroesophageal varices (GEV) are a major and feared complication of PH, occurring in up to 60% of patients with cirrhosis [1]. Bleeding from GEV occurs as a severe and life-threatening complication of PH [2], with an extremely high risk of death. In particular, bleeding from GEV still has a mortality rate of 10%-15%, despite the clinical progress [3]. Prevention and treatment of variceal bleeding largely depends on the timely diagnosis and risk stratification of GEV [4, 5]. Esophagogastroduodenoscopy (EGD) remains the gold standard diagnostic method for GEV and should be performed to screen for the presence of GEV in all patients who are first diagnosed with liver cirrhosis, in accordance with the recent Baveno VI consensus [5]. However, a variable proportion of cirrhotic patients will not have GEV, as 30%-40% of all varices and 6%-20% of HRV are seen in compensated cirrhosis [3, 6]. Thus, screening all cirrhotic patients with EGD leads to a large number of unnecessary endoscopies, which increases the healthcare costs and the financial burden to the families and societies [7], and has a severe influence on the quality of life of patients. In addition, EGD is invasive, expensive, poorly accepted by patients, and unavailable in developing countries and rural areas [8]. Consequently, there have recently been significant updates in the noninvasive prediction of GEV, especially the use of

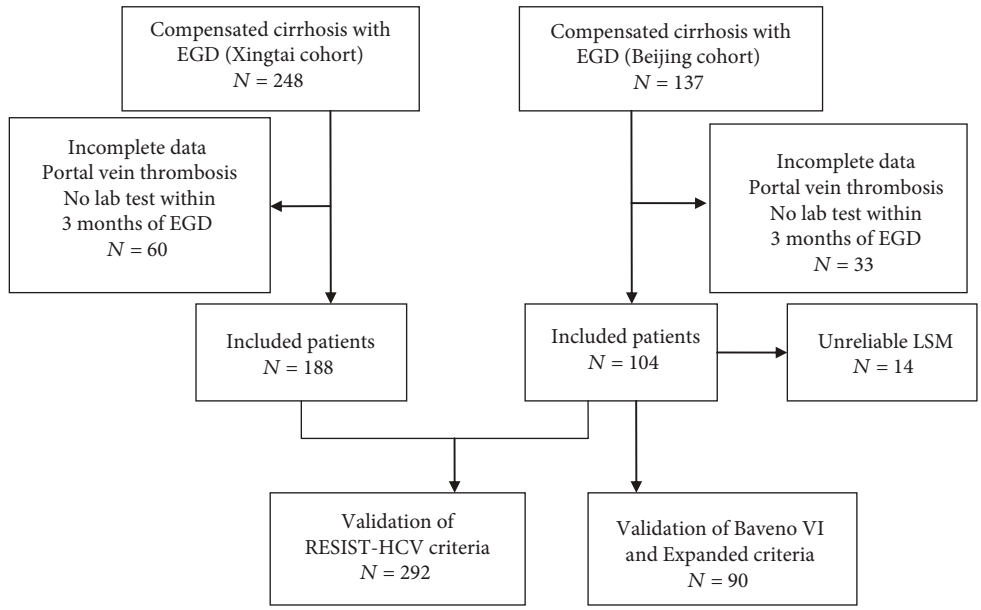

Figure 1: Flow chart of patients included in this study. Abbreviations: EGD: esophagogastroduodenoscopy; LSM: liver stiffness measurement.

noninvasive tests (NITs) to assess the likelihood of GEV and HRV [9]. NITs such as assessment of platelet count, spleen diameter, and liver stiffness can help identify patients at very low risk of having HRV or GEV [9–12]. Among them, the Baveno VI criteria (liver stiffness measurement (LSM) < 20 kPa and platelet count > 150000/μL) are the most widely studied and employed, and these criteria are associated with<5% chance of missing HRV and can spare about 30% of EGD in compensated patients [5]. However, as transient elastography (TE) is not widely available in all liver units, the Baveno VI criteria cannot be applied in many clinical settings. The development of noninvasive criteria that do not include TE is desirable. Therefore, an easy-to-use Rete Sicilia Selezione Terapia hepatitis C virus (RESIST-HCV) criteria, which uses only platelet count and serum albumin, have been proposed to exclude HRV in compensated cirrhosis by Calvaruso et al. [13]. By using these criteria, the spared EGD rate and the missed HRV rate were 31.4% and 1.6%, respectively [13].

The primary aim of this study was to validate the performance and safety of the RESIST-HCV criteria compared to screening endoscopy for HRV. The secondary aim was to assess the performance of the Baveno VI and Expanded Baveno VI criteria.

2. Materials and Methods

2.1. Study Population. This was a retrospective study involving all patients with compensated cirrhosis who underwent EGD from January 2018 to January 2020 and who were referred to Xingtai People's Hospital or Beijing Shijitan Hospital. The data collected included LSM (measured by TE), laboratory tests, liver ultrasonography findings, liver function tests, platelet counts, and EGD results.

2.2. Ethics. This study was conducted in compliance with the Declaration of Helsinki and approved by the Ethics Committees at Xingtai People's Hospital and Beijing Shijitan Hospital. Given the retrospective nature of this study, obtaining informed consent was not applicable.

2.3. Inclusion Criteria. Patients with Child-Pugh A and B cirrhosis with NITs (laboratory tests, reliable LSM, and ultrasonography) performed within 3 months of EGD were included in the study. A diagnosis of cirrhosis was established based on the history of chronic liver disease, clinical manifestations (especially PH-related complications), liver and spleen ultrasonography and computed tomography findings, presence of GEV on EGD, LSM > 10 kPa, and previous liver biopsy if available.

2.4. Exclusion Criteria. Exclusion criteria were the occurrence of decompensation events (ascites, hepatic encephalopathy, Child-Pugh C, previous variceal bleeding, esophageal varices (EV) band ligation, portal vein thrombosis, transjugular intrahepatic portosystemic shunt, and hepatocellular carcinoma), current use of nonselective beta-blockers and antiplatelet agents, anticoagulation, and incomplete data.

2.5. Liver Stiffness Measurements. TE was only available for the Beijing cohort. LSM was assessed according to the manufacturer's FibroScan standard procedure [14, 15], performed by one expert operator (Li Li) at Beijing Shijitan Hospital (>100 procedures). LSM was considered valid when there were at least 10 measurements with an interquartile range to median ratio (IQR/M) ≤ 30% [16]. Patients fasted for four hours before the procedure.

2.6. Upper Gastrointestinal Endoscopy. Two experienced endoscopists reviewed all the endoscopic findings and assessed the presence and size of GEV independently, without knowledge of the TE and blood test results and clinical data. The presence and size of EV were assessed according to the proposed guidelines [5]. Gastroesophageal varices were defined as low-

Table 1: Main characteristics of the study.

Variables	Total cohort, $n = 292$	Beijing cohort, $N = 104$	Xingtai cohort, $N = 188$
Male (%)	187 (64.0)	57 (54.8)	130 (69.1)
Age (years)	52 (43-60.5)	52 (43-60)	52.5 (43-61.5)
BMI	23.2 (21.4-25.2)	23.2 (21.4-25.2)	NA
Etiology			
Hepatitis B	174 (59.6)	36 (34.6)	138 (73.4)
Hepatitis C	4 (1.4)	0 (0)	4 (2.1)
PBC	8 (2.7)	5 (4.8)	3 (1.6)
Alcohol	12 (4.1)	11 (10.6)	1 (0.5)
Others	93 (31.8)	52 (50.0)	41 (21.8)
Child-Pugh			
A	245 (83.9)	84 (80.8)	161 (85.6)
B	47 (16.1)	20 (19.2)	27 (14.4)
Platelets ($10^3/\mu$L)	111 (80-162.5)	97 (68-139.5)	118.5 (88.5-171)
ALT (IU/L)	26.9 (19-41.9)	22 (16-29.5)	31.5 (21.3-50)
ALB (g/L)	41 (36.3-44.1)	37.5 (34.5-41.1)	42.2 (38.5-45.7)
Bilirubin (μM/L)	20.1 (14-30.1)	21 (15.1-30.6)	19 (14-28.8)
INR	1.2 (1.1-1.3)	1.2 (1.1-1.4)	1.2 (1.1-1.3)
TE† LSM (kPa)	19.8 (12-34.8)	19.8 (12-34.8)	NA
High-risk varices (%)	47 (16.1)	19 (18.3)	28 (14.9)
Any varices	142 (48.6)	54 (51.9)	88 (46.8)
With RESIST-HCV criteria	109	26	83
With RESIST-HCV criteria who had HRV	1	1	0

Continuous variables are expressed as median (interquartile range) unless indicated. †TE was available in 90 patients. Abbreviations: ALB: albumin; ALT: alanine aminotransferase; HRV: high-risk varices; INR: international normalized ratio; LSM: liver stiffness measurement; PBC: primary biliary cholangitis; TE: transient elastography.

risk varices (LRV) or high-risk varices (HRV). HRV were defined by a medium or large size or the presence of high-risk stigmata (cherry red spots and red wale marks) [5].

2.7. Laboratory Markers. Blood samples were drawn in the fasting state and handled according to the standard procedures of each hospital. The index blood samples chosen for assessing the proposed criteria were the closest to the screening endoscopy (within 3 months).

2.8. Statistical Analysis. Continuous data were all expressed as median with interquartile range (IQR), as none were normally distributed. Categorical data were expressed as numbers and percentages. A two-tailed P value of less than 0.05 was considered statistically significant. LSM and laboratory data were compared between patients with and without HRV; continuous data were compared using Mann–Whitney U test, and Fisher's exact test was used for proportions for categorical data. The rate of spared EGD was calculated as the ratio of the numbers of patients with EGD that could be spared to the total number of patients. The missed HRV rate was defined by the rate of patients with missed HRV among the patients with spared EGD. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS, Chicago, IL).

3. Results

3.1. Study Population. Over the study period, 137 compensated cirrhotic patients in the Beijing cohort and 248 patients from the Xingtai cohort underwent EGD. After excluding incomplete data, portal vein thrombosis, and unavailable lab tests within 3 months of EGD, a total of 292 patients from the two cohorts were included to validate the RESIST-HCV criteria, and 90 patients from the Beijing cohort were included to validate the Baveno VI and Expanded Baveno VI criteria. The flowchart of this study is shown in Figure 1. The baseline characteristics of the 292 patients with compensated cirrhosis are shown in Table 1. Overall, HRV was present in 16.1% (47 of 292 cases). The etiology of the underlying liver disease was hepatitis B virus (HBV) in 174 (59.6%), alcohol-related liver disease (ALD) in 12 (4.1%), primary biliary cholangitis (PBC) in 8 (2.7%), hepatitis C virus (HCV) in 4 (1.4%), and 93 others (31.8%); these others included unknown causes in 52, Budd-Chiari syndrome in 14, autoimmune hepatitis in 11, nonalcoholic fatty liver disease (NAFLD) in 9, drug reaction in 6, and overlap syndrome in 1. The majority of patients were Child-Pugh A (245; 83.9%), with 47 cases (16.1%) who were Child-Pugh B. In total, 90 patients had a reliable LSM from Beijing Shijitan Hospital. There were 109 (37.3%) patients who fulfilled the RESIST-HCV criteria ruling out the presence of HRV and could have avoided screening endoscopy. Only one patient with HRV was missed. The missed HRV rate was 0.9% and

the NPV was 99.1%. The above data is summarized in Table 1. When compared to patients without HRV, those with HRV had lower platelet count (77×10^9/L ($57.5 - 96 \times 10^9$/L) vs. 119×10^9/L ($90 - 169 \times 10^9$/L); $P < 0.001$) and lower serum albumin (37.2 g/L (33.2-40.9 g/L) vs. 41.6 g/L (36.8-44.4 g/L); $P < 0.001$), as seen in Table 2.

3.2. Diagnostic Accuracy of RESIST-HCV Criteria for HRV. The RESIST-HCV criteria combine platelet count > 120000/μL and albumin > 36 g/L. In the combined cohort, 109 (37.3%) cases met these criteria, of whom 1 (0.9%) had HRV. Among the 183 (62.7%) cases that did not meet these criteria, 46 (25.1%) had HRV (Figure 2). The combination of platelet count and albumin using the recommended cut-off values to predict HRV gave a sensitivity of 97.9%, specificity of 44.1%, PPV of 25.1%, and NPV of 99.1% (Table 3). One case (0.9%) of HRV was missed (Figure 2), and the case had liver cirrhosis secondary to HBV, and the platelet count and albumin were 142000/μL and 40.5 g/L, respectively.

3.3. Analysis of the Avoidance of the Baveno VI, Expanded BavenoVI Criteria, and RESIST-HCV Criteria. Using the RESIST-HCV criteria, we classified all patients into low risk (those who fulfilled these criteria) and high risk (those who did not fulfill these criteria). The RESIST-HCV criteria could spare 37.3% (109 of 292) of EGDs, with a 0.9% (1 of 109) missed HRV rate and NPV of 99.1% (Table 3). Of the 90 patients who had reliable LSM from the Beijing cohort, 14 (15.6%) and 21 (23.3%) patients met the Baveno VI and the Expanded BavenoVI criteria, respectively, and 0% (0 of 14) and 4.8% (1 of 21) of HRV were missed, respectively. The RESIST-HCV criteria had the best performance with an area under receiving operator characteristics curve (AUROC) of 0.710 (Table 3).

Table 2: Comparison of the total population with HRV vs. the population without HRV.

Variables	HRV, $N = 47$	Non-HRV, $n = 245$	P^*
Male (%)	30 (63.8)	157 (64.1)	0.974[a]
Age (years)	50 (44.5-58)	53 (43-61)	0.697
BMI	22 (21.1-23.4)	23.2 (21.5-25.2)	0.126
Etiology			0.363[a]
Hepatitis B	34	140	
Hepatitis C	0	4	
PBC	1	7	
Alcohol	2	10	
Others	10	83	
Child-Pugh			0.291[a]
A	37	208	
B	10	37	
Platelets ($10^3/\mu$L)	77 (57.5-96)	119 (90-169)	<0.001
ALT (IU/L)	29.3 (21-37)	26.1 (18-42)	0.465
ALB (g/L)	37.2 (33.2-40.9)	41.6 (36.8-44.4)	<0.001
Bilirubin (μM/L)	22.3 (15.5-33.2)	20 (14-28.1)	0.129
INR	1.3 (1.2-1.4)	1.2 (1.1-1.3)	<0.001
TE[†] LSM (kPa)	30.1 (14-35.6)	19.6 (12-34.8)	0.405

[*]Statistical comparison between the presence and absence of high-risk varices using Mann–Whitney U test unless indicated. [a]Statistical comparison between the presence and absence of high-risk varices using Chi-square test or Fisher's exact test. [†]TE was available in 90 patients. Abbreviations: ALB: albumin; HCV: hepatitis C virus; INR: international normalized ratio; LSM: liver stiffness measurement; PBC: primary biliary cholangitis; TE: transient elastography.

4. Discussion

In the present study, we validated the recently published RESIST-HCV criteria [13] that use only platelet count and serum albumin level to identify patients who are at low risk of HRV and can safely avoid endoscopic screening, saving time and reducing costs. This is the first validation performed in Chinese patients. Interestingly, the main etiology of cirrhosis was HBV, which makes this study different from a previous study [13], where the main etiology was HCV. Compared with the study with HCV predominance, our study demonstrated that these criteria had a similar diagnostic accuracy for HBV-related cirrhosis patients. Applying these criteria in our study would have spared 37.3% of endoscopies, with a 0.9% missed HRV rate.

As expected, in our study, the RESIST-HCV criteria could safely avoid 37.3% (109 of 292) of EGDs, while maintaining the missed HRV rate below 5%, which was similar to the recent study by Calvaruso et al. [13]. In the large cohort of 1381 cirrhotic patients with HCV, the RESIST-HCV criteria spared 31.4% of EGDs and showed a 1.6% false-negative rate for the medium and large varices [13]. Similarly, the RESIST-HCV criteria failed to identify one (0.9%) HBV patient with HRV. To our knowledge, the RESIST-HCV criteria are clearly able and safe to stratify compensated cirrhotic patients for HRV risk. In the present study, the RESIST-HCV criteria were the most accurate diagnostic tool for ruling out HRV patients.

We further validated the Baveno VI and the Expanded Baveno VI criteria. In this study, the Baveno VI criteria were safe and 15.6% of patients could have avoided endoscopy, while the risk of missing HRV was 0%, and the NPV was 100%. The spared EGD rate (15.6%) was comparable with that reported in previous studies [7, 13, 17–20]. In addition, our data demonstrated an acceptable rate of missing HRV. The Expanded Baveno VI criteria would have spared 23.3% of unnecessary EGDs and missed 4.8% of HRV. The number of spared EGDs is lower than the number reported in previous studies [7, 13, 14, 19]. The lower number of spared EGDs may be explained by the bias in selection of patients and the high prevalence of HRV, which may have led to a low NPV and influenced the diagnostic performance [18]. Besides, the sample size ($n = 90$) is rather small. In addition, identifying and classifying varices in cirrhosis by different endoscopists may be inconsistent due to differences in technique and the experience of doctors [21].

Our study had a few limitations. First, the study was retrospective and the EV size and high-risk stigmata were evaluated by two experienced endoscopists. However, this issue was present in other studies [11, 13, 18–20, 22]. Most studies

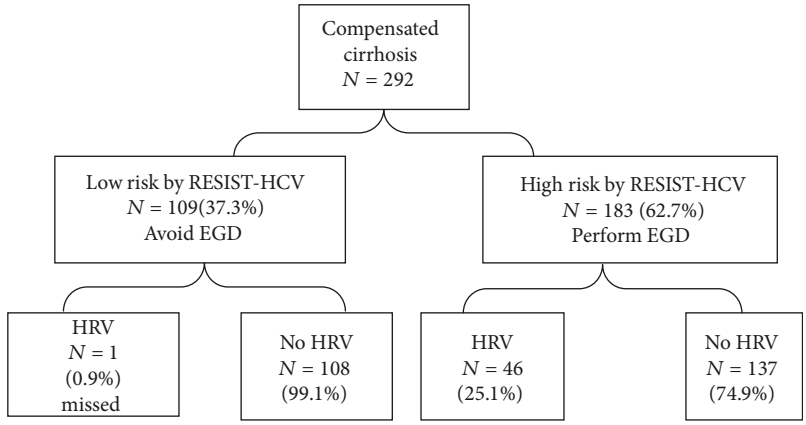

Figure 2: Application of RESIST-HCV criteria to a real-world cohort with compensated cirrhosis. Abbreviations: EGD: esophagogastroduodenoscopy; HRV: high-risk varices.

Table 3: Need for EGD based on noninvasive criteria for ruling out high-risk varices.

Characteristics	Pooled cohort (292patients)		
	B6C ($n = 90$)	EB6C ($n = 90$)	RESIST-HCV ($n = 292$)
AUROC	0.599	0.615	0.710
[a]Spared EGD, *n* (%)	14 (15.6)	21 (23.3)	109 (37.3)
[b]Missed HRV, *n* (%)	0 (0)	1 (4.8)	1 (0.9)
NPV (%)	100	95.2	99.1
Sen (%)	100	94.7	97.9
Spe (%)	19.7	28.2	44.1
PPV (%)	25	26.1	25.1

Abbreviations: AUROC: area under receiving operator characteristics curve; B6C: Baveno VI criteria; EB6C: expanded Baveno VI criteria; EGD: esophagogastroduodenoscopy; HRV: high-risk varices; NPV: negative predictive value; PPV: positive predictive value; Sen: sensitivity; Spe: specificity. [a]The spared EGD rate was calculated as the ratio between the number of patients with EGD that could be spared and the total number of patients. [b]The missed HRV rate was defined as the rate of patients with missed HRV among the patients with spared EGD.

that attempted to noninvasively rule out HRV, however, were retrospective and did not include an assessment of EV size [23]. Reassuringly, the diagnostic performance of the RESIST-HCV criteria was consistent with the performance reported in the recently published study [13]. Second, LSM measured by TE is useful for the assessment of HRV. However, TE was only available for 90 patients from Beijing Shijitan Hospital. Third, the present study lacked internal and external validation sets. Further validation in larger cohorts is needed.

5. Conclusions

Our study validated the RESIST-HCV criteria which could identify low-risk patients who can safely circumvent surveillance endoscopy for HRV screening for more than 30% of EGDs by using simple-to-use laboratory parameters not requiring TE. However, we have to acknowledge that a small proportion of HRV cases will be missed with an acceptable rate. Prospective validation of these criteria would be required to prove its diagnostic performance for HRV in other populations and various etiologies.

Abbreviations

ALT: Alanine aminotransferase
LSM: Liver stiffness measurement
TE: Transient elastography
HRV: High-risk varices
HCV: Hepatitis C virus
EGD: Esophagogastroduodenoscopy
PPV: Positive predictive value
NPV: Negative predictive value
pH: Portal hypertension
GEV: Gastroesophageal varices
NITs: Noninvasive tests.

Data Availability

The original data can be obtained from the correspondence author.

Ethical Approval

After review by the ethics committee, the health, rights, and privacy of the subjects were fully protected (approval letter no. 2020[017]).

Consent

Given the retrospective nature of this study and that all data were processed anonymously, obtaining an informed consent was not applicable.

Conflicts of Interest

All authors have no conflict of interest to disclose.

Authors' Contributions

Zhihui Duan designed the study, collected data, analysed data, performed the statistical analyses, wrote the manuscript, and approved final manuscript. Li Li collected data, wrote the manuscript, and approved the final manuscript. Zhihui Duan and Li Li are co-first authors. Shengyun Zhou and Jinlong Li collected data and contributed to the drafting and final approval of the manuscript. Zhihui Duan and Li Li contributed equally to this work.

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Review Article

Current Status and Prospects of Spontaneous Peritonitis in Patients with Cirrhosis

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Spontaneous bacterial peritonitis (SBP) is a common cirrhotic ascites complication which exacerbates the patient's condition. SBP is caused by gram-negative bacilli and, to a lesser extent, gram-positive cocci. Hospital-acquired infections show higher levels of drug-resistant bacteria. Geographical location influences pathogenic bacteria distribution; therefore, different hospitals in the same country record different bacteria strains. Intestinal changes and a weak immune system in patients with liver cirrhosis lead to bacterial translocation thus causing SBP. Early diagnosis and timely treatment are important in SBP management. When the treatment effect is not effective, other rare pathogens should be explored.

1. Introduction

Spontaneous bacterial peritonitis (SBP) is a common complication in patients with liver cirrhosis and is recorded in 10–30% of hospitalized patients with cirrhotic ascites leading to sepsis or even death [1–4]. Studies show that bacterial translocation plays a key role in the occurrence and development of SBP [5, 6]. Bacterial translocation is caused by disorder of gut microflora, increased intestinal permeability, and host immunodeficiency [7, 8]. Although gram-negative bacilli are the main cause of SBP, infections due to gram-positive bacteria drug-resistant bacteria have been reported [9–11]. Therefore, it is important to understand the epidemiology and pathogenesis of SBP and develop effective therapy approaches.

2. Epidemiology

Geographical location affects SBP pathogen distribution with variations recorded among different hospitals in the same country. Gram-negative bacilli are the main SBP-causing pathogens, but infections of gram-positive cocci [12, 13], fungi, and some other rare pathogens cannot be ignored [14–18]. Increased use of broad-spectrum antibiotics and prophylactic quinolones has led to the emergence of multidrug-resistant bacteria, especially in hospital-acquired infections [19–22]. Only 50-60% of SBP patients have positive ascites culture; therefore, pathogen identification is challenging [23]. These limitations hamper development of effective anti-infection therapy.

2.1. Asia. Li et al. [24] retrospectively analyzed 288 Chinese patients with spontaneous peritonitis from 2011 to 2013 and isolated 306 pathogenic bacteria, among which gram-negative bacteria, gram-positive bacteria, and fungi accounted for 58.2%, 27.8%, and 2.9% of the isolates. The main pathogenic bacteria were *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus,* and *Staphylococcus aureus*. Of the 306 pathogenic bacteria, 99 cause nosocomial infections and 207 were community-acquired and play a role in other infection pathogenesis. *Escherichia coli* and *K. pneumoniae* produce more broad-spectrum β-lactamase in nosocomial infections compared with nonnosocomial infections. Piperacillin/tazobactam combination is a more effective therapy for nonhospital infections than nosocomial infections caused by *E. coli*. The authors reported that the pathogenic bacteria causing abdominal infection in patients with liver cirrhosis were mainly gram-negative, and the drug resistance rate of

nosocomial infection was significantly higher compared with the rate for nonnosocomial infection.

In another retrospective study, Ding et al. [25] analyzed the etiology of 334 Chinese patients with SBP from 2012 to 2016 and arrived at a similar conclusion. A total of 334 pathogenic bacteria were isolated, including 178 gram-negative bacteria and 138 gram-positive bacteria. The main pathogens were *E. coli*, *K. pneumoniae*, and *Enterococcus faecium*. The proportion of *Enterococci* in patients with hospital-acquired SBP was significantly higher than in those with community-acquired SBP. Pathogens isolated from nosocomial infections showed significantly higher resistance to first-line recommended drugs and were associated with poor prognosis.

In a retrospective cohort study in South Korea, Cheong et al. [21] analyzed the microbial characteristics of 236 patients with SBP from 2000 to 2007: *E. coli* accounted for 43.2%, *Klebsiella* accounted for 14.0% while *Streptococcus* accounted for 9.8% of the total bacteria population. The resistance rate of G^- to third generation cephalosporins and quinolones for hospital-acquired infections was significantly higher compared with that for community-acquired infections. In another study, Choi et al. [15] found 43 cases of SBP caused by *Aeromonas aerobicus* as a result of weather changes between 1997 and 2006. Hwang et al. [26] reported that *Candida* infection was the main causative agent of fungal spontaneous peritonitis in Korea from 2000 to 2005.

2.2. Europe. In a Spanish retrospective study from 2001 to 2009, 34.6% of the 200 SBP patients showed community-acquired infections while 26.8% of these infections were hospital acquired. The third-generation cephalosporin resistance rate was 7.1% for the community-acquired infections and 40.9% for the hospital-acquired infections. These drug-resistant cases were mainly a result of gram-negative bacilli and *Enterococci* that produce extended-spectrum β-lactamases. Previous use of cephalosporins, diabetes, upper gastrointestinal bleeding, and nosocomial-acquired infections are risk factors for the development of drug-resistant bacterial infections [27]. Fernandez et al. [28] analyzed bacterial infection in 507 Spanish patients with liver cirrhosis and ascites admitted to hospital during 2005–2007 and 2010–2011 in a prospective study. 35% of hospital-acquired patients had higher number of drug-resistant strains compared with those with community-acquired infections (4%). Moreover, SBP mortality caused by drug-resistant bacteria was significantly higher.

Friedrich et al. [29] retrospectively analyzed the etiology of the first occurrence of SBP in 311 German patients with liver cirrhosis from 2007 to 2013. Gram-positive bacteria accounted for 47.8% of the total infections, gram-negative bacteria accounted for 44.9% while fungi accounted for 7.2% of the infections. In this study, *Enterobacter*, *Enterococcus*, and *Staphylococcus* were the most common isolates. Third-generation cephalosporins were effective in 70.2% of non-hospital-acquired SBP patients and in 56.3% of hospital-acquired SBP patients. In another prospective study from Germany, Lutz et al. [30] analyzed 86 German SBP patients from 2012 to 2016 and obtained similar results. *E. coli*, *Klebsiella*, *Enterococcus*, and *Streptococcus* were the most common isolates. The resistance rate of nosocomial bacteria was higher than that of healthcare-related bacteria.

Bert et al. [31] analyzed 95 cases of hospital-acquired and community-acquired bacterial peritonitis in France from 1998 to 1999. A total of 78 pathogenic bacteria were isolated, of which 34 were *Streptococcus* spp. and 23 were *E. coli*. Streptococci are more common in community-acquired infections while gram-negative bacteria are more common in hospital-acquired infections. Another prospective observational study in France in 2005, involving 331 patients with SBP at 25 medical centers, revealed 222 gram-negative bacilli, mainly *E. coli*, *Enterobacter*, *K. pneumoniae*, and *P. aeruginosa*; 148 gram-positive cocci, mainly *Streptococcus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus aureus* while all 19 strains of fungi were *Candida albicans* [32]. Imipenem is an effective treatment for *P. aeruginosa* hospital-acquired infections [32].

Piroth et al. [33] retrospectively analyzed 114 strains of SBP in five hospitals in France from 2006 to 2007. *Staphylococci* and *E. coli* were the most common pathogens. Notably, 28% patients infected by the *E. coli* strain showed resistance to amoxicillin+clavulanic acid, and 27% of patients infected with *S. aureus* were resistant to methicillin. An observational study carried out in France in 2010 and 2011 showed that of the 57 confirmed SBP cases, gram-positive cocci (64.9%) were the main causative pathogens, including coagulase-negative *Staphylococci*, *Enterococci*, *Streptococci*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [13]. Another study on SBP patients in France reported that gram-positive bacteria were the dominant strains, accounting for 70% of nosocomial infections [34].

Gunjaca and Francetić [35] prospectively studied 108 cases of cirrhosis in Croatia, where SBP prevalence was 21% and the mortality was 26%. The pathogens causing SBP were mainly gram-negative bacteria such as *E. coli*, methicillin-resistant *S. aureus* (MRSA), and *Acinetobacter*.

Alexopoulou et al. [36] retrospectively carried out a study on 47 SBP patients in Greece from 2008 to 2011. Twenty-eight patients had medically related infections and 15 were treated with quinolone prophylaxis. Gram-positive coccus was the most commonly isolated pathogen. Nine isolates were multidrug-resistant bacteria, including *K. pneumoniae*-producing carbapenemase and *E. coli*- and *P. aeruginosa*-producing ultrabroad spectrum β-lactamase. Higher number of gram-negative bacteria was reported in hospital-associated infections compared with gram-positive cocci. Another Greek prospective study from 2012 to 2014 included 130 SBP patients with a 30-day follow-up. The results showed that gram-positive cocci (GPC) were the causative agents for half of the cases. Multidrug-resistant (MDR) strains comprised 20.8% of the total cases while 10% were extensively drug resistant (XDR). Drug-resistant bacteria showed a significant increase in mortality rates [37].

2.3. America. Chaulk et al. [38] retrospectively analyzed 192 Canadian SBP patients from 2003 to 2011. Among them, 77 patients had culture-positive infection with gram-positive bacteria causing 57% of these cases. The antibiotic

Table 1: Pathogens associated with spontaneous peritonitis in cirrhosis.

Country/author/year	Pathogens	Type of study	G^-	G^+	HA SBP	CA SBP
China/Li et al./2011-2013	306	Retrospective	58.2%	27.8%	99	207
China/Ding et al./2102-2016	334	Retrospective	52.3%	41.3%	155	179
Korea/Cheong/2000-2007	236	Retrospective	72.9%	22.9%	126	110
Germany/Friedrich/2007-2013	114	Retrospective	44.9%	47.8%	—	—
France/Bert/1998-1999	78	Retrospective	44.9%	51.3%	39	39
France/Montravers/2005.1-2005.7	829	Prospective	41%	27%	540	289
France/Piroch/2010-2011	268	Prospective	34%	64.9%	109	159
Canada/Chaulk/2003-2011	77	Retrospective	27%	57%	52	25

G^-: gram-negative bacteria; G^+: gram-positive bacteria; HA: hospital acquired; CA: community acquired; SBP: spontaneous bacterial peritonitis.

resistance rate was 8% in community-acquired infections and 41% in hospital-acquired infections (Table 1).

Ardolino et al. [39] retrospectively studied 160 SBP cases in the United States from 2005 to 2015. This study reports that gram-negative bacteria were mainly *E. coli*. The sensitivity rate to ceftriaxone was 71%. Gram-positive cocci including *Enterococci*, *Streptococcus*, and *Staphylococcus* accounted for 37.5% of the cases. 71% of *Enterococci* were resistant to vancomycin, and MRSA accounted for 80% of the infections.

Reddy et al. [40] reported a rare case of SBP caused by the *Salmonella enteritis* group b in a patient with liver cirrhosis in the United States. Wu and Giri [41] first reported a case of SBP caused by *Haemophilus paraphilus*. Later, the patient also developed tuberculous peritonitis, a combination that had not been reported before. Emily and Maraj [42] reported cases of SBP with *Lactobacillus* as the pathogen. *Lactobacillus paracasei* was isolated from the abdominal cavity of a 73-year-old American man with liver cirrhosis. This strain was resistant to carbapenem antibiotics. Further, the patient eventually developed hepatorenal syndrome and succumbed to acute renal failure. Toyoshima et al. [43] reported SBP cases caused by *Listeria monocytogenes* in two patients with liver cirrhosis in Brazil. Third-generation cephalosporins are not effective for *Listeria* infections.

2.4. Africa. Oladimeji et al. [44] conducted a retrospective analysis of 31 patients with ascites in Nigeria from 2009 to 2010. In these SBP patients, the main pathogens were *E. coli* and *Klebsiella*. The gram-positive bacteria implicated in SBP infections were mainly *Streptococcus* and *Staphylococcus aureus*. Zaki et al. [45] explored the bacterial and fungal causes of SBP in an Egyptian population comprising 100 SBP patients. In this population, the pathogens were mainly gram-positive coccus (48.8%), gram-negative bacillus (12.2%), and 7.3% were *Mycobacterium tuberculosis*. Mohamed et al. [46] performed SBP screening on 3000 cirrhosis patients with ascites and pleural effusion in Egypt. SBP prevalence in patients with cirrhosis was reported to be 1.6% with the main causative pathogens being *E. coli* and *K. pneumoniae.*

3. Pathogenesis

Intestinal flora is considered as an important component of the intestinal barrier [47]. Changes to the gut microbiota are implicated in the SBP occurrence and progression [48–51]. Therefore, exploring the role of intestinal flora on SBP pathogenesis is the key in development of effective prevention and treatment strategies. For patients with liver cirrhosis, bacterial translocation (BT) as a result of intestinal gram-negative *Enterobacteriaceae* infections is the main cause of SBP occurrence and development [6, 52, 53]. Previous studies have shown that gastrointestinal stasis due to portal hypertension in patients with liver cirrhosis, intestinal bacterial overgrowth due to low levels of bile acid and gastric acid, delayed intestinal transport, altered intestinal permeability, and immune dysfunction promote BT and ultimately SBP [5, 7, 8] (Figure 1).

3.1. Small Intestinal Bacterial Overgrowth (SIBO). Cirrhosis results in small intestinal bacterial overgrowth [54–56], especially in patients with ascites and SBP history [57]. Overgrowth of small intestinal bacteria is implicated in bacterial translocation and SBP [58]. In a previous study, Bauer et al. reported that small intestinal bacterial overgrowth (SIBO) in patients with cirrhosis has no effect on spontaneous bacterial peritonitis [59]. However, in a subsequent study, he carried out quantitative culturing of jejunal secretion in 53 cirrhosis patients with a 1-year follow-up. In his findings, he reported that SIBO was present in 59% of the cirrhosis patients he examined and was associated with systemic endotoxemia [60]. Fukui et al. [61] also reported an increase in gram-negative bacteria represented by *E. coli* resulting in high levels of lipopolysaccharides (LPS) and endotoxemia in patients with liver disease. BT or microbial translocation is defined as the migration of surviving microorganisms or bacterial products (i.e., bacterial LPS, peptidoglycans, and lipopeptides) from the intestinal lumen to the mesenteric lymph nodes and other external intestinal sites [62–66]. In addition, studies have shown that small bowel transport is significantly longer in patients with SIBO [67]. Animal experiments by Pérez-Paramo et al. [68] reported that intestinal overgrowth and severe impairment of intestinal permeability in cirrhotic rats with ascites cause bacterial translocation and SIBO was associated with insufficient intestinal motility. In recent studies, gastrointestinal stasis due to portal hypertension, relative lack of bile and gastric acid secretion, intestinal dyskinesia, and long-term use of broad-spectrum antibiotics in patients with liver cirrhosis are implicated in increased intestinal aerobic bacteria and colonic bacterial migration to the jejunum and duodenum.

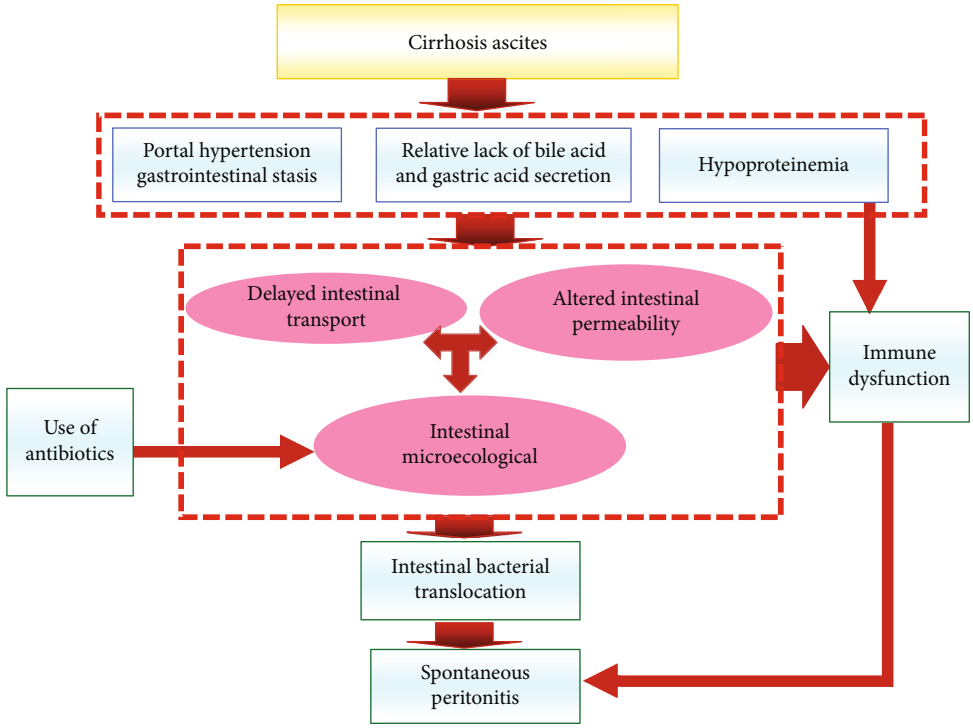

Figure 1: The pathogenesis of spontaneous peritonitis.

These changes further cause SIBO and promote BT, which is implicated in SBP prognosis in patients with liver cirrhosis [7]. Notably, the most common pathogenic microorganisms were isolated from the intestinal flora of cirrhotic ascites in SBP patients [69]. Interestingly, quantitative metagenomics analysis showed that some of the bacteria in SIBO were oral strains. Qin et al. [70] proposed that oral symbiotic bacteria in liver cirrhosis patients invaded the intestine as a result of bile secretion changes in these patients. The changes in bile secretion created a more favorable environment for the survival of foreign bacteria in the intestinal tract. Pardo et al. [54] also reported that cisapride increases BT from the oral cavity to the cecum. The use of cisapride in cirrhotic rats showed reduction of SIBO and occurrence of BT.

3.2. Altered Intestinal Permeability. The human intestinal mucosa mechanical barrier is the first barrier against BT and consists of intestinal epithelial cells and cell-to-cell connections [71–73]. The intestinal barrier system of intestinal epithelial cells prevents the transportation of a large number of bacteria and bacterial products; therefore, few bacteria and bacterial products reach the liver [74]. Tight junctions between cells are the key in maintaining integrity of the intestinal barrier, and reduction in density of these tight junctions impairs the function of the intestinal barrier [75, 76]. Assimakopoulos et al. [77] reported that expression levels of proteins associated with tight junctions in intestinal epithelial cells were lower in cirrhosis patients compared with patients with decompensated cirrhosis. Animal experiments [78] show that the intestinal mucosa of rats with liver cirrhosis shows signs of atrophy, shortening, and villus rupture. Capsule endoscopy studies show abnormal changes in the mucosa of the small intestine in cirrhosis patients [79] while pathological examination shows shortening and atrophy of the small intestine [80, 81]. However, Du Plessis et al. [82] reported that electron microscopy showed complete epithelial barriers in patients with decompensated cirrhosis, implying that the epithelial barrier was functionally altered but structurally normal in cirrhosis. The contrasting findings may be due to differences in methodology and the relatively small number of studies/patients [83]. Assimakopoulos et al. [84] performed duodenal biopsies on healthy controls and patients with cirrhosis and decompensated cirrhosis. In this study, patients with decompensated and decompensated cirrhosis had decreased intestinal mucosa mitosis and increased cell apoptosis compared with the control group. Intestinal permeability changes with progression of cirrhosis and occurrence of SIBO, with increased intestinal permeability of bacteria and their products resulting in BT [83, 85, 86]. Several studies report that cirrhosis and ascites patients have significantly high intestinal permeability, while the intestinal permeability of patients with Child–Pugh C is significantly higher than the permeability of those with Child–Pugh with A and B cirrhosis [87, 88]. For patients with SBP history, intestinal permeability is higher and can lead to severe sepsis complications [89, 90].

3.3. Delayed Bowel Transit. Studies show that liver cirrhosis changes intestinal motility [91]. Delayed movements of the small intestine can lead to SIBO and eventually cause BT [92]. A radiological examination by Kalaitzakis et al. [93] showed that intestinal transit time was prolonged in 38% patients with liver cirrhosis. Chen et al. [94] used a noninvasive hydrogen breath test and found that the intestinal transit

time of patients with decompensated cirrhosis was significantly longer compared with that of patients with decompensated cirrhosis. Further, the intestinal transit time was positively correlated with the severity of cirrhosis [95]. The small intestine transit delay and SIBO interact are associated and activate each other [71]. Perez-Paramo et al. [68] reported that nonselective beta blocker (NSBB (propranolol)) treatment in cirrhotic animals significantly reduces portal vein pressure and accelerates intestinal transport. The rate of bacterial overgrowth and metastasis in liver cirrhosis cases is low; therefore, intestinal bacteria overgrowth is positively correlated with insufficient intestinal motility. Propranolol accelerates intestinal transport and reduces bacterial overgrowth and transfer rates. However, Mandorfer et al. [96] found that although NSBB can reduce the risk of portal vein pressure and esophageal varix bleeding in patients with liver cirrhosis, it can increase the rate of hemodynamic disorders and liver-renal syndrome in patients with liver cirrhosis and SBP. Animal experiment results show that cisapride accelerates the transit time, improves the permeability of the small intestine, and reduces BT [97].

3.4. Impaired Local and Systemic Immune Function. Although the intestinal immune system is the last line of defense in microbial invasion, it the most important line of defense against intestinal microbial invasion. The interaction between intestinal flora and mucosal immune system is dynamic and complex [98]. Under normal physiological conditions, the microbiome can maintain a delicate balance with the mucosal immune system, which is extremely important for the host health [99]. Changes in the intestinal microenvironment causes excessive growth of opportunistic pathogenic bacteria and the reduction of symbiotic bacteria in critically ill patients. The changes aggravate mucosal immune dysfunction, promote the increase of intestinal BT, and eventually lead to intestinal infection [100–103].

Bacteria occur in the intestinal lymphoid tissue but do not harm the body, as they are usually effectively cleared by phagocytes [104]. Damage to the body's defense mechanisms also promotes subsequent infection of fluid in the peritoneal cavity [54]. Immune disorders in patients with cirrhosis are known as cirrhosis-associated immune dysfunction (CAID) [105]. Cirrhosis-related immune dysfunction and immunodeficiency are dynamic and result from liver inflammation driven primarily by monocytes/macrophages. The liver's mononuclear-phagocytic system function in patients with cirrhosis is impaired, leading to a decrease in the body's immune function and opsonin activity in the ascites [106]. This further reduces the level of bacteria removal leading to the body's inability to effectively remove pathogenic bacteria eventually causing bacterial translocation and ultimately results in SBP. Phagocytosis of hepatic macrophages in cirrhosis patients is lower compared with that in the healthy control group and is correlated with the severity of liver disease [107–110]. In addition, severe malnutrition in patients with cirrhosis also affects their immune system. Diet and nutrition are key factors in host-microbe interactions while starvation adversely affects intestinal mucosal integrity, epithelial cell proliferation, and mucin and antimicrobial peptide synthesis. Hodin et al. [111] observed autophagy of Paneth cells in starved mice due lack of enteral nutrition and decreased expression of antibacterial products. The poor nutrition weakened the protective effect on BT, thereby causing BT. Therefore, improving the nutritional status of patients with advanced cirrhosis improves the body's immune function and reduces the BT and SBP incidences. Albumin is specifically synthesized in the liver and is implicated in a myriad of functions such as the binding and transport of substances, the regulation of endothelial function, antioxidant and clearance properties, and the regulation of inflammatory responses. Serum albumin levels are low in liver cirrhosis patients due to synthetic defects, and structural and functional changes due to posttranscriptional modifications hinder their ability to perform physiological functions [112, 113].

4. Treatment

For patients with decompensated liver cirrhosis, spontaneous peritonitis can lead to further decompensation and multiple organ failure; therefore, SBP therapy is important for these patients. However, current methods are limited to antibiotic treatment, which leads to increases in drug-resistant bacteria and nonclassical pathogen infections [9–11]. Therefore, understanding the mechanism of SBP development, antibiotic treatment, new adjuvant treatment methods, and multiple treatment coordination are needed to minimize the occurrence of infection, reduce bacterial resistance, and improve survival.

4.1. Antibiotic Treatment. If the patient is clinically suspected of developing SBP, ascites culture should be performed immediately along with initiation of antibiotic treatment to reduce complications and improve survival [114, 115]. Third-generation broad-spectrum cephalosporin, cefixime, is the first-line treatment option for out-of-hospital SBP infection, with a recommended dose of 2 g/8 h (6 g/day) for 5 days [116, 117], which can be extended to 7 days [118]. Fluoroquinolones have good oral bioavailability and can be used as therapy for uncomplicated SBP [119]. Third-generation cephalosporin antibiotics and quinolones have been used to control SBP infection with high levels of clinical efficacy. However, long-term application increases the risk of bacterial resistance and double infection. Notably, *Enterobacteriaceae* family shows increased resistance to cephalosporins, particularly in nosocomial infections [120, 121]. Long-term preventive norfloxacin treatment reduces the risk of gram-negative infections but increases the risk of hospital-acquired *Staphylococcal* infections [122]. Therefore, considering that the distribution of SBP varies with geographic region and the proportion of drug-resistant pathogens is high, when selecting first-line empirical antibiotic treatment, the epidemic situation of drug-resistant bacteria should be based on the local situation [10]. Piperacillin/tazobactam is the first-line treatment for nosocomial SBP infection in areas with low resistance. Meropenem is recommended in hospitals with a high positive rate of ESBLs produced by *Enterobacteria* [30]. In areas with high prevalence of MRSA and

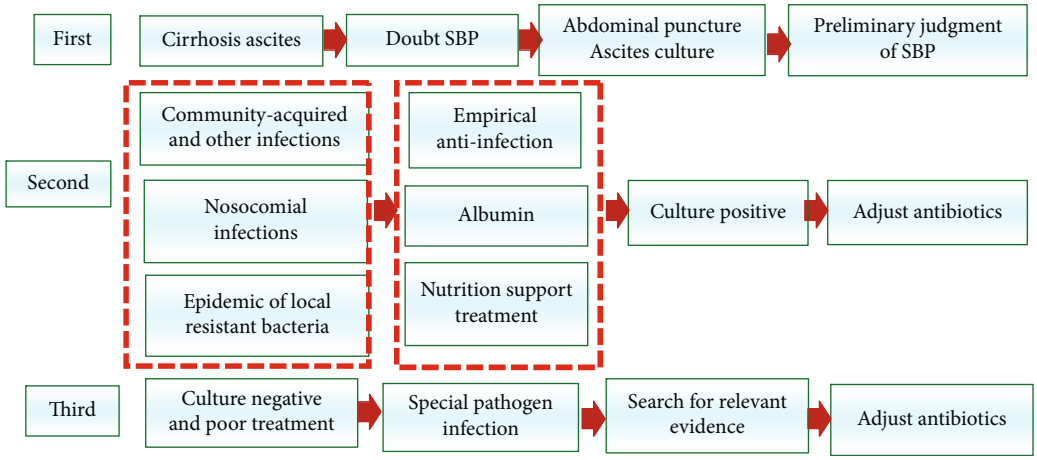

Figure 2: Treatment procedure of spontaneous peritonitis.

vancomycin-sensitive *Enterococcus* (VSE), a combination of meropenem and vancomycin or teicoplanin is recommended, while linezolid is recommended in case of vancomycin-resistant *Enterococcus* (VRE) [19]. In areas with high resistance to third-generation cephalosporins, meropenem combined with daptomycin can be used to improve patient survival of the nosocomial SBP [123]. If the ascites culture is positive, non-broad-spectrum antibiotics should be selected according to the drug sensitivity results to reduce the emergence of drug-resistant bacteria [115]. When antibiotic therapy fails in patients with spontaneous peritonitis, the possibility of fungal or other rare pathogens should be considered [14, 26, 124].

4.2. Gut Microecological Intervention. Intestinal bacteria are the main source of infections in patients with decompensated cirrhosis; therefore, norfloxacin is often used to clear the intestines for preventive treatment. However, antibiotic prevention can lead to increase in drug-resistant bacteria [125, 126]. Therefore, prevention is limited to a small number of patients with a high risk of infection. Probiotics can competitively inhibit adhesion to epithelial cells through competitive nutrients, reduce intestinal pH, and secrete antibacterial compounds to inhibit the growth of harmful pathogenic microorganisms. On the contrary, probiotics improve the intestinal mucosal barrier function and regulate the liver's natural killing of T lymphocytes [127]. Studies have reported that probiotics can reduce BT and effectively prevent the occurrence of hepatic encephalopathy [128]. Rat models with cirrhosis show that probiotics reduce BT, proinflammatory response status, formation of ascites, and oxidative damage in the ileum [129]. In a previous study, *Bifidobacterium* was shown to reduce the expression of proinflammatory chemokine receptors in the lymphocytes of mice with liver cirrhosis. Thus, the intestinal permeability of mice treated with *Bifidobacterium* was reduced while the liver function and inflammatory response improved [65]. The use of probiotics in liver-damaged rats alters the host's intestinal environment and reduces the occurrence of BTs [6, 130]. In a randomized double-blind controlled experiment, Gupta et al. [66] reported that the hepatic vein pressure gradient in the probiotic group was significantly lower compared with the propranolol group and that the addition of probiotics increased the effectiveness of propranolol treatment. However, a randomized controlled trial by Pande et al. [131] showed that the addition of probiotics to norfloxacin had no significant effect on SBP prevention in cirrhosis and ascites patients. Although more studies should be carried out needed to support the application of probiotic therapy in the prevention or management of SBP, previous studies report that probiotic therapy is effective in managing gastrointestinal diseases.

4.3. Immunity Therapy. In addition to intestinal targeting methods, immunotherapy methods have been developed to reduce the susceptibility of patients with decompensated cirrhosis to infection. In addition to antibiotics, albumin is a key therapy for SBP patients as it restores the immune function and improves survival [132]. Studies have found that infusion of human albumin reduces immunosuppression and the risk of infection in patients with acute decompensated cirrhosis [9, 133]. Combination of antibiotics and albumin significantly reduces serum and ascites cytokines and LPS levels in patients with SBP [134]. Caraceni et al. [135] evaluated 440 patients with decompensated liver cirrhosis who received standard treatment or standard treatment plus albumin. The 18-month survival rate of the treatment group was significantly higher compared with that of the standard treatment group. Sort et al. [136] randomly divided 126 patients with SBP; the mortality rate of the antibiotic plus albumin group was lower compared with that of the antibiotic group. Although the role of albumin is beneficial, not all patients with SBP can be treated with albumin, and patients with bile $< 68.4\,\mu$mol/L and creatinine $< 88.4\,\mu$mol/L cannot receive albumin treatment [136, 137]. Most patients with advanced liver cirrhosis are malnourished, which can easily lead to BT and SBP [138]. Patients with liver cirrhosis should optimize nutrition, avoid raw foods and coarse superfoods, limit sodium intake, eat small meals, and include 1.2-1.5 g of protein daily [139]. Cytokine treatments can improve the function of existing immune cells, significantly increase peripheral

white blood cell counts, and improve the prognosis of patients with decompensated cirrhosis [140, 141]; however, more experimental and clinical evidence is needed.

5. Conclusion

Spontaneous bacterial peritonitis causes high mortality rates and occurs in 7-31% of hospitalized patients with cirrhosis and ascites [142]. Patients susceptible to SBP need rigorous evaluation to optimize nutrition and avoid unnecessary drug treatment [12]. When patients with cirrhosis and ascites are hospitalized for gastrointestinal and parenteral diseases, ascites analysis should be performed whether symptoms are present or not. The long-term use of antibiotics has led to the emergence of multidrug-resistant bacteria and recent changes in the bacterial spectrum, including increased incidence of SBP associated with gram-positive cocci. Therefore, patients with cirrhosis and ascites should be monitored keenly and early diagnosis and treatment of SBP are important to prevent poor prognosis. A good understanding of the epidemiology of the region is the key to the correct choice of antibiotics. When encountering cases with poor treatment results, it is necessary to consider the possibility of other rare pathogens such as fungi and adjust the treatment strategy. Therapy approaches should include improved nutrition support to enhance the immunity of patients and comprehensive treatment should be considered for better results (Figure 2). SBP prevention should focus on stabilizing the intestinal environment, restoring the balance of intestinal flora, and reducing the occurrence of BT.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

Li YT wrote the paper. Huang JR and Peng ML have revised the paper for final approval.

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Research Article

No Association between Ischemic Stroke and Portal Vein Thrombosis in Liver Cirrhosis

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Background and Aims. There seems to be a higher risk of ischemic stroke and portal vein thrombosis in liver cirrhosis. Both of them may be associated with hypercoagulability. We aim to explore the association between ischemic stroke and portal vein thrombosis in liver cirrhosis. *Study Design and Methods*. We selected patients from our prospectively established database of liver cirrhosis from December 2014 to July 2019. The difference between patients with and without stroke was compared. A 1 : 1 propensity score matching (PSM) analysis was performed to adjust the effect of age, sex, Child-Pugh score, and MELD score on our statistical results. *Results*. There were 349 cirrhotic patients in the cross-sectional study. The prevalence of stroke, ischemic stroke, hemorrhagic stroke, and portal vein thrombosis was 8.88% (31/349), 8.31% (29/349), 1.15% (4/349), and 28.65% (100/349) in liver cirrhosis, respectively. Patients with ischemic stroke were significantly older and had significantly higher proportions of alcohol abuse, smoking, and arterial hypertension and higher levels of white blood cell and low-density lipoprotein. However, statistical analyses with and without PSM did not find any significant association between ischemic stroke and portal vein thrombosis in patients with liver cirrhosis. *Conclusion*. Ischemic stroke might not be associated with portal vein thrombosis in liver cirrhosis.

1. Introduction

Stroke, an acute cerebrovascular disease, is the leading cause of death and disability worldwide, especially in Asia [1]. It has been traditionally considered that stroke, especially atherosclerotic ischemic stroke, is closely associated with hypercoagulability, such as increased homocysteine and lipoprotein(a) and antiphospholipid antibodies [2]. Liver cirrhosis, which often has an increased level of factor VIII (FVIII) and decreased level of protein C (PC) and mean lifetime of platelet, has been also considered as a potential risk factor of stroke [3, 4]. Besides, it is associated with the development of venous thromboembolism, including portal vein thrombosis [5].

Development of stroke and portal vein thrombosis in liver cirrhosis seems to share several common pathophysiological mechanisms or risk factors. First, both of them relate to the hypercoagulability of cirrhosis [3], which is characterized as reduced PC in combination with increased FVIII [6]. Second, both stroke and portal vein thrombosis relate to portal hypertension. Reduced portal vein flow velocity and portosystemic collateral shunt are common characteristics of portal hypertension, which aggravate the development of portal vein thrombosis [7]. Ascites and esophageal variceal bleeding are major clinical manifestations of portal hypertension, which result in the decrease of effective circulating blood volume and hypovolemia of organs, thereby leading to ischemic stroke [8]. Third, diabetic patients have

Table 1: Comparison between patients with and without stroke.

Variables	No. pts	Stroke Median (range) or frequency (percentage)	No. pts	No stroke Median (range) or frequency (percentage)	p value
Age (years)	31	60.21 (38.72-77.30)	318	54.21 (20.57-88.73)	*0.003*
Gender (male)	31	25 (80.6%)	318	230 (72.3%)	0.319
Systolic blood pressure (mmHg)	31	125.00 (90.00-173.00)	317	122.00 (83.00-193.00)	0.594
Diastolic blood pressure (mmHg)	31	75.00 (50.00-117.00)	317	75.00 (34.00-118.00)	0.844
Etiology of liver diseases					
Hepatitis B virus infection	31	10 (32.3%)	318	125 (39.3%)	0.442
Hepatitis C virus infection	31	1 (3.2%)	318	20 (6.3%)	0.494
Alcohol abuse	31	20 (64.5%)	318	139 (43.7%)	*0.026*
Drug related	31	4 (12.9%)	318	21 (6.6%)	0.194
Budd-Chiari syndrome	31	0 (0.0%)	318	1 (0.3%)	0.755
Autoimmune liver diseases	31	0 (0.0%)	318	22 (6.9%)	0.130
Clinical presentations at admission					
Hepatic encephalopathy	31	1 (3.2%)	318	8 (2.5%)	0.812
Acute gastrointestinal bleeding	31	9 (29.0%)	318	101 (31.8%)	0.755
Ascites (no/mild/moderate-severe)	31	16 (51.6%)/8 (25.8%)/7 (22.6%)	318	127 (39.9%)/108 (34.0%)/83 (26.1%)	0.440
History					
History of venous thrombus	31	0 (0.0%)	318	7 (2.2%)	0.404
History of hematological diseases	31	1 (3.2%)	318	5 (1.6%)	0.499
History of diabetes mellitus	31	8 (25.8%)	318	51 (16.0%)	0.166
History of arterial hypertension	31	12 (38.7%)	318	41 (12.9%)	*<0.001*
History of smoking	31	21 (67.7%)	318	135 (42.5%)	0.007
History of cardiac diseases	31	5 (16.1%)	318	23 (7.2%)	0.082
Laboratory tests					
Red blood cell (10^{12}/L)	31	3.33 (1.15-5.20)	318	3.27 (1.45-5.46)	0.432
Hemoglobin (g/L)	31	101.00 (37.00-174.00)	318	92.50 (28.00-156.00)	0.104
White blood cell (10^9/L)	31	4.50 (1.30-22.70)	318	3.40 (0.70-20.80)	*0.001*
Platelet (10^9/L)	31	86.00 (37.00-377.00)	318	73.00 (19.00-470.00)	0.169
Total bilirubin (μmol/L)	31	21.10 (5.70-132.70)	318	22.00 (5.20-281.10)	0.775
Direct bilirubin (μmol/L)	31	8.90 (2.00-78.20)	318	9.45 (2.00-210.40)	0.920
Indirect bilirubin (μmol/L)	31	11.90 (3.60-76.00)	318	11.30 (3.20-93.80)	0.677
Albumin (g/L)	31	32.50 (22.10-44.50)	316	32.35 (14.20-50.60)	0.482
Alanine aminotransferase (U/L)	31	20.06 (7.74-176.68)	318	24.34 (4.23-613.24)	0.396
Aspartate aminotransferase (U/L)	31	30.73 (9.74-143.00)	318	34.33 (9.63-761.63)	0.824
Alkaline phosphatase (U/L)	31	88.23 (28.83-337.00)	318	94.40 (31.00-983.93)	0.641
γ-Glutamyl transpeptidase (U/L)	31	70.00 (10.00-1779.18)	318	42.64 (7.54-1283.02)	0.121
Blood urea nitrogen (mmol/L)	31	5.85 (3.52-47.25)	314	5.31 (0.64-24.80)	0.226
Serum creatinine (μmol/L)	31	70.90 (42.82-267.63)	314	64.07 (23.83-178.55)	0.228
Potassium (mmol/L)	31	4.01 (2.80-5.41)	317	3.86 (2.42-5.28)	0.066
Sodium (mmol/L)	31	138.00 (134.10-145.50)	317	138.90 (118.00-152.90)	0.574
Homocysteine (μmol/L)	16	10.49 (6.70-31.79)	170	9.16 (1.59-102.81)	0.145
Total cholesterol (mmol/L)	20	3.68 (1.82-6.58)	208	3.11 (1.14-6.29)	0.148
Triglyceride (mmol/L)	20	0.83 (0.41-6.22)	208	0.85 (0.35-4.81)	0.661
High-density lipoprotein (mmol/L)	20	0.93 (0.47-1.39)	208	0.94 (0.24-2.29)	0.766
Low-density lipoprotein (mmol/L)	20	2.12 (0.99-4.37)	208	1.65 (0.47-4.06)	*0.013*
Lipoprotein α (mg/L)	20	88.70 (17.90-466.40)	208	63.20 (3.60-911.40)	0.108
Prothrombin time (seconds)	31	15.30 (12.70-20.40)	314	15.70 (10.30-28.00)	0.188
International normalized ratio	31	1.23 (0.99-1.75)	314	1.27 (0.89-2.77)	0.164

Table 1: Continued.

Variables	No. pts	Stroke Median (range) or frequency (percentage)	No. pts	No stroke Median (range) or frequency (percentage)	*p* value
Activated partial thromboplastin time (seconds)	31	40.20 (19.80-53.80)	314	39.90 (23.10-71.30)	0.808
D-dimer (mg/L)	19	1.32 (0.16-10.56)	252	0.86 (0.10-46.17)	0.873
Antithrombin III (%)	11	65.30 (40.00-84.00)	118	63.00 (21.00-123.00)	0.933
Protein C activity (%)	6	57.30 (49.80-75.10)	54	59.65 (24.00-119.30)	0.721
Protein S activity (%)	6	61.55 (55.60-73.30)	54	63.50 (20.70-123.60)	0.873
Child-Pugh score	31	7.00 (5.00-10.00)	313	7.00 (5.00-13.00)	0.837
MELD score	31	10.46 (7.23-18.00)	312	10.41 (6.43-30.03)	0.736
Portal vein thrombosis	31	8 (25.8%)	318	92 (28.9%)	0.713
LPV		4 (12.9%)		25 (7.9%)	0.332
RPV		2 (6.5%)		31 (9.7%)	0.549
MPV		4 (12.9%)		52 (16.4%)	0.617
Confluence of SMV and SV		5 (16.1%)		35 (11.0%)	0.393
SMV		2 (6.5%)		51 (16.0%)	0.156
SV		0 (0.0%)		13 (4.1%)	0.251

No. pts: number of patients; MELD: model for the end-stage liver diseases; LPV: left portal vein; RPV: right portal vein; MPV: main portal vein; SMV: superior mesenteric vein; SV: splenic vein.

approximately 2-4 times higher risk of ischemic stroke than those with normal glucose levels [9]. Meanwhile, diabetes is also an independent risk factor of portal vein thrombosis [10]. Fourth, antiphospholipid syndrome may increase the risk of ischemic stroke as well as portal vein thrombosis. Irregular thickening of the valve leaflets secondary to antiphospholipid syndrome and lupus anticoagulants are significant risk factor of stroke [11]. Meanwhile, anticardiolipin antibodies levels were higher in liver cirrhosis with portal vein thrombosis than that without portal vein thrombosis [12, 13]. Fifth, Helicobacter pylori not only acts as a promoter of antiphospholipid syndrome through chronic inflammation [14] but also stimulates the production of plasminogen activator inhibitor-2, which has an effect on increasing the risk of ischemic stroke and portal vein thrombosis [15]. Sixth, methylenetetrahydrofolate reductase (MTHFR) mutation is a common risk factor of stroke and portal vein thrombosis, which relates to endothelial damage [16]. MTHFR activity is reduced in the patients with MTHFR mutations, thereby leading to the deficiency of folate and hyperhomocysteinemia. MTHFR is responsible for catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folate cycle, which further produces the active form of folate for the remethylation of homocysteine to methionine [17]. There is a significant association between hyperhomocysteinemia and stroke [18]. An elevated homocysteine concentration significantly increases the risk of stroke [19]. Folic acid supplementation is effective in stroke prevention [20]. And MTHFR A1298C mutation is associated with increased risk of ischemic stroke [21]. On the other hand, MTHFR C677T mutation may increase the risk of portal vein thrombosis in cirrhotic patients [22]. The prevalence of hyperhomocysteinemia is significantly higher in cirrhotic patients with portal vein thrombosis than those without portal vein thrombosis [22]. Taken together, we hypothesized that cirrhotic patients with stroke might have an increased risk of portal vein thrombosis. Herein, this retrospective study was aimed at elucidating this issue.

2. Methods

2.1. Study Design. The study population was selected from our prospectively established database of cirrhotic patients without malignancy of the Department of Gastroenterology of our hospital from December 2014 to July 2019. All included patients must undergo abdominal enhanced computed tomography or magnetic resonance and endoscopy at their first enrollment. Age, sex, and the etiologies of liver cirrhosis were not limited. Repeated admissions of the same patients were excluded. Patients with abdominal surgery, including splenectomy, and splenic arterial embolization were excluded. Patients in whom a history of stroke cannot be accurately evaluated were excluded. Patients in whom the location of portal vein thrombosis cannot be evaluated due to missing images were also excluded. The study protocol was approved by the Medical Ethics Committee of our hospital. The ethical approval number was k(2019)39. The patient's informed consent was not required in our retrospective study.

2.2. Medical Data. The data were collected as follows.

(1) Demographic information: age and sex

(2) Systolic and diastolic blood pressure at admission

(3) Etiologies of liver diseases: hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, drug related

Table 2: Comparison between patients with ischemic stroke and those without stroke.

Variables	No. pts	Ischemic stroke Median (range) or frequency (percentage)	No. pts	No stroke Median (range) or frequency (percentage)	p value
Age (years)	29	61.08 (38.72-77.30)	318	54.21 (20.57-88.73)	*0.002*
Gender (male)	29	23 (79.3%)	318	230 (72.3%)	0.418
Systolic blood pressure (mmHg)	29	124.00 (90.00-173.00)	317	122.00 (83.00-193.00)	0.975
Diastolic blood pressure (mmHg)	29	73.00 (50.00-108.00)	317	75.00 (34.00-118.00)	0.700
Etiology of liver diseases					
Hepatitis B virus infection	29	10 (34.5%)	318	125 (39.3%)	0.610
Hepatitis C virus infection	29	1 (3.4%)	318	20 (6.3%)	0.539
Alcohol abuse	29	19 (65.5%)	318	139 (43.7%)	*0.024*
Drug related	29	4 (13.8%)	318	21 (6.6%)	0.152
Budd-Chiari syndrome	29	0 (0.0%)	318	1 (0.3%)	0.762
Autoimmune liver diseases	29	0 (0.0%)	318	22 (6.9%)	0.143
Clinical presentations at admission					
Hepatic encephalopathy	29	1 (3.4%)	318	8 (2.5%)	0.762
Acute gastrointestinal bleeding	29	8 (27.6%)	318	101 (31.8%)	0.643
Ascites (no/mild/moderate-severe)	29	15 (51.7%)/7 (24.1%)/7 (24.1%)	318	127 (39.9%)/108 (34.0%)/83 (26.1%)	0.424
History					
History of venous thrombus	29	0 (0.0%)	318	7 (2.2%)	0.420
History of hematological diseases	29	1 (3.4%)	318	5 (1.6%)	0.458
History of diabetes mellitus	29	8 (27.6%)	318	51 (16.0%)	0.113
History of arterial hypertension	29	11 (37.9%)	318	41 (12.9%)	*<0.001*
History of smoking	29	20 (69.0%)	318	135 (42.5%)	*0.006*
History of cardiac diseases	29	5 (17.2%)	318	23 (7.2%)	0.058
Laboratory tests					
Red blood cell (10^{12}/L)	29	3.33 (1.15-5.20)	318	3.27 (1.45-5.46)	0.471
Hemoglobin (g/L)	29	101.00 (37.00-174.00)	318	92.50 (28.00-156.00)	0.126
White blood cell (10^9/L)	29	4.90 (1.30-22.70)	318	3.40 (0.70-20.80)	*<0.001*
Platelet (10^9/L)	29	90.00 (37.00-377.00)	318	73.00 (19.00-470.00)	0.068
Total bilirubin (μmol/L)	29	25.10 (5.70-132.70)	318	22.00 (5.20-281.10)	0.606
Direct bilirubin (μmol/L)	29	9.00 (2.00-78.20)	318	9.45 (2.00-210.40)	0.892
Indirect bilirubin (μmol/L)	29	12.40 (3.60-76.00)	318	11.30 (3.20-93.80)	0.576
Albumin (g/L)	29	31.10 (22.10-44.50)	316	32.35 (14.20-50.60)	0.401
Alanine aminotransferase (U/L)	29	21.33 (7.74-176.68)	318	24.34 (4.23-613.24)	0.643
Aspartate aminotransferase (U/L)	29	31.44 (9.74-143.00)	318	34.33 (9.63-761.63)	0.902
Alkaline phosphatase (U/L)	29	92.49 (28.83-337.00)	318	94.40 (31.00-983.93)	0.921
γ-Glutamyl transpeptidase (U/L)	29	70.00 (10.00-1779.18)	318	42.64 (7.54-1283.02)	0.073
Blood urea nitrogen (mmol/L)	29	5.82 (3.52-47.25)	314	5.31 (0.64-24.80)	0.271
Serum creatinine (μmol/L)	29	70.90 (42.82-267.63)	314	64.07 (23.83-178.55)	0.253
Potassium (mmol/L)	29	4.05 (2.80-5.41)	317	3.86 (2.42-5.28)	*0.047*
Sodium (mmol/L)	29	137.90 (134.10-145.50)	317	138.90 (118.00-152.90)	0.430
Homocysteine (μmol/L)	15	10.47 (6.70-31.79)	170	9.16 (1.59-102.81)	0.179
Total cholesterol (mmol/L)	19	3.77 (1.82-6.58)	208	3.11 (1.14-6.29)	0.104
Triglyceride (mmol/L)	19	0.86 (0.47-6.22)	208	0.85 (0.35-4.81)	0.414
High-density lipoprotein (mmol/L)	19	0.92 (0.47-1.39)	208	0.94 (0.24-2.29)	0.762
Low-density lipoprotein (mmol/L)	19	2.12 (0.99-4.37)	208	1.65 (0.47-4.06)	*0.008*
Lipoprotein α (mg/L)	19	91.70 (17.90-466.40)	208	63.20 (3.60-911.40)	0.114
Prothrombin time (seconds)	29	15.30 (12.70-20.40)	314	15.70 (10.30-28.00)	0.198
International normalized ratio	29	1.23 (0.99-1.75)	314	1.27 (0.89-2.77)	0.170

Table 2: Continued.

Variables	No. pts	Ischemic stroke Median (range) or frequency (percentage)	No. pts	No stroke Median (range) or frequency (percentage)	p value
Activated partial thromboplastin time (seconds)	29	40.00 (19.80-53.80)	314	39.90 (23.10-71.30)	0.952
D-dimer (mg/L)	19	1.32 (0.16-10.56)	252	0.86 (0.10-46.17)	0.873
Antithrombin III (%)	11	65.30 (40.00-84.00)	118	63.00 (21.00-123.00)	0.933
Protein C activity (%)	6	57.30 (49.80-75.10)	54	59.65 (24.00-119.30)	0.721
Protein S activity (%)	6	61.55 (55.60-73.30)	54	63.50 (20.70-123.60)	0.873
Child-Pugh score	29	7.00 (5.00-10.00)	313	7.00 (5.00-13.00)	0.749
MELD score	29	10.46 (7.23-18.00)	312	10.41 (6.43-30.03)	0.892
Portal vein thrombosis	29	7 (24.1%)	318	92 (28.9%)	0.584
LPV		3 (10.3%)		25 (7.9%)	0.638
RPV		2 (6.9%)		31 (9.7%)	0.616
MPV		4 (13.8%)		52 (16.4%)	0.720
Confluence of SMV and SV		5 (17.2%)		35 (11.0%)	0.314
SMV		2 (6.9%)		51 (16.0%)	0.190
SV		0 (0.0%)		13 (4.1%)	0.267

No. pts: number of patients; MELD: model for the end-stage liver diseases; LPV: left portal vein; RPV: right portal vein; MPV: main portal vein; SMV: superior mesenteric vein; SV: splenic vein.

liver diseases, Budd-Chiari syndrome, and autoimmune liver diseases, etc.

(4) Clinical presentations at admission: hepatic encephalopathy, acute gastrointestinal bleeding, and ascites

(5) Medical history: venous thrombus, hematological diseases, diabetes mellitus, arterial hypertension, smoking, and cardiac diseases

(6) Laboratory tests: red blood cell, hemoglobin, white blood cell, platelet, total bilirubin, direct bilirubin, indirect bilirubin, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, blood urea nitrogen, serum, creatinine, potassium, sodium homocysteine, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, lipoprotein α, prothrombin time, international normalized ratio (INR), activated partial thromboplastin time, D-dimer, antithrombin III, PC activity, and PS activity

(7) Child-Pugh and model for the end-stage liver diseases (MELD) score

2.3. Definitions. Stroke was defined as previous history of stroke as well as a diagnosis of stroke at their enrollment. Type of stroke was divided into ischemic and hemorrhagic stroke according to previous medical history, clinical manifestations, and/or the results of brain imaging examination. Location of portal vein thrombosis was determined by our study group via the images of enhanced computed tomography or magnetic resonance. Portal system vessels include left portal vein (LPV), right portal vein (RPV), main portal vein (MPV), confluence of superior mesenteric vein (SMV) and splenic vein (SV), SMV, and SV.

2.4. Statistical Analyses. Continuous and categorical variables were expressed by median (range) and frequency (percentage), respectively. The difference between patients with and without stroke was compared using Mann-Whitney U test and χ^2 test, as appropriate. A 1 : 1 propensity score matching (PSM) analysis was performed to adjust the effect of age, sex, Child-Pugh score, and MELD score on our statistical results. If a p value was less than 0.05, it would be considered statistically significant. SPSS statistical software, version 22.0 (IBM Corp, Armonk, NY, USA), was employed to perform statistical analyses.

3. Results

3.1. Patients' Characteristics. Overall, 349 cirrhotic patients were included, of whom 31 (8.88%) had stroke and 318 did not have stroke. Among the 31 patients with stroke, 29 (8.31%), 4 (1.15%), and 2 (0.57%) patients had ischemic stroke, hemorrhagic stroke, and hemorrhagic combined with ischemic stroke, respectively. The prevalence of portal vein thrombosis was 28.65% (100/349). The prevalence of thrombosis within LPV, RPV, MPV, confluence of SMV and SV, SMV, and SV was 8.31% (29/349), 9.46% (33/349), 16.05% (56/349), 11.46% (40/349), 15.19% (53/349), and 3.72% (13/349), respectively.

3.2. Comparison between Liver Cirrhosis with and without Stroke. Compared with the patients without stroke, those with stroke were significantly older (60.21 versus 54.21 years, $p = 0.003$) and had significantly higher proportions of alcohol abuse (64.5% versus 43.7%, $p = 0.026$), smoking (67.7% versus 42.5%, $p = 0.007$), and arterial hypertension (38.7% versus 12.9%, $p < 0.001$) and higher levels of white blood cell (4.50 versus 3.40×10^9/L, $p = 0.001$) and low-density

Table 3: Characteristics of cirrhotic patients with ischemic stroke and those without stroke after propensity score matching.

Variables	Overall ($n = 56$)	Ischemic stroke ($n = 28$)	No stroke ($n = 28$)	p value
Age (years)	59.68 (35.18-77.30)	60.65 (38.72-77.30)	58.44 (35.18-75.72)	0.258
Gender (male)	47 (83.9%)	22 (78.6%)	25 (89.3%)	0.275
Systolic blood pressure (mmHg)	125.00 (83.00-173.00)	124.50 (90.00-173.00)	126.50 (83.00-158.00)	0.491
Diastolic blood pressure (mmHg)	75.50 (44.00-108.00)	72.00 (50.00-108.00)	81.00 (44.00-107.00)	0.161
Etiology of liver diseases				
Hepatitis B virus infection	23 (41.1%)	10 (35.7%)	13 (46.4%)	0.415
Hepatitis C virus infection	1 (1.8%)	1 (3.6%)	0 (0.0%)	0.313
Alcohol abuse	35 (62.5%)	18 (64.3%)	17 (60.7%)	0.783
Drug related	6 (10.7%)	4 (14.3%)	2 (7.1%)	0.388
Budd-Chiari syndrome	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA
Autoimmune liver diseases	1 (1.8%)	0 (0.0%)	1 (3.6%)	0.313
Clinical presentations at admission				
Hepatic encephalopathy	2 (3.6%)	1 (3.6%)	1 (3.6%)	1.000
Acute gastrointestinal bleeding	13 (23.2%)	7 (25.0%)	6 (21.4%)	0.752
Ascites (no/mild/moderate-severe)	27 (48.2%)/14 (25.0%)/15 (26.8%)	15 (53.6%)/6 (21.4%)/7 (25.0%)	12 (42.9%)/8 (28.6%)/8 (28.6%)	0.710
History				
History of venous thrombus	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA
History of hematological diseases	1 (1.8%)	1 (3.6%)	0 (0.0%)	0.313
History of diabetes mellitus	12 (21.4%)	8 (28.6%)	4 (14.3%)	0.193
History of arterial hypertension	13 (23.2%)	10 (35.7%)	3 (10.7%)	0.027
History of smoking	39 (69.6%)	20 (71.4%)	19 (67.9%)	0.771
History of cardiac diseases	8 (14.3%)	5 (17.9%)	3 (10.7%)	0.445
Laboratory tests				
Red blood cell (10^{12}/L)	3.52 (1.15-5.20)	3.34 (1.15-5.20)	3.69 (1.74-5.08)	0.456
Hemoglobin (g/L)	109.00 (37.00-174.00)	102.00 (37.00-174.00)	110.50 (58.00-156.00)	0.812
White blood cell (10^9/L)	4.25 (1.30-22.70)	4.70 (1.30-22.70)	4.05 (1.30-20.80)	0.063
Platelet (10^9/L)	84.50 (37.00-423.00)	88.00 (37.00-377.00)	75.00 (37.00-423.00)	0.961
Total bilirubin (μmol/L)	24.25 (5.70-132.70)	25.30 (5.70-132.70)	24.15 (6.60-47.00)	0.718
Direct bilirubin (μmol/L)	9.55 (2.00-78.20)	9.30 (2.00-78.20)	9.60 (2.50-27.00)	0.961
Indirect bilirubin (μmol/L)	12.45 (3.60-76.00)	12.60 (3.60-76.00)	12.35 (4.10-27.70)	0.967
Albumin (g/L)	32.55 (22.10-46.20)	30.75 (22.10-44.50)	33.95 (24.50-46.20)	0.201
Alanine aminotransferase (U/L)	24.79 (7.53-176.68)	21.66 (7.74-176.68)	25.59 (7.53-140.00)	0.793
Aspartate aminotransferase (U/L)	33.94 (9.74-166.49)	34.22 (9.74-143.00)	33.94 (13.94-166.49)	0.857
Alkaline phosphatase (U/L)	98.86 (28.83-337.00)	93.60 (28.83-337.00)	102.83 (52.28-337.00)	0.481
γ-Glutamyl transpeptidase (U/L)	60.87 (10.00-1779.18)	69.50 (10.00-1779.18)	56.00 (10.93-552.26)	0.611
Blood urea nitrogen (mmol/L)	5.62 (2.96-47.25)	5.81 (3.52-47.25)	5.38 (2.96-20.15)	0.502
Serum creatinine (μmol/L)	66.64 (42.82-267.63)	68.96 (42.82-267.63)	64.19 (44.50-117.53)	0.870
Potassium (mmol/L)	3.99 (2.74-5.41)	4.03 (2.80-5.41)	3.89 (2.74-4.75)	0.517
Sodium (mmol/L)	138.50 (133.50-145.50)	137.95 (134.10-145.50)	139.65 (133.50-145.20)	0.210
Prothrombin time (seconds)	15.30 (12.70-20.40)	15.30 (12.70-20.40)	15.25 (12.00-18.90)	0.774
International normalized ratio	1.24 (0.93-1.75)	1.24 (0.99-1.75)	1.24 (0.93-1.64)	0.883
Activated partial thromboplastin time (seconds)	40.10 (19.80-53.80)	40.10 (19.80-53.80)	40.30 (27.50-50.80)	0.961
Child-Pugh score	7.00 (5.00-10.00)	7.00 (5.00-10.00)	7.00 (5.00-13.00)	0.259
MELD score	10.43 (6.43-18.00)	10.52 (7.23-18.00)	10.32 (6.43-15.58)	0.676
Portal vein thrombosis	14 (25.0%)	7 (25.0%)	7 (25.0%)	1.000

MELD: model for the end-stage liver diseases.

lipoprotein (2.12 versus 1.65 mmol/L, $p = 0.013$) (Table 1). There was no difference in the prevalence of portal vein thrombosis and location of portal vein thrombosis between patients with and without stroke.

3.3. Comparison between Cirrhotic Patients with Ischemic Stroke and Those without Stroke. Compared with the patients without stroke, those with ischemic stroke were significantly older (61.08 versus 54.21 years, $p = 0.002$) and had significantly higher proportions of alcohol abuse (65.5% versus 43.7%, $p = 0.024$), smoking (69.0% versus 42.5%, $p = 0.006$), and arterial hypertension (37.9% versus 12.9%, $p < 0.001$) and higher levels of white blood cell (4.90 versus 3.40×10^9/L, $p < 0.001$), potassium (4.05 versus 3.86 mmol/L, $p = 0.047$), and low-density lipoprotein (2.12 versus 1.65 mmol/L, $p = 0.008$) (Table 2). There was no significant difference in the prevalence of portal vein thrombosis and location of portal vein thrombosis between patients with and without stroke.

3.4. PSM Analysis between Cirrhotic Patients with Ischemic Stroke and Those without Stroke. Twenty-eight patients were matched in each group after a 1 : 1 PSM analysis (Table 3). There was no significant difference in demographics, etiology of liver disease, laboratory tests, clinical presentations, Child-Pugh score, and MELD score between the two groups. Notably, we still did not find any significant association between ischemic stroke and portal vein thrombosis.

4. Discussion

Recently, a large population-based cohort study using Danish National Patient Registry demonstrated that splanchnic vein thrombosis significantly increased the risk of bleeding and arterial cardiovascular events as compared to patients with venous thromboembolism and general population [23]. In this study, splanchnic vein thrombosis referred to venous thrombosis within the portal, hepatic, mesenteric, and splenic veins; and arterial cardiovascular events included unstable angina pectoris, acute myocardial infarction, and ischemic stroke. They included 1,915 patients with splanchnic vein thrombosis, of whom 1,711 patients had portal vein thrombosis. They found that patients with splanchnic vein thrombosis had higher risk of bleeding and arterial cardiovascular events up to 1 year after diagnosis. In contrast to these findings, we did not find any significant association between stroke and portal vein thrombosis in liver cirrhosis, even in the PSM analysis. There were several potential reasons for this unexpected phenomenon. First, HCV infection, which should be regarded as a possible causative factor in the antiphospholipid syndrome with an increased prevalence of anticardiolipin antibodies [24] and an aggravated risk of hypercoagulability, is more prevalent in Western countries; by comparison, in our Chinese patients, HBV infection is the major etiology of liver cirrhosis, and the prevalence of HCV infection was only 6.02% (21/349) in our patients, which might lead to a low probability of antiphospholipid syndrome. Second, because Helicobacter pylori is an indirect risk factor of hypercoagulation and is not routinely detected in liver cirrhosis, our study did not have such data and could not compare the difference in the prevalence of Helicobacter pylori infection between patients with and without stroke. Third, our study demonstrated that the level of homocysteine was higher in patients with stroke or ischemic stroke than in patients without stroke, but the difference was not significant. This finding should be validated by expanding the number of patients who underwent laboratory tests of homocysteine levels.

Our study had several limitations. First, the number of our study population, especially patients with ischemic stroke, might not be adequate. Among the patients admitted to our Department of Gastroenterology, most of stroke events are usually asymptomatic. Therefore, in our retrospective observational study, the imaging examination of the brain has not been performed in every cirrhotic patient to evaluate the risk of asymptomatic stroke. Indeed, the imaging examination of the brain in every cirrhotic patient might not be approved by any ethical committee. Herein, the history of asymptomatic stroke, such as lacunar infarction, might be missed. Second, in the present study, we selected study population from a prospectively established database, in which only the patients undergoing enhanced computed tomography or magnetic resonance are included. Although enhanced computed tomography or magnetic resonance can evaluate more precisely the existence, extent, and degree of portal vein thrombosis, there was a potential bias in patient selection in this setting. Third, hypercoagulability is not evaluated in all patients. Fourth, Helicobacter pylori infection, which may be associated with both portal vein thrombosis and stroke, has not been studied in our study.

In conclusion, our study could not establish any association between stroke and portal vein thrombosis in liver cirrhosis. Certainly, well-designed large-scale prospective cohort studies will be necessary to confirm this finding.

Abbreviations

FVIII: Factor VIII
PC: Protein C
MTHFR: Methylenetetrahydrofolate reductase
HBV: Hepatitis B virus
HCV: Hepatitis C virus
INR: International normalized ratio
MELD: Model for the end-stage liver diseases
LPV: Left portal vein
RPV: Right portal vein
MPV: Main portal vein
SMV: Superior mesenteric vein
SV: Splenic vein
PSM: Propensity score matching.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure

The abstract was published in Asian-Pacific Association for the Study of the Liver (APASL) 2020 Conference. Please see the following link: https://link.springer.com/content/pdf/10.1007/s12072-020-10030-4.pdf.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Kexin Zheng, Xiaozhong Guo, and Xingshun Qi were responsible for conceptualization. Kexin Zheng, Fangfang Yi, Le Wang, and Xingshun Qi were responsible for data curation. Kexin Zheng and Xingshun Qi were responsible for the formal analysis, investigation, methodology, software, and writing of the original draft. Xiaozhong Guo and Xingshun Qi were responsible for the funding acquisition, project administration, and supervision. Xingshun Qi provided resources and was responsible for the validation and visualization. Kexin Zheng, Xiaozhong Guo, Fangfang Yi, Le Wang, Andrea Mancuso, and Xingshun Qi were responsible for the writing, reviewing and editing.

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Research Article

No Benefit of Hemostatic Drugs on Acute Upper Gastrointestinal Bleeding in Cirrhosis

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Background and Aims. Acute upper gastrointestinal bleeding (AUGIB) is one of the most life-threatening emergency conditions. Hemostatic drugs are often prescribed to control AUGIB in clinical practice but have not been recommended by major guidelines and consensus. The aim of this study was to investigate the therapeutic effect of hemostatic drugs on AUGIB in cirrhosis. *Methods*. All cirrhotic patients with AUGIB who were admitted to our hospital from January 2010 to June 2014 were retrospectively included. Patients were divided into hemostatic drugs and no hemostatic drug groups. A 1 : 1 propensity score matching (PSM) analysis was performed by adjusting age, gender, etiology of liver disease, Child-Pugh score, MELD score, hematemesis, red blood cell transfusion, vasoactive drugs, antibiotics, proton pump inhibitors, and endoscopic variceal therapy. Primary outcomes included 5-day rebleeding and in-hospital mortality. *Results*. Overall, 982 cirrhotic patients with AUGIB were included (870 in hemostatic drugs group and 112 in no hemostatic drug group). In overall analyses, hemostatic drugs group had a significantly higher 5-day rebleeding rate (18.10% versus 5.40%, $P = 0.001$) than no hemostatic drug group; in-hospital mortality was not significantly different between them (7.10% versus 4.50%, $P = 0.293$). In PSM analyses, 172 patients were included (86 patients in each group). Hemostatic drugs group still had a significantly higher 5-day rebleeding rate (15.10% versus 5.80%, $P = 0.046$); in-hospital mortality remained not significantly different (7.00% versus 3.50%, $P = 0.304$) between them. Statistical results remained in PSM analyses according to the type of hemostatic drugs. *Conclusions*. The use of hemostatic drugs did not improve the in-hospital outcomes of cirrhotic patients with AUGIB.

1. Introduction

Acute upper gastrointestinal bleeding (AUGIB) is a life-threatening and frequent complication in cirrhosis with its mortality approaching 5-20% [1–4]. About 70% of AUGIB episodes in cirrhosis are due to esophageal variceal rupture secondary to portal hypertension [5]. The primary goals of therapy of AUGIB in liver cirrhosis are initial control of bleeding and prevention of early rebleeding [1, 5, 6]. According to the current guidelines, the mainstay pharmacological management of AUGIB should be the use of vasoactive drugs (terlipressin and somatostatin or its analogues), which can reduce portal blood flow and portal pressure [6–8].

Traditionally, it has been considered that variceal rupture bleeding is potentially more dangerous in cirrhosis due to the underlying coagulation abnormalities [9, 10]. In clinical practice, though not recommended, treating physicians arbitrarily prescribe hemostatic drugs, which act on vasculature or coagulation cascade, as adjuvants for control of bleeding [11]. However, the therapeutic effect of hemostatic drugs on AUGIB remains uncertain. The results of a recent meta-analysis showed that antifibrinolytic agents were deleterious in patients with acute or chronic liver disease and AUGIB [12]. Herein, we conducted a retrospective study to investigate the effect of hemostatic drugs on AUGIB in patients with liver cirrhosis.

2. Methods

The study was approved by the Medical Ethical Committee of the General Hospital of Northern Theater Command with an approval number [number K (2019)32] and was performed according to the Declaration of Helsinki.

2.1. Study Design. In this retrospective study, a total of 1026 cirrhotic patients with AUGIB who were consecutively admitted to the General Hospital of Northern Theater Command from January 2010 to June 2014 were screened. The inclusion criteria were as follows: (1) a diagnosis of liver cirrhosis, and (2) a diagnosis of AUGIB presenting with hematemesis and/or melena at admission. The exclusion criteria were as follows: (1) no episodes of gastrointestinal bleeding within 5 days before admission, and (2) only positive occult blood test. Age, sex, source of gastrointestinal bleeding, cause of liver disease, and malignancy were not limited. Repeated admission was not excluded. Finally, 982 patients were included in our study.

The following data was extracted from our retrospective database: demographic data (i.e., age and gender), etiology of liver disease, presence of hematemesis and/or melena at admission, and laboratory tests (i.e., red blood cell, hemoglobin, white blood cell, platelet count, total bilirubin, direct bilirubin, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, blood urea nitrogen, creatinine, potassium, sodium, prothrombin time, activated partial thromboplastin time, and international normalized ratio [INR]). The Child-Pugh score and model for end-stage liver disease (MELD) score were calculated. The use of red blood cell transfusion and antibiotics as well as the use of vasoactive drugs (i.e., somatostatin and/or octreotide) and proton pump inhibitors (PPIs) were recorded. The grade of esophageal varices was evaluated [13]. The use of endoscopic variceal therapy, Sengstaken-Blakemore tube, and splenectomy with or without devascularization were also recorded.

Hemostatic drugs employed in our study included drugs acting on vascular wall or platelet (i.e., norepinephrine, carbazochrome sodium sulfonate, and Yunnan Baiyao), antifibrinolytic drugs (i.e., ethylenediamine diacetoacetic), thrombin (i.e., lyophilizing thrombin powder), hemocoagulase (i.e., snake venom hemocoagulase), and procoagulant drugs (i.e., vitamin K). Modes of administration included intravenous, oral, and topical administration. The mechanisms and indications of various hemostatic drugs are shown in Supplementary Table 1.

According to the use of these hemostatic drugs during hospitalization, we divided the patients into hemostatic drugs and no hemostatic drug groups. The major outcomes included a 5-day rebleeding rate and in-hospital mortality. Five-day rebleeding was defined as the recurrence of hematemesis and fresh melena within 5 days after the initial bleeding episode was completely controlled [3].

2.2. Statistical Analyses. Continuous variables were expressed as mean ± standard deviation and median (range). Categorical variables were expressed as frequency (percentage). Nonparametric Mann-Whitney *U* test was used for continuous variables, and chi-square test was used for categorical variables to compare the differences between hemostatic drugs and no hemostatic drug groups. A 1 : 1 propensity score matching (PSM) analysis was used. Matching factors included age, gender, etiology of liver diseases, which mainly include hepatitis B, hepatitis C, alcohol abuse, drug abuse, and autoimmunity, Child-Pugh score, MELD score, hematemesis, red blood cell transfusion, vasoactive drugs, antibiotics, PPIs, and endoscopic variceal therapy. After exclusion of patients with malignancy and those who underwent surgery, subgroup analyses were conducted in patients with Child-Pugh class B and C, MELD score > 15 [14], and use of endoscopic variceal therapy and antibiotics. All statistical analyses were performed with IBM SPSS software version 20.0 (IBM Corp, Armonk, NY, USA) and Stata/SE 12.0 (Stata Corp, College Station, TX, USA) software. A histogram demonstrating the frequency of various hemostatic drugs used during the study period was drawn by the Excel version 10.0 (Microsoft Corp, Redmond Washington, USA).

3. Results

3.1. Overall Analyses. A total of 982 patients with cirrhosis and AUGIB were included in our study. Baseline characteristics of patients at admission were shown in Table 1. The median age was 56.01 years (range: 6.28-95.13 years), and most patients were male ($n = 688$, 70.1%). Hepatitis B virus ($n = 399$, 40.6%) was the most common etiology of cirrhosis. One hundred and eighty-nine patients (19.2%) had malignancy, including 160 patients with liver cancer and 29 patients with extrahepatic cancer (i.e., lung cancer, breast cancer, gastric cancer, and rectal cancer). Most patients were in Child-Pugh class B (476/982, 52.80%). The median MELD score was 6.60 (range: -7.52-40.95). Endoscopy was performed in 702 patients. Detailed information regarding the grade of esophageal varices on endoscopy was clearly available in 563 patients. Hemostatic drugs group had a higher proportion of hematemesis at admission, lower levels of red blood cell, platelet count, albumin, and alkaline phosphatase, and higher levels of blood urea nitrogen, potassium, prothrombin time, and INR than no hemostatic drug group.

Blood transfusion was given in 640 (65.20%) patients, of whom 611 (62.20%) received red blood cell transfusion with a median of 4 units (range: 1.00-33.00). Somatostatin and/or

Table 1: Overall analysis of the difference between hemostatic drugs and no hemostatic drugs group.

Variables	No. Pts	Overall	No. Pts	Hemostatic drug group	No. Pts	No hemostatic drug group	*P* value
Age (years)	982	56.01 (6.28-95.13) 56.45 ± 11.87	870	56.01 (6.28-95.13) 56.51 ± 11.76	112	55.45 (24.92-84.77) 56.04 ± 12.76	0.536
Sex (male) (%)	982	688 (70.10%)	870	608 (69.90%)	112	80 (71.40%)	0.737
Cancer (%)	982	189 (19.20%)	870	169 (19.40%)	112	20 (17.90%)	0.692
Liver cancer (%)	982	160 (16.30%)	870	142 (16.30%)	112	18 (16.10%)	0.946
Extrahepatic cancer (%)	982	29 (3.00%)	870	27 (3.10%)	112	2 (1.80%)	0.438
Clinical features of AUGIB (%)							
Hematemesis (%)	982	587 (59.80%)	870	541 (62.20%)	112	46 (41.10%)	<0.001
Melena (%)	982	770 (78.40%)	870	675 (77.60%)	112	95 (84.80%)	0.080
Hematemesis and melena (%)	982	375 (38.20%)	870	346 (39.80%)	112	29 (25.90%)	0.004
Etiology of liver diseases	982		870		112		0.305
HBV (%)	982	399 (40.60%)	870	359 (41.30%)	112	40 (35.70%)	0.260
HCV (%)	982	83 (8.50%)	870	75 (8.60%)	112	8 (7.10%)	0.597
Alcohol abuse (%)	982	354 (36.00%)	870	315 (36.20%)	112	39 (34.80%)	0.774
HBV+alcohol abuse (%)	982	106 (10.80%)	870	94 (10.80%)	112	12 (10.70%)	0.977
HCV+alcohol abuse (%)	982	18 (1.80%)	870	14 (1.60%)	112	4 (3.60%)	0.145
Other or unknown etiology (%)	982	186 (18.90%)	870	159 (18.30%)	112	27 (24.10%)	0.138
Endoscopic evaluation of EV (%)	982	563 (57.30%)	870	503 (57.80%)	112	60 (53.60%)	0.393
No EV (%)	982	34 (3.50%)	870	26 (3.00%)	112	8 (7.10%)	0.024
Mild EV (%)	982	27 (2.70%)	870	26 (3.00%)	112	1 (0.90%)	0.202
Moderate EV (%)	982	66 (6.70%)	870	60 (6.90%)	112	6 (5.40%)	0.540
Severe EV (%)	982	437 (44.50%)	870	392 (45.10%)	112	45 (40.20%)	0.328
Laboratory tests							
Red Blood Cell (10^{12}/L)	973	2.54 (0.79-5.94) 2.62 ± 0.70	864	2.52 (0.79-5.49) 2.59 ± 0.69	109	2.81 (1.21-5.08) 2.81 ± 0.71	0.002
Hemoglobin (g/L)	974	72.00 (19.00-180.00) 75.10 ± 22.58	865	72.00 (19.00-180.00) 74.75 ± 22.47	109	75.00 (31.00-170.00) 77.88 ± 23.38	0.165
White blood cell (10^9/L)	974	4.90 (0.80-46.10) 6.17 ± 4.75	865	4.90 (0.80-46.10) 6.19 ± 4.76	109	4.50 (1.10-30.70) 6.02 ± 4.69	0.658
Platelet (10^9/L)	974	76.00 (9.00-842.00) 95.96 ± 77.81	865	75.00 (9.00-775.00) 93.75 ± 74.28	109	84.00 (17.00-842.00) 113.51 ± 100.29	0.010
Total bilirubin (μmol/L)	964	20.70 (3.30-553.60) 31.34 ± 39.68	855	20.60 (3.30-553.60) 31.38 ± 40.46	109	22.60 (5.90-241.40) 31.04 ± 33.14	0.857
Albumin (g/L)	949	29.90 (9.60-49.30) 29.69 ± 6.75	841	29.60 (9.60-49.30) 29.43 ± 6.69	108	31.45 (13.60-48.40) 31.66 ± 6.94	0.002
Alanine aminotransferase (U/L)	962		853		109		0.764

Table 1: Continued.

Variables	No. Pts	Overall	No. Pts	Hemostatic drug group	No. Pts	No hemostatic drug group	*P* value
		24.00 (5.00-1064.00) 37.64 ± 61.52		24.00 (5.00-1064.00) 37.66 ± 62.94		24.00 (5.00-438.00) 37.40 ± 49.23	
Aspartate aminotransferase (U/L)	962	32.00 (7.00-1487.00) 58.93 ± 117.01	853	32.00 (7.00-1487.00) 58.87 ± 117.48	109	31.00 (13.00-994.00) 59.39 ± 113.76	0.906
Alkaline phosphatase (U/L)	962	74.90 (1.30-889.00) 99.86 ± 88.33	853	73.00 (1.30-889.00) 97.68 ± 85.57	109	84.00 (28.00-868.00) 116.92 ± 106.48	0.002
Gamma-glutamyl transpeptidase (U/L)	962	40.00 (5.00-1168.00) 87.40 ± 126.42	853	39.00 (5.00-1168.00) 83.96 ± 119.53	109	48.00 (10.00-994.00) 114.36 ± 169.35	0.126
Blood urea nitrogen (mmol/L)	932	7.81 (1.54-55.01) 9.12 ± 5.99	827	7.94 (1.54-49.19) 9.28 ± 5.85	105	6.19 (2.22-55.01) 7.92 ± 6.90	<0.001
Serum creatinine (μmol/L)	930	60.20 (20.00-1189.00) 72.69 ± 69.59	825	61.00 (20.00-1189.00) 72.43 ± 66.07	105	57.00 (28.00-919.00) 74.77 ± 93.11	0.154
Potassium (mmol/L)	952	4.07 (2.13-7.87) 4.11 ± 0.55	845	4.09 (2.13-7.87) 4.13 ± 0.57	107	4.00 (2.79-5.80) 4.00 ± 0.43	0.003
Sodium (mmol/L)	952	138.60 (83.00-160.80) 138.17 ± 5.14	845	138.70 (83.00-160.10) 138.22 ± 5.14	107	138.00 (118.70-160.80) 137.84 ± 5.21	0.146
Prothrombin time (seconds)	922	16.20 (10.80-62.80) 17.29 ± 4.82	816	16.30 (11.00-62.80) 17.44 ± 4.89	106	14.95 (10.80-40.90) 16.13 ± 4.17	<0.001
INR	920	1.31 (0.77-7.96) 1.45 ± 0.58	814	1.33 (0.79-7.96) 1.46 ± 0.59	106	1.18 (0.77-4.19) 1.32 ± 0.48	<0.001
Child-Pugh score	901	7.00 (5.00-15.00) 7.78 ± 2.00	798	7.00 (5.00-15.00) 7.81 ± 1.95	103	7.00 (5.00-14.00) 7.51 ± 2.30	0.187
Child-Pugh class A/B/C (%)	901	256 (28.40%)/476 (52.80%)/169 (18.80%)	798	215 (26.90%)/433 (54.30%)/150 (18.80%)	103	41 (39.80%)/43 (41.70%)/19 (18.40%)	0.018
MELD score	895	6.60 (-7.52-40.95) 7.59 ± 6.71	795	6.74 (-7.44-39.17) 7.76 ± 6.64	100	5.21 (-7.52-40.95) 6.24 ± 7.09	0.056
Vasoactive drugs (%)	982	892 (90.80%)	870	815 (93.70%)	112	77 (68.80%)	<0.001
Somatostatin (%)	982	814 (82.90%)	870	755 (86.80%)	112	59 (50.40%)	<0.001
Octreotide (%)	982	379 (38.60%)	870	332 (38.20%)	112	47 (42.00%)	0.436
Proton-pump inhibitors (%)	982	967 (98.50%)	870	864 (99.30%)	112	103 (92.00%)	<0.001
Red blood cell transfusion (%)	982	611 (62.20%)	870	561 (64.50%)	112	50 (44.60%)	<0.001
Antibiotics (%)	982	468 (47.70%)	870	416 (47.80%)	112	52 (46.40%)	0.782
5-day rebleeding (%)	981	163 (16.60%)	869	157 (18.10%)	112	6 (5.40%)	0.001
In-hospital death (%)	982	67 (6.80%)	870	62 (7.10%)	112	5 (4.50%)	0.293

Abbreviations: Pts: patients; HBV: hepatitis B virus; HCV: hepatitis C virus; AUGIB: acute upper gastrointestinal bleeding; INR: international normalized ratio; APTT: activated partial thromboplastin time; MELD: model for end-stage liver disease; EV: esophageal varices.

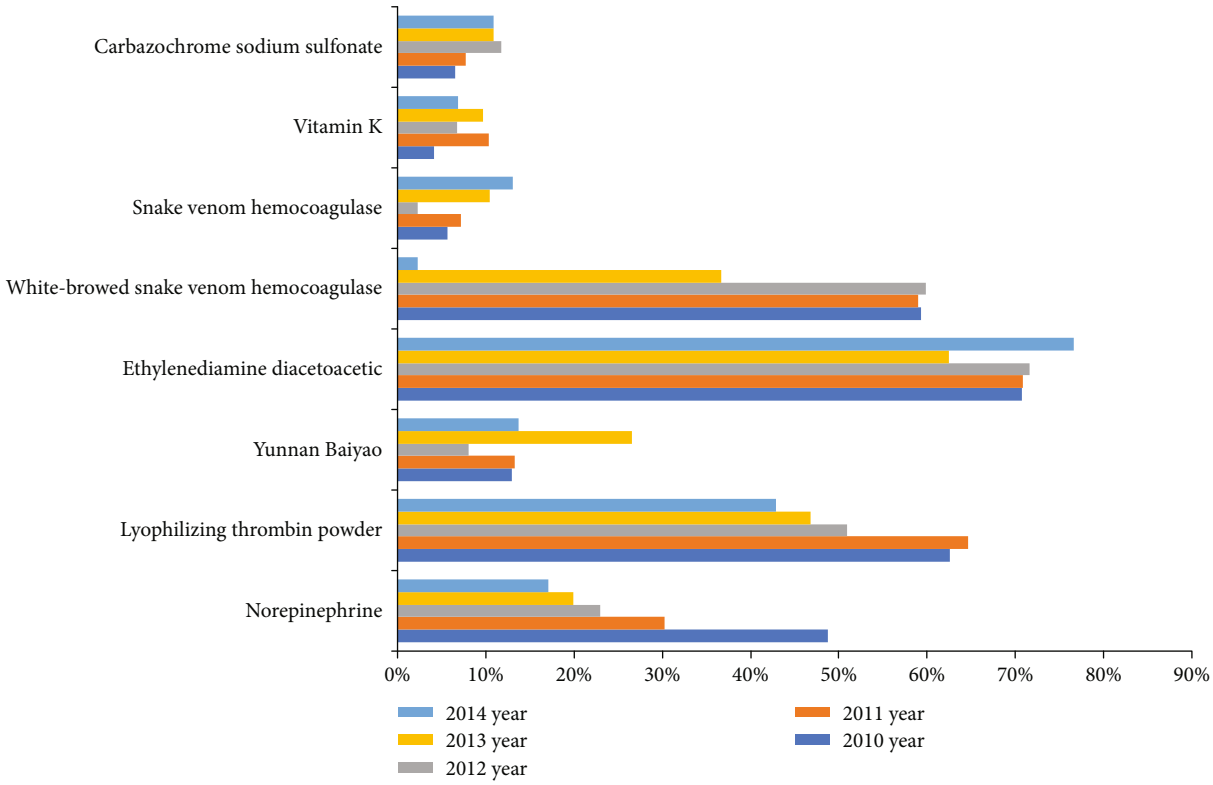

Figure 1: A trend in the frequency of hemostatic drugs used in our study.

octreotide were given in 892 (90.80%) patients. PPIs were given in 967 (98.50%) patients. Antibiotics were given in 468 (47.70%) patients. Endoscopic variceal therapy was performed in 574 (58.50%) patients. Sengstaken-Blakemore tube placement was given in 20 (1.90%) patients. Splenectomy with and without devascularization was performed in 9 (0.9%) patients. Hemostatic drugs group was more likely to receive blood transfusion, red blood cell transfusion, somatostatin and/or octreotide, and PPIs than no hemostatic drug group.

Among the hemostatic drugs, ethylenediamine diacetoacetic, white-browed snake venom hemocoagulase, and lyophilizing thrombin powder were common hemostatic drugs with a high utilization rate of up to 60%-70%. By contrast, carbazochrome sodium sulfonate, vitamin K, and snake venom hemocoagulase were uncommon hemostatic drugs with a relatively low utilization rate of about 10%. There was a trend in a lower utilization rate of norepinephrine, white-browed snake venom hemocoagulase, and lyophilizing thrombin powder over time. By contrast, there was a trend in a higher utilization rate of carbazochrome sodium sulfonate and snake venom hemocoagulase over time (Figure 1).

The 5-day rebleeding rate was 16.6% ($n = 163$), and in-hospital mortality was 6.8% ($n = 67$). Hemostatic drugs group had a significantly higher 5-day rebleeding rate than no hemostatic drug group (18.10% versus 5.40%, $P = 0.001$). In-hospital mortality was not significantly different between the two groups (7.10% versus 4.50%, $P = 0.293$). The causes of death included uncontrolled bleeding ($n = 40$), uncontrolled bleeding with hepatic encephalopathy ($n = 5$), end-stage liver disease ($n = 20$), and advanced hepatocellular carcinoma ($n = 2$).

3.2. PSM Analyses

3.2.1. PSM Analyses of Any Hemostatic Drug. A total of 172 patients were included in PSM analyses. In the hemostatic drugs group ($n = 86$), most patients ($n = 74$) started using hemostatic drugs from the day at admission until the bleeding stopped or death, and average duration of hemostatic drugs was 6.99 days (range: 1-41 days); rebleeding occurred in 13 patients during hospitalization, all of which developed after the use of hemostatic drugs. Compared with no hemostatic drug group, hemostatic drugs group had a significantly higher incidence of 5-day rebleeding (15.10% versus 5.80%, $P = 0.046$). In-hospital mortality was statistically similar between the two groups (7.00% versus 3.50%, $P = 0.304$) (Table 2).

3.2.2. PSM Analyses of Ethylenediamine Diacetoacetic. A total of 160 patients were included in PSM analyses. In the ethylenediamine diacetoacetic group ($n = 80$), rebleeding occurred in 10 patients during hospitalization, all of which developed after the use of ethylenediamine diacetoacetic. There was no significant difference in the incidence of 5-day rebleeding (13.30% versus 5.30%, $P = 0.092$) or in-hospital mortality (5.30% versus 4.00%, $P = 0.699$) between the two groups (Supplementary Table 2).

3.2.3. PSM Analyses of Lyophilizing Thrombin Powder. A total of 140 patients were included in PSM analyses. In the

Table 2: PSM analysis of difference between hemostatic drugs and no hemostatic drug groups.

Variables	No. Pts	Hemostatic drugs group	No. Pts	No hemostatic drug group	*P* value
Age (years)	86	55.60 (30.37-89.23) 57.00 ± 11.39	86	55.78 (24.92-84.77) 55.65 ± 11.73	0.504
Sex (male) (%)	86	60 (69.80%)	86	62 (72.10%)	0.737
Cancer (%)	86	10 (11.60%)	86	16 (18.60%)	0.201
Liver cancer (%)	86	8 (9.30%)	86	15 (17.40%)	0.117
Extrahepatic cancer (%)	86	2 (2.30%)	86	1 (1.20%)	0.560
Clinical features of AUGIB (%)					
Hematemesis (%)	86	33 (38.40%)	86	36 (41.90%)	0.641
Melena (%)	86	72 (83.70%)	86	74 (86.00%)	0.670
Both hematemesis and melena (%)	86	19 (22.10%)	86	24 (27.90%)	0.379
Etiology of liver diseases					
HBV (%)	86	22 (25.60%)	86	32 (37.20%)	0.100
HCV (%)	86	5 (5.80%)	86	7 (8.10%)	0.549
Alcohol abuse (%)	86	33 (38.40%)	86	34 (39.50%)	0.876
HBV+alcohol abuse (%)	86	6 (7.00%)	86	11 (12.80%)	0.201
HCV+alcohol abuse (%)	86	3 (3.50%)	86	4 (4.70%)	0.700
Other or unknown etiology (%)	86	35 (40.70%)	86	29 (33.70%)	0.344
Endoscopic evaluation of EV (%)	86	53 (61.60%)	86	49 (57.00%)	0.535
No EV (%)	86	5 (5.80%)	86	7 (8.10%)	0.549
Mild EV (%)	86	3 (3.50%)	86	1 (1.20%)	0.312
Moderate EV (%)	86	6 (7.00%)	86	5 (5.80%)	0.755
Severe EV (%)	86	39 (45.30%)	86	36 (41.90%)	0.646
Laboratory tests					
Red Blood Cell (10^{12}/L)	86	2.58 (0.93-5.07) 2.67 ± 0.73	86	2.68 (1.21-4.22) 2.71 ± 0.62	0.438
Hemoglobin (g/L)	86	73.00 (31.00-157.00) 78.64 ± 24.96	86	73.50 (42.00-122.00) 74.99 ± 19.85	0.608
White blood cell (10^9/L)	86	4.05 (1.00-25.20) 5.35 ± 4.29	86	4.20 (1.10-30.70) 5.70 ± 4.62	0.400
Platelet (10^9/L)	86	70.50 (9.00-775.00) 97.90 ± 96.51	86	82.00 (17.00-842.00) 111.50 ± 105.11	0.103
Total bilirubin (μmol/L)	86	20.10 (4.80-553.60) 35.42 ± 64.87	86	23.25 (5.90-241.40) 30.03 ± 30.64	0.968
Albumin (g/L)	85	30.90 (17.20-49.30) 30.60 ± 6.47	85	31.20 (13.60-48.00) 31.58 ± 7.12	0.337
Alanine aminotransferase (U/L)	86	26.00 (6.00-184.00) 32.23 ± 24.92	86	23.00 (5.00-438.00) 37.58 ± 53.46	0.548
Aspartate aminotransferase (U/L)	86	35.50 (8.00-228.00) 49.35 ± 42.70	86	30.50 (13.00-994.00) 60.43 ± 125.71	0.218
Alkaline phosphatase (U/L)	86	76.30 (36.00-707.00) 114.00 ± 102.19	86	92.50 (28.00-450.00) 104.13 ± 61.66	0.381
Gamma-glutamyl transpeptidase (U/L)	86	55.50 (8.00-1168.00) 131.82 ± 209.08	86	49.50 (10.00-994.00) 103.97 ± 154.16	0.454
Blood urea nitrogen (mmol/L)	86	7.37 (2.07-24.92) 8.54 ± 4.91	86	6.20 (2.22-55.01) 8.04 ± 7.18	0.177
Serum Creatinine (μmol/L)	86	55.00 (24.00-449.00) 65.36 ± 49.07	86	57.00 (28.00-919.00) 72.37 ± 97.01	0.565
Potassium (mmol/L)	85	4.07 (2.13-5.48) 4.08 ± 0.55	85	4.00 (2.79-5.80) 4.02 ± 0.45	0.430

Table 2: Continued.

Variables	No. Pts	Hemostatic drugs group	No. Pts	No hemostatic drug group	*P* value
Sodium (mmol/L)	85	139.40 (128.90-147.30) 139.08 ± 3.77	85	137.60 (122.60-146.50) 137.51 ± 4.47	0.023
Prothrombin time (seconds)	86	15.60 (12.90-47.00) 16.71 ± 4.44	86	15.25 (10.80-40.90) 16.37 ± 4.44	0.280
INR	86	1.25 (0.97-5.21) 1.38 ± 0.53	86	1.20 (0.77-4.19) 1.34 ± 0.51	0.219
Child-Pugh score	86	7.00 (5.00-14.00) 7.63 ± 2.05	86	7.00 (5.00-14.00) 7.52 ± 2.33	0.460
Child-Pugh class A/B/C (%)	86	29 (33.70%)/42 (48.80%)/15 (17.50%)	86	35 (40.70%)/35 (40.70%)/16 (18.60%)	0.585
MELD score	86	5.27 (-6.44-32.06) 6.28 ± 6.84	86	5.09 (-7.52-40.95) 6.17 ± 7.05	0.861
Endoscopic variceal treatment (%)	86	45 (52.30%)	86	38 (44.20%)	0.285
Vasoactive drugs (%)	86	67 (77.90%)	86	65 (75.60%)	0.718
Somatostatin (%)	86	60 (69.80%)	86	49 (57.70%)	0.082
Octreotide (%)	86	31 (36.00%)	86	40 (46.50%)	0.163
Proton-pump inhibitors (%)	86	83 (96.50%)	86	84 (97.70%)	0.650
Antibiotics (%)	86	41 (47.70%)	86	43 (50.00%)	0.760
Red blood cell transfusion (%)	86	47 (54.70%)	86	44 (51.20%)	0.647
5-day rebleeding (%)	86	13 (15.10%)	86	5 (5.80%)	0.046
In-hospital death (%)	86	6 (7.00%)	86	3 (3.50%)	0.304

Abbreviations: Pts: patients; HBV: hepatitis B virus; HCV: hepatitis C virus; AUGIB: acute upper gastrointestinal bleeding; INR: international normalized ratio; APTT: activated partial thromboplastin time; MELD: model for end-stage liver disease; EV: esophageal varices.

lyophilizing thrombin powder group ($n = 70$), rebleeding occurred in 10 patients during hospitalization, all of which developed after the use of lyophilizing thrombin powder. There was no significant difference in the incidence of 5-day rebleeding (14.30% versus 5.70%, $P = 0.091$) or in-hospital mortality (2.90% versus 2.90%, $P = 1.000$) between the two groups (Supplementary Table 3).

3.2.4. PSM Analyses of White-Browed Snake Venom Hemocoagulase. A total of 128 patients were included in PSM analyses. In the white-browed snake venom hemocoagulase group ($n = 64$), rebleeding occurred in 10 patients during hospitalization, all of which developed after the use of white-browed snake venom hemocoagulase. There was no significant difference in the incidence of 5-day rebleeding (12.50% versus 4.70%, $P = 0.115$) or in-hospital mortality (3.10% versus 3.10%, $P = 1.000$) between the two groups (Supplementary Table 4).

3.2.5. PSM Analyses of Snake Venom Hemocoagulase. A total of 62 patients were included in PSM analyses. In the snake venom hemocoagulase group ($n = 31$), rebleeding occurred in 6 patients during hospitalization, all of which developed after the use of snake venom hemocoagulase. There was no significant difference in the incidence of 5-day rebleeding (19.40% versus 9.70%, $P = 0.279$) or in-hospital mortality (9.70% versus 3.20%, $P = 0.301$) between the two groups (Supplementary Table 5).

3.2.6. PSM Analyses of Yunnan Baiyao. A total of 98 patients were included in PSM analyses. In the Yunnan Baiyao group ($n = 49$), rebleeding occurred in 13 patients during hospitalization, all of which developed after the use of Yunnan Baiyao. The incidence of 5-day rebleeding was significantly higher in the Yunnan Baiyao group (26.50% versus 4.10%, $P = 0.002$). There was no significant difference in the in-hospital mortality (12.20% versus 4.10%, $P = 0.140$) between the two groups (Supplementary Table 6).

3.2.7. PSM Analyses of Norepinephrine. A total of 96 patients were included in PSM analyses. In the norepinephrine group ($n = 48$), rebleeding occurred in 10 patients (6 patients were treated with norepinephrine during the endoscopic variceal therapy procedure and 4 patients were treated with norepinephrine orally) during hospitalization, all of which developed after the use of norepinephrine. The incidence of 5-day rebleeding was significantly higher in the norepinephrine group (20.80% versus 6.20%, $P = 0.037$). In-hospital mortality was significantly lower in the norepinephrine group (0.00% versus 8.30%, $P = 0.041$) (Supplementary Table 7).

3.2.8. PSM Analyses of Carbazochrome Sodium Sulfonate. A total of 62 patients were included in PSM analyses. In the carbazochrome sodium sulfonate group ($n = 31$), rebleeding occurred in 3 patients during hospitalization, all of which developed after the use of carbazochrome sodium sulfonate. There was no significant difference in the incidence of 5-day rebleeding (9.70% versus 3.20%, $P = 0.301$) or in-

hospital mortality (6.50% versus 0.00%, $P = 0.151$) between the two groups (Supplementary Table 8).

3.2.9. PSM Analyses of Vitamin K. A total of 64 patients were included in PSM analyses. In the vitamin K group ($n = 32$), rebleeding occurred in 10 patients during hospitalization, all of which developed after the use of vitamin K. The incidence of 5-day rebleeding was significantly higher in the vitamin K group (31.20% versus 6.20%, $P = 0.010$). There was no significant difference in the in-hospital mortality (15.60% versus 3.10%, $P = 0.086$) between the two groups (Supplementary Table 9).

3.3. Subgroup Analyses

3.3.1. Subgroup Analyses of Patients with Child-Pugh Class B and C after Excluding Patients with Malignancy and Those Undergoing Surgery. After excluding patients with malignancy and those undergoing surgery, 523 patients had Child-Pugh class B and C. Hemostatic drugs group had a significantly higher incidence of 5-day rebleeding (18.10% versus 1.90%, $P = 0.003$), but there was no significant difference in the in-hospital mortality (7.40% versus 3.80%, $P = 0.323$) between the two groups.

3.3.2. Subgroup Analyses of Patients with MELD Score > 15 after Excluding Patients with Malignancy and Those Undergoing Surgery. After excluding patients with malignancy and those undergoing surgery, 79 patients had a MELD score > 15. Hemostatic drugs group had a significantly higher incidence of 5-day rebleeding (15.90% versus 4.30%, $P = 0.003$), but there was no significant difference in the in-hospital mortality (6.10% versus 3.30%, $P = 0.278$) between the two groups.

3.3.3. Subgroup Analyses of Patients Having Esophageal Varices on Endoscopy after Excluding Patients with Malignancy and Those Undergoing Surgery. After excluding patients with malignancy and those undergoing surgery, 471 patients had esophageal varices on endoscopy. There was no significant difference in the incidence of 5-day rebleeding (14.90% versus 8.30%, $P = 0.218$) or in-hospital mortality (3.50% versus 2.10%, $P = 0.596$) between the two groups.

3.3.4. Subgroup Analyses of Patients Receiving Endoscopic Variceal Therapy and Antibiotics after Excluding Patients with Malignancy and Those Undergoing Surgery. After excluding patients with malignancy and those undergoing surgery, 243 patients received both endoscopic variceal therapy and antibiotics. There was no significant difference in the incidence of 5-day rebleeding (18.90% versus 9.50%, $P = 0.285$) or in-hospital mortality (4.50% versus 0.00%, $P = 0.321$) between the two groups.

3.3.5. Subgroup Analyses of Patients with Liver Cancer. There were 160 patients with liver cancer. There was no significant difference in the incidence of 5-day rebleeding (26.10% versus 11.10%, $P = 0.164$) or in-hospital mortality (12.00% versus 11.10%, $P = 0.915$) between the two groups.

4. Discussion

Hemostatic drugs have never been recommended by major practice guidelines and consensus for the management of AUGIB in liver cirrhosis [1]. This is primarily because previous studies did not find any benefit of hemostatic drugs for AUGIB [15–20], which is consistent with our findings. Notably, our overall, PSM, and subgroup analyses suggested that neither 5-day rebleeding rate nor in-hospital mortality was improved by the use of hemostatic drugs. This finding can be explained by the fact that AUGIB in cirrhosis is mainly caused by hemodynamic alterations of portal hypertension, but not coagulation disorder [21–23]. A well-known effect of vasoactive drugs is the visceral vasoconstriction, thus decreasing the portal pressure, so these drugs are the first-line choice of therapy for acute variceal bleeding [23]. By comparison, hemostatic drugs cannot act on portal pressure reduction.

Our study specifically analyzed the effect of different hemostatic drugs in patients with cirrhosis and AUGIB. The findings from PSM analyses performed according to the type of hemostatic drugs were similar to those from overall analysis (Figure 2).

Tranexamic acid is one of the most widely employed antifibrinolytic drugs [24]. A meta-analysis showed that the use of tranexamic acid might not reduce the mortality of AUGIB [15]. Tranexamic acid was administered to few patients in our study, but the majority of our patients received ethylenediamine diacetoacetic which has the same mechanism as tranexamic acid. Therefore, the findings of the previous meta-analysis might be comparable to our finding that ethylenediamine diacetoacetic did not improve the in-hospital outcome of cirrhosis with AUGIB.

The ε-aminocaproic acid is another antifibrinolytic drug. Gunawa and Runyon reported a potential benefit of ε-aminocaproic acid for hyperfibrinolysis, defined as abnormal euglobulin lysis time < 120 min, in liver cirrhosis [16]. Among the 37 cirrhotic patients with hyperfibrinolysis who developed bleeding episodes and received ε-aminocaproic acid, the hemostatic successful rate was 92% (34/37). However, a control group without ε-aminocaproic acid was lacking and the findings might be inconclusive. By comparison, euglobulin lysis time was not regularly measured in our study, and the use of ethylenediamine diacetoacetic in our patients did not depend on the fibrinolysis status. Therefore, our study could not evaluate the benefits of ethylenediamine diacetoacetic in patients with hyperfibrinolysis.

Thrombin can directly affect the conversion from fibrinogen to fibrin clots and acts on the coagulation cascade [25]. A previous study demonstrated that endoscopic injection of human thrombin was effective for gastric variceal bleeding [26]. Additionally, an Indian prospective study including 20 patients with gastric variceal bleeding showed that endoscopic injection of human thrombin was effective and the hemostatic successful rate was 100% [18]. However, in the two studies, endoscopic injection was the only mode of administration, and only gastric and ectopic varices were treated. By comparison, our patients received oral or local spray of lyophilizing

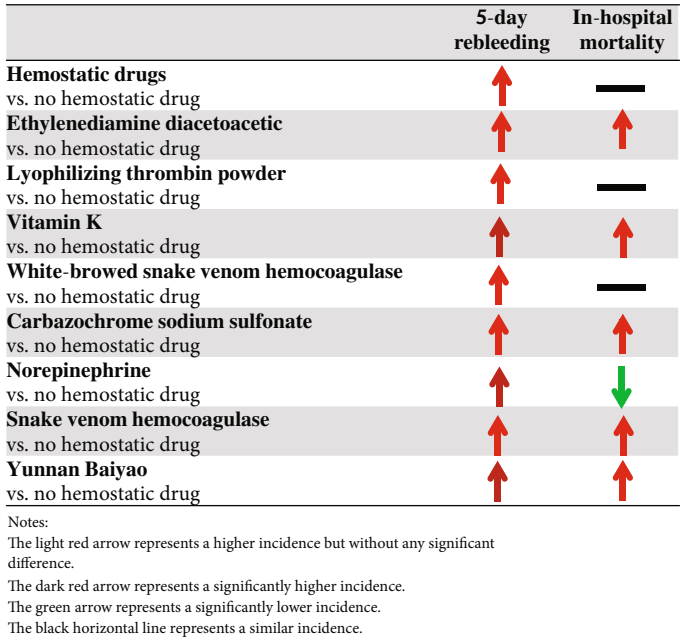

Figure 2: An overview of our findings.

thrombin powder, and a majority of our patients had esophageal varices.

Hemocoagulase, which is extracted from the venom of a snake, such as *Brothrops atrox* and *Agkistrodon blomhoffii ussurensis*, has a thrombin-like effect [27]. Recently, a randomized controlled trial demonstrated that the topical spray of hemocoagulase might not significantly increase the rate of hemostatic success as compared with traditional 8% norepinephrine (100% versus 94.0%, $P = 0.060$) [17]. By comparison, in our study, no hemostatic drug was employed as the control group, and intravenous infusion of hemocoagulase was the only mode of administration. Notably, compared with local spray, intravenous infusion can cause hypofibrinogenemia, which may aggravate bleeding. Indeed, this phenomenon has been observed in several case reports [28–30].

Recombinant factor VIIa (rFVIIa) is not sufficiently supported by the current evidence for the management of acute variceal bleeding [6]. Two randomized controlled trials assessed the efficacy of rFVIIa for acute variceal bleeding in patients with cirrhosis [19, 31]. The first study assessed 245 cirrhotic patients with AUGIB by assessing a composite endpoint, which consisted of failure to control bleeding within 24 hours, failure to prevent rebleeding between 24 hours and day 5, or death within 5 days. Compared with placebo, rFVIIa significantly improved the composite endpoint (8% versus 23%, $P = 0.03$) in the subgroup analysis of Child-Pugh B class and C patients with variceal bleeding, despite the overall analysis found that the endpoint was not significantly different between rFVIIa and placebo groups (14% versus 16%, $P = 0.72$). Then, the investigators further selected a total of 256 cirrhotic patients with Child-Pugh class B and C and variceal bleeding in a second study to evaluate the same endpoint. Compared with placebo, rFVIIa did not add any significant benefit (23% versus 20%, OR = 0.80, $P = 0.37$) and had a lower rate of the composite endpoint (13%). Our meta-analysis of the two trials suggested that the difference in the endpoint was not significant between placebo and rFVIIa groups. Therefore, the effect of rFVIIa in cirrhotic patients with AUGIB remains controversial [32].

Vitamin K participates into the formation of coagulation factors II, VII, IX, and X in the liver and is usually used as a supplementary intervention [33]. Cirrhosis reduces the ability of synthesizing vitamin K-dependent clotting factors [34]. But intravenous infusion of vitamin K is not recommended to correct the coagulation abnormalities in cirrhosis with bleeding [20]. Indeed, vitamin K failed to achieve a remarkable benefit in the reduction of INR in cirrhosis patients [35]. Similarly, our study also suggested that intravenous infusion of vitamin K brings no benefit for treating gastrointestinal bleeding in patients with cirrhosis.

Due to the retrospective nature of this study, several limitations should be acknowledged. First, not all patients had Child-Pugh and MELD scores due to missing laboratory data. But we conducted the subgroup analyses according to the Child-Pugh class and MELD score. Second, not all patients underwent endoscopy to determine the presence and severity of gastroesophageal varices. But we conducted the subgroup analysis according to the use of endoscopic variceal therapy. Third, different hemostatic drugs during hospitalization were often combined. Fourth, a decision on the use of hemostatic drugs was arbitrarily made by our physicians. But we attempted to conduct the PSM analysis by adjusting 15 confounding factors that are associated with patients' outcomes. Finally, some hemostatic drugs were domestic, such as ethylenediamine diacetoacetic. Other hemostatic drugs were traditional Chinese medicine, such as Yunnan Baiyao. Both of them were not available in the West. The new topical hemostatic powder represents a

user-friendly and effective tool in the management of upper gastrointestinal bleeding during endoscopic therapy procedures [36]. However, it has not been available at our hospital.

In conclusion, the effect of hemostatic drugs on AUGIB in cirrhotic patients was unsatisfactory, because the use of hemostatic drugs did not decrease the 5-day rebleeding rate or the in-hospital mortality in cirrhotic patients with AUGIB. Notably, most of the rebleeding events occurred after the initial use of hemostatic drugs. Recent advances in the management of AUGIB should be acknowledged. Future studies should employ more recent data to validate our findings. Additionally, considering the limitations of our study, well-designed randomized controlled trials are still needed in future.

Abbreviations

AUGIB: Acute upper gastrointestinal bleeding
INR: International normalized ratio
MELD: Model for end-stage liver disease
HCC: Hepatocellular carcinoma
PPIs: Proton pump inhibitors
PSM: Propensity score matching
rFVIIa: Recombinant factor VIIa
OR: Odds ratio

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure

The abstract was published in the Asian-Pacific Association for the Study of Liver (APASL) 2020 Conference as a poster presentation. Please see the following link https://link.springer.com/content/pdf/10.1007/s12072-020-10030-4.pdf.

Conflicts of Interest

The authors declare that there is no conflict of interest in this study.

Authors' Contributions

Y.A. wrote the protocol, reviewed, and searched the literature, collected the data, performed the statistical analysis, and drafted the manuscript. Z.B., Y.L., and Y.W. collected and check the data. Z.B., X.X., X.G., F.G.R., and C.A.P. gave critical comments and revised the manuscript. X.Q. conceived the work, wrote the protocol, reviewed, and searched the literature, performed the statistical analysis, interpreted the data, and revised the manuscript. All authors read and approved the final manuscript. Yang An, Zhaohui Bai, Xiangbo Xu, and Xiaozhong Guo are co-first authors.

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Supplementary Materials

Supplementary Table 1: Mechanisms and indications of different hemostatic drugs. Supplementary Table 2: PSM analysis of difference between ethylenediamine diacetoacetic and no hemostatic drug groups. Supplementary Table 3: PSM analysis of difference between lyophilizing thrombin powder and no hemostatic drug groups. Supplementary Table 4: PSM analysis of difference between white-browed snake venom hemocoagulase and no hemostatic drug groups. Supplementary Table 5: PSM analysis of difference between snake venom hemocoagulase and no hemostatic drug groups. Supplementary Table 6: PSM analysis of difference between Yunnan Baiyao and no hemostatic drug groups. Supplementary Table 7: PSM analysis of difference between norepinephrine and no hemostatic drug groups. Supplementary Table 8: PSM analysis of difference between carbazochrome sodium sulfonate and no hemostatic drug groups. Supplementary Table 9: PSM analysis of difference between vitamin K and no hemostatic drug groups. *(Supplementary Materials)*

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Research Article

The Risk of Acute Kidney Injury in Hepatitis B Virus-Related Acute on Chronic Liver Failure with Tenofovir Treatment

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Aims. Tenofovir (TDF) is an antiviral drug with potential risk of kidney injury. The study is aimed at comparing the incidence of acute kidney injury (AKI) between TDF and entecavir (ETV) treatment in hepatitis B virus- (HBV-) related acute on chronic liver failure (ACLF). *Methods*. Treatment-naive patients with HBV-related ACLF were included. Propensity score matching was used to balance the baseline characteristics between ETV and TDF groups. The risk of AKI and the efficacy of TDF and ETV were compared. *Results*. A total of 95 cases with HBV-related ACLF were included in this study, with 74.74% of male and a mean age of 47.01 ± 14.71 years. The antiviral therapy was initiated within 2 days after admission, with 39 cases on the TDF group and 56 on the ETV group. Patients in the TDF group had higher AST, hemoglobin, and serum sodium levels and lower MELD-Na score. After propensity matching, 39 cases of TDF and 39 of ETV were included in the final analysis. No difference was found in the changes of creatinine and cystatin C from baseline to 4 weeks after treatment between ETV and TDF groups. AKI was developed in 1 (2.56%) patient in the ETV group and 2 (5.13%) in the TDF group within one month ($P = 0.556$). Survival analysis revealed no significant difference in the 6-month mortality between the two groups ($P = 0.813$). Cox analysis showed that the type of antiviral drug or the development of AKI was not an independent risk factor for the outcomes. *Conclusions*. Compared to ETV, TDF did not increase the risk of AKI nor the mortality in patients with HBV-related ACLF in the short time.

1. Introduction

Hepatitis B virus (HBV) is a major health problem with 3.5% of the population being chronically infected globally [1]. Patients with chronic HBV infection may suffer from various hepatic complications, such as cirrhosis, liver failure, and hepatocellular carcinoma [2]. Acute on chronic liver failure (ACLF) is defined as a precipitating event in a patient with chronic liver disease, leading to jaundice and coagulopathy complicated by clinical ascites and/or encephalopathy [3]. Patients with ACLF due to HBV reactivation (HBV-ACLF) have extremely poor prognosis, with a reported short-term mortality ranging from 29.7% to 40% within 28 days [4–6]. Acute kidney injury (AKI) is common in ACLF and may develop within a very short period and lead to a poor outcome in ACLF [7].

The management of HBV-ACLF includes antiviral therapy, artificial liver support system, alternative therapies, and liver transplantation [8]. The antiviral therapy is the most evident treatment among them. Currently, tenofovir (TDF) and entecavir (ETV) are both recommended as the first-line antiviral agents for their potent antiviral activity and high genetic barrier for drug resistance [9, 10]. However, TDF has also been demonstrated to have potential kidney toxicity by several observational studies and case reports [11–14]. It is unclear whether or not the use of TDF may increase the risk of AKI in ACLF. The aim of this study was to compare the risk of AKI and the mortality between ETV and TDF groups in HBV-ACLF.

2. Patients and Methods

2.1. Patients. We retrospectively reviewed cases of HBV-related ACLF hospitalized in the First Affiliated Hospital of Fujian Medical University between January 2016 and November 2018. Treatment-naive patients who were diagnosed with

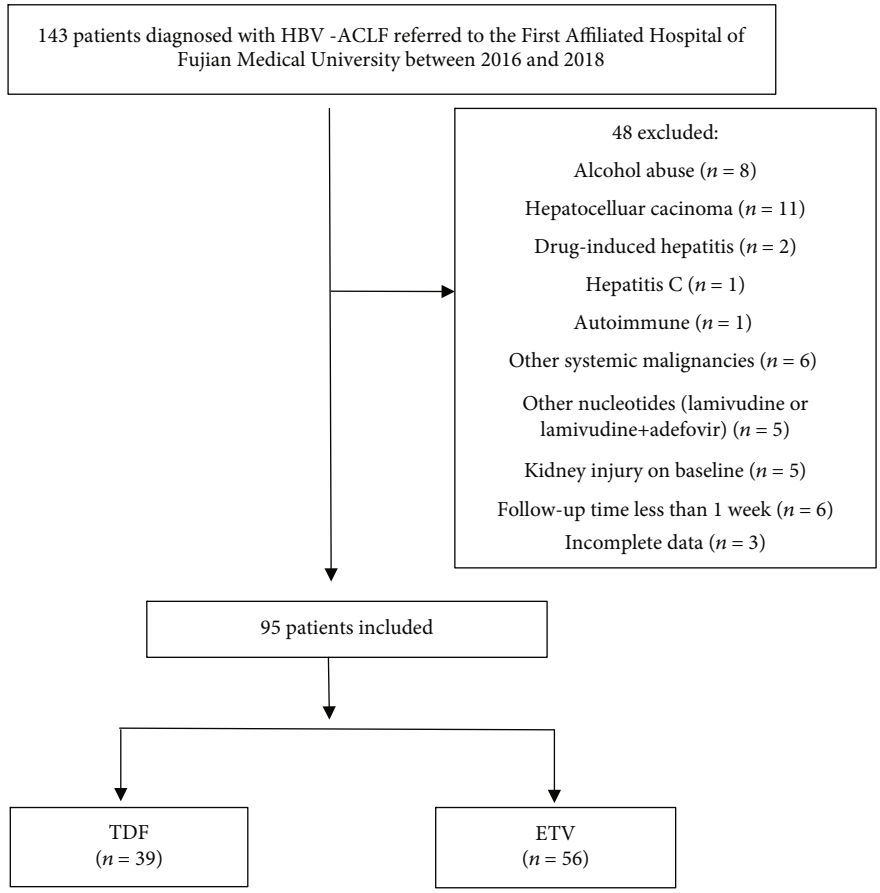

Figure 1: Flow chart of patient selection.

ACLF and received TDF or ETV therapy after hospitalization were included in this study. The exclusion criteria were as follows: (1) patients with kidney injury on baseline; (2) patients with nucleotide treatment other than ETV or TDF; (3) patients with malignant tumor; (4) patients concomitant with other liver diseases such as alcoholic liver disease, autoimmune hepatitis, drug-induced liver injury, or other viral infections (hepatitis A, C, and E virus or HIV infection); (5) patients with missing data; and (6) patients who died or were lost to follow-up within one week after admission.

The diagnosis of ACLF was based on the definition by the Asian Pacific Association for the Study of the Liver (APASL) [3]: jaundice (a serum bilirubin level of ≥5 mg/dL) and coagulopathy (an international normalized ratio (INR) of ≥1.5 or prothrombin activity of <40%). The definition of AKI was based on the criteria by the International Club of Ascites (ICA), which is an increase in serum creatinine (sCr) ≥ 0.3 mg/dL (≥26.5 μmol/L) within 48 hours or a percentage increase in sCr ≥ 50% from baseline which is known, or presumed, to have occurred within the prior 7 days. A value of sCr obtained in the previous 3 months, when available, can be used as baseline sCr. In patients with more than one value within the previous 3 months, the value closest to the admission time to the hospitalization was used [15].

2.2. Treatments. During hospitalization, all patients received supportive treatments including nutrition support, albumin, and other medications that aimed to protect the liver. In patients with liver failure, plasma exchange was given if necessary. Antiviral therapy with TDF or ETV was started immediately when HBV-DNA was detected.

2.3. Data Collection and Follow-Up. The clinical and laboratory data were collected on admission, including the presence of ascites or hepatic encephalopathy (HE), the presence of underlying cirrhosis, total bilirubin (TBIL), albumin, alanine aminotransferase (ALT), aspartate transaminase (AST), international normalized ratio (INR), serum creatinine (sCr), cystatin C, glomerular filtration rate (GFR), serum sodium (Na), hemoglobin, platelets, white blood cell (WBC), Child-Turcotte-Pugh (CTP) score, model for end-stage liver disease (MELD) score, chronic liver failure-sequential organ failure assessment (CLIF-SOFA), hepatitis B surface antigen (HBsAg) levels, hepatitis B e antigen (HBeAg), and HBV DNA levels. Patients were divided into ETV and TDF groups according to the antiviral treatment.

The renal function was reexamined in all survival patients on 4 weeks after antiviral treatment. The survival status was followed up until 2019. For patients being transferred to local hospital, the survival status was collected upon

Table 1: Baseline characteristics of study population.

Variable	Unmatched			Matched		
	ETV group ($n = 56$)	TDF group ($n = 39$)	P value	ETV group ($n = 39$)	TDF group ($n = 39$)	P value
Age (years)	47.80 ± 14.16	44.33 ± 15.87	0.266	45.97 ± 14.10	44.33 ± 15.87	0.631
Male, *n* (%)	42 (75.00%)	29 (74.36%)	0.944	30 (76.92%)	29 (74.36%)	0.792
Ascites, *n* (%)	44 (78.57%)	31 (79.49%)	0.914	29 (74.36%)	31 (79.49%)	0.591
HE, *n* (%)	9 (16.07%)	5 (12.82%)	0.884	7 (17.95%)	5 (12.82%)	0.530
Cirrhosis, *n* (%)	39 (69.62%)	29 (74.36%)	0.787	26 (66.67%)	29 (74.36%)	0.456
TBIL (mmol/L)	282.15 ± 131.00	259.64 ± 120.26	0.396	274.60 ± 138.61	259.64 ± 120.26	0.612
ALT (U/L)	624.61 ± 571.32	861.64 ± 691.44	0.071	724.79 ± 601.63	861.64 ± 691.44	0.354
AST (U/L)	419.04 ± 372.70	645.00 ± 629.04	0.031	490.10 ± 405.92	645.00 ± 629.04	0.200
Albumin (g/L)	30.05 (27.85-32.80)	30.00 (27.90-34.00)	0.934	29.80 (27.40-33.30)	30.00 (27.90-34.00)	0.768
INR	2.14 ± 0.89	1.96 ± 0.55	0.249	1.94 ± 0.59	1.96 ± 0.55	0.864
BUN (mmol/L)	4.33 ± 2.00	3.58 ± 1.52	0.052	4.07 ± 1.84	3.58 ± 1.52	0.203
sCr (μmol/L)	59.81 ± 12.35	57.86 ± 13.87	0.474	59.23 ± 11.24	57.86 ± 13.87	0.633
Cystatin C (mg/L)	1.11 ± 0.41	1.00 ± 0.21	0.128	1.06 ± 0.26	1.00 ± 0.21	0.301
GFR (mL/min)	93.00 ± 18.71	96.72 ± 23.24	0.392	94.49 ± 19.79	96.72 ± 23.24	0.650
HBsAglog10 (ng/mL)	3.10 ± 1.08	2.98 ± 1.07	0.595	3.28 ± 1.12	2.98 ± 1.07	0.243
HBeAg-positive, *n* (%)	26 (46.43%)	22 (52.79%)	0.454	19 (48.72%)	22 (52.79%)	0.496
HBVDNAlog10 (IU/mL)	5.11 ± 2.00	5.34 ± 1.68	0.560	5.39 ± 1.95	5.34 ± 1.68	0.903
Na (mmol/L)	136.16 ± 3.70	138.12 ± 2.93	0.007	136.62 ± 3.91	138.12 ± 2.93	0.058
WBC ($\times 10^9$/L)	6.38 ± 3.24	7.21 ± 3.57	0.247	6.68 ± 3.45	7.21 ± 3.57	0.510
HGB (g/L)	119.07 (102.25-136.50)	132.67 (119.00-147.00)	0.011	124.00 (111.00-143.00)	132.67 (119.00-147.00)	0.147
Platelets ($\times 10^9$/L)	106.95 ± 52.22	118.97 ± 60.22	0.303	116.03 ± 53.48	118.97 ± 60.22	0.820
CTP score	10.48 ± 1.87	10.36 ± 2.12	0.766	10.18 ± 1.90	10.36 ± 2.12	0.694
MELD score	20.25 ± 6.80	18.33 ± 5.20	0.139	18.22 ± 4.94	18.33 ± 5.20	0.928
MELD-Na score	21.68 ± 7.81	18.74 ± 5.70	0.047	19.46 ± 6.15	18.74 ± 5.70	0.593
CLIF-SOFA score	7.25 ± 1.73	7.05 ± 1.96	0.603	6.97 ± 1.67	7.05 ± 1.96	0.852
Diabetes, *n* (%)	8 (14.29%)	3 (7.70%)	0.508	5 (12.82%)	3 (7.70%)	0.709
Hypertension, *n* (%)	7 (12.50%)	2 (5.13%)	0.395	4 (10.26%)	2 (5.13%)	0.671

HE: hepatic encephalopathy; TBIL: total bilirubin; ALT: alanine aminotransferase; AST: aspartate transaminase; INR: international normalized ratio; BUN: blood urea nitrogen; sCr: serum creatinine; GFR: glomerular filtration rate; HBV: hepatitis B virus; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen; WBC: white blood cell; HGB: hemoglobin; CTP: Child-Turcotte-Pugh; MELD: model for end-stage liver disease; CLIF-SOFA: chronic liver failure-sequential organ failure assessment.

phone contact. The primary outcome was the incidence of AKI within 1 month; the secondary outcome was death or liver transplantation.

2.4. Statistical Analyses. The continuous variables were reported as mean ± standard deviation or medium (interquartile rage), while categorical variables were reported as percentage. The Student *t*-test was used for the comparisons of continuous variables, and the chi-squared test was used for the comparison of categorical variables [16]. Propensity score matching (PSM) analysis was performed to minimize the probability of selection bias [17]. The Cox proportional hazard model was used to analyze the risk factors of mortality. The log-rank test was used to compare the risks between groups. All statistical analyses were performed using SPSS software version 24.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Patient Characteristics. A total of 143 patients were diagnosed with ACLF during the study period, among whom 48 patients were excluded due to various reasons (Figure 1). Ninety-five cases were eligible for the final analysis, including 56 cases with ETV therapy and 39 cases with TDF therapy (Figure 1). The average age was 47.01 ± 14.71 years old, and 71 (74.74%) of them were male. The median follow-up time of the overall population was 531 days (range 14-1207 days). There were 20 patients who died during this time

Table 2: Index changes between ETV and TDF groups after 2-week treatment.

	ETV ($n = 39$)	TDF ($n = 39$)	P (ETV vs. TDF)
HBVDNA			
Before treatment	5.39 ± 1.95	5.34 ± 1.68	
After 2 weeks	3.36 ± 1.13	3.22 ± 1.10	
Reduction	2.03 ± 1.52	2.12 ± 1.01	$P = 0.776$
P (baseline vs. 2 weeks)	<0.001	<0.001	
ALT			
Before treatment	724.79 ± 601.63	861.64 ± 691.44	
After 2 weeks	130.90 ± 278.18	119.51 ± 112.05	
Reduction	593.90 ± 540.26	742.13 ± 689.12	$P = 0.294$
P (baseline vs. 2 weeks)	<0.001	<0.001	
TBIL			
Before treatment	274.60 ± 138.61	259.64 ± 120.26	
After 2 weeks	239.89 ± 250.38	223.54 ± 124.94	
Reduction	34.71 ± 234.75	36.09 ± 105.37	$P = 0.973$
P (baseline vs. 2 weeks)	0.362	0.039	

Table 3: Comparison changes in serum creatinine and cystatin C between the ETV and TDF group.

	ETV ($n = 39$)	TDF ($n = 39$)	P (ETV vs. TDF)
sCr			
Before treatment	59.23 ± 11.24	57.86 ± 13.87	
After 2 weeks	61.06 ± 12.69	58.82 ± 11.56	
Changes from baseline to 2 weeks	−1.57 ± 5.95	−0.96 ± 10.32	0.748
P (baseline vs. 2 weeks)	0.080	0.565	
After 4 weeks	61.71 ± 12.14	60.92 ± 16.52	
Changes from baseline to 4 weeks	−2.68 ± 8.96	−2.17 ± 11.81	0.837
P (baseline vs. 4 weeks)	0.072	0.285	
Cystatin C			
Before treatment	1.06 ± 0.26	1.00 ± 0.21	
After 2 weeks	1.18 ± 0.32	1.11 ± 0.24	
Changes from baseline to 2 weeks	−0.12 ± 0.31	−0.11 ± 0.16	0.810
P (baseline vs. 2 weeks)	0.02	<0.001	
After 4 weeks	1.15 ± 0.16	1.28 ± 0.30	
Changes from baseline to 4 weeks	−0.08 ± 0.39	−0.25 ± 0.25	0.237
P (baseline vs. 4 weeks)	0.044	0.011	

period, with a median survival time of 26 days. The baseline characteristics are shown in Table 1. Patients in the TDF group had higher AST, hemoglobin, and serum sodium levels and lower MELD-Na score. There was no difference in other baseline characteristics, including age, sex, HBV DNA levels, MELD score, and the presence of underlying cirrhosis.

We performed PSM to balance the baseline factors. After PSM, there were 39 cases with ETV treatment and 39 cases with TDF treatment that were finally included. The baseline characteristics were comparable between the two groups after PSM. There were 15 patients in this PSM cohort who died during this follow-up, with a median survival time of 35 days.

3.2. Virological and Serological Responses in TDF and ETV Groups. Significant reductions in HBV-DNA, bilirubin, and ALT were observed in both TDF and ETV groups after two weeks of treatment, with no difference in the reduction level between the two groups (Table 2). The HBV-DNA undetectable rate after 2 weeks of antiviral therapy was 28.21% (11/39) in the ETV group and 35.90% (14/39) in the TDF group ($P = 0.467$).

Table 4: The clinical features of the AKI patients.

	A	B	C
Age	61	51	46
Sex	Male	Female	Male
sCr (baseline) (μmol/L)	64	64	67
sCr (after treatment) (μmol/L)	113	104	105
Antivirus therapy	ETV	TDF	TDF
Cirrhosis	Yes	Yes	Yes
Hypertension	Yes	Yes	No
Diabetes	Yes	No	No
Pneumonia	Yes	Yes	Yes
Outcome	Death	Survival	Survival

3.3. The Dynamic Changes of Renal Function in TDF and ETV Groups. Slight increases in sCr were found in both TDF and ETV groups after treatment. However, no significant difference in the change of sCr within 2 weeks or 4 weeks was found within each group or between two groups. Significant difference in the change of cystatin C within 2 weeks or 4 weeks was found within each group, but no significant difference in the dynamic changes of cystatin C between ETV and TDF groups (Table 3). Patients were followed up for 1 month, and AKI was developed in 1 (2.56%) patient in the ETV group and 2 (5.13%) patients in the TDF group. This difference was not statistically significant ($P = 0.556$). All of these 3 patients with AKI had cirrhotic background and pneumonia on admission. Two of them had diabetes. The patients with AKI in the ETV group died at 8 weeks after admission. The other two patients in the TDF group survived (Table 4).

3.4. The Mortality in Overall Study Population and Predictors for Mortality. A total of 15/78 (19.23%) patients died within 6 months. Survival analysis revealed no significant difference in the 6-month mortality between two groups ($P = 0.813$). The results of univariate analysis showed that age, HE, HBeAg positive, MELD score-Na, CTP score, and SOFA score were related to the overall mortality.

Before multivariate analysis, collinearity diagnostics was conducted to assess the sources of collinearity among MELD-Na, CTP, and SOFA scores. The result showed that the tolerance of all variables > 0.1 and the variance inflation factor < 5, indicating limited collinearity among the above variables. As the presence of cirrhosis, HBV DNA, and AKI and gender had been reported to be important predictive factors for the prognosis of ACLF [18–21], those were included in multivariate analysis as well.

The results of multivariate Cox regression analysis showed that the age (HR = 1.103, 95% CI: 1.038-1.172, $P = 0.002$), CTP score (HR = 1.990, 95% CI: 1.210-3.271, $P = 0.007$), SOFA score (HR = 3.000, 95% CI: 1.366-3.171, $P < 0.001$), and cirrhosis (HR = 47.232, 95% CI: 5.538-402.802, $P < 0.001$) were independent risk factors for mortality (Table 5). The types of antiviral drug and the development of AKI were not independently associated with the outcome (Figure 2 and Table 5).

4. Discussion

This study compared the impact of TDF and ETV in renal function in patients with HBV-ACLF. The results showed that TDF did not increase the risk of AKI nor the mortality in patients with HBV-related ACLF within 6 months.

Both TDF and ETV are currently recommended as the first-line treatment for chronic hepatitis B (CHB) for their high efficacy and low resistance rate [9, 22–24]. Previous studies have demonstrated that TDF and ETV have similar effectiveness in treatment-naive CHB patient [25–27]. However, some reports indicate that TDF might lead to a higher incidence of AKI compared to ETV in CHB patients [28, 29]. As AKI is common in ACLF [30], renal injury associated with TDF use has raised some concerns [31]. However, in this single-center study, we found that the use of TDF did not increase the risk of AKI within one month of treatment. This might be due to the short follow-up period of this study. As reported previously, renal injury associated with TDF use usually develops after at least one year of treatment. A recent real-world study from Korea showed that TDF therapy did decrease overall renal function in CHB patients during the first two years of TDF use [13]. Therefore, long-term follow-up might be helpful to access the renal impairment in ACLF patients with different antiviral therapies.

It is worth noticing that all three patients suffering from AKI had bacterial infection and two of them had comorbidities like diabetes and hypertension. Hypertension and diabetes are both well-known risk factors for chronic kidney injury. The bacterial infection is also a main trigger for AKI in liver failure [32]; thus for patients who had AKI in this cohort, the impact of the other complication/comorbidities might overwhelm the influence of antiviral drugs. Prospective studies with longer follow-up period are greatly needed to reveal the real relationship between AKI and TDF in ACLF patients.

Cystatin C is a sensitive marker for renal impairment [33]. In this study, no significant difference in the change of sCr within 1 month was found in both TDF and ETV groups, while there was significant difference in the change of cystatin C in both groups. Cystatin C levels may be more sensitive for evaluating the renal impairment in ACLF [34]. However, in terms of the impact of different antiviral drugs on renal function, the changes of cystatin C were similar as those of sCr, which further consolidated that TDF had limited influence on renal function in an ACLF population in a short-term period.

The efficacy of different antiviral drugs in ACLF remains controversial. Wan et al. [35] showed that TDF was superior to ETV in the treatment of HBV-ACLF; however, more studies showed no difference between these two groups [27, 36, 37]. The results of our study were in consistence with most studies showing that TDF was not superior to ETV regarding the HBV DNA suppression or mortality.

There are several limitations of this study. Firstly, the data of HBV-DNA levels, liver function, and kidney function

Table 5: Cox analysis of risk factors for mortality.

Variable	Univariate analysis (95% CI)	*P* value	Multivariate analysis (95% CI)	*P* value
Age	1.044 (1.010-1.079)	0.011	1.103 (1.038-1.172)	0.002
Male	1.276 (0.360-4.522)	0.706	1.200 (0.277-6.340)	0.830
HE	3.291 (1.123-9.644)	0.030	7.156 (0.740-69.170)	0.089
HBeAg-positive	8.356 (1.884-37.062)	0.005	10.611 (1.314-85.709)	0.027
Cirrhosis	1.253 (0.428-3.667)	0.681	47.232 (5.538-402.802)	<0.001
Antivirus therapy	0.885 (0.321-2.442)	0.814		
AKI	1.617 (0.213-12.302)	0.642	5.394 (0.535-54.420)	0.153
lgHBsAg (ng/mL)	0.874 (0.551-1.388)	0.570		
lgHBV-DNA (IU/mL)	0.920 (0.687-1.233)	0.577	0.925 (0.563-1.522)	0.760
ALT (U/L)	1.000 (1.000-1.001)	0.407		
sCr (μmol/L)	0.985 (0.879-1.104)	0.795		
WBC (10^{12}/L)	0.993 (0.857-1.151)	0.929		
PLT (10^9/L)	0.997 (0.988-1.007)	0.598		
Meld-Na score	1.107 (1.020-1.201)	0.015	0.972 (0.837-1.128)	0.704
CTP score	1.743 (1.266-2.400)	0.001	1.990 (1.210-3.271)	0.007
SOFA score	2.146 (1.528-3.013)	<0.001	3.000 (1.621-5.553)	<0.001

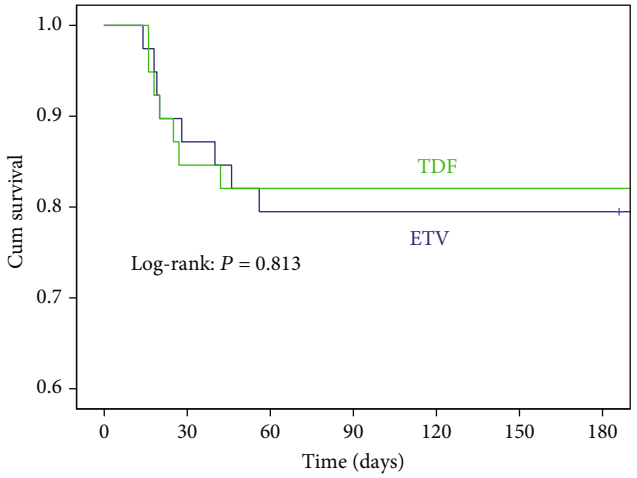

Figure 2: Cumulative survival of ETV and ETV within 6 months.

is largely missing after 3 months because most survival patients were transferred to a local hospital after recovery; thus, the long-term changes of renal function were unclear. Secondly, the incidence rate of AKI was low and the sample size relatively small, which may easily lead to false-negative results. Further study with larger sample size is needed to guarantee the results.

In summary, our study showed that compared with ETV, TDF did not increase the risk of AKI nor the mortality in patients with HBV-related ACLF within a short-term period.

Data Availability

The data in this study are available from the corresponding author on reasonable request.

Ethical Approval

The study protocol has been approved by the Institutional Ethics Committee of the First Affiliated Hospital of Fujian Medical University. The clinical activities being reported are consistent with the principles of the Declaration of Helsinki.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kai Zhang, Jiaofeng Huang, and Mingfang Wang collected and analyzed the data. Kai Zhang and Su Lin wrote the primary draft. Yueyong Zhu and Su Lin did the study design and revised the final article for important intellectual content. All authors read and approved the final version of the manuscript.

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