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Unveiling miRNA–Gene Regulatory Axes as Promising Biomarkers for Liver Cirrhosis and Hepatocellular Carcinoma

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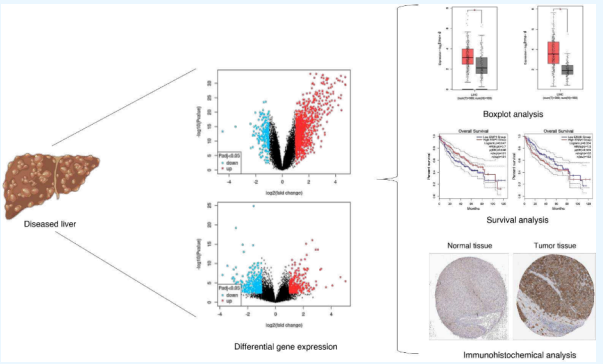
ABSTRACT: Liver cirrhosis, a severe scarring condition of the liver with the potential to progress to hepatocellular carcinoma (HCC), necessitates the development of reliable biomarkers for early detection due to the asymptomatic nature of its early stages. Recent discoveries in microRNAs (miRNAs) hold promise for a noninvasive test, with the potential to significantly improve patient outcomes. Building upon these promising findings, this study investigates gene expression data, identifying distinct sets of DEGs and DEMs using GEO2R. Subsequently, a gene−miRNA network was constructed using Cytoscape to explore potential interactions between DEMs and their target genes (DEGs). Boxplot analysis was carried out to identify and validate differences in gene expression between healthy and diseased tissues. This analysis revealed four significantly differentially expressed genes: CAV1, PEA15, EMP1, and ENAH. Notably, subsequent survival analysis demonstrated that EMP1 and ENAH significantly impact overall patient survival. Intriguingly, the constructed network identified several potential regulatory axes: hsa-miR-191-5p/ENAH, hsa-miR-3158-3p/ENAH, hsa-miR-371a-5p/ENAH, and hsa-miR-6753-5p/EMP1. Crucially, a direct comparison of DEGs and DEMs between liver cirrhosis and HCC pinpointed AGO3, NCOA3, and TNPO1, along with their regulatory elements, as potential key drivers of HCC development in cirrhotic patients, underscoring their importance as targets for early diagnostic and therapeutic strategies. Finally, immunohistochemical (IHC) analysis not only validates our findings but also reiterates the novelty of the identified genes. Overall, elucidating the role of these novel genes and regulatory elements could pave the way for an earlier and more accurate diagnosis of liver diseases.



1. INTRODUCTION

Liver diseases pose a significant threat to global health, causing about 2 million deaths annually.[1] Cirrhosis constitutes a significant contributor to global mortality, accounting for an estimated 2.4% of worldwide deaths in the year 2019.[2] Furthermore, cirrhosis poses a grave risk for individuals by dramatically increasing their chances of developing HCC, the most common type of primary liver cancer.[3] While curative approaches like surgical resection and liver transplantation exist for early-stage HCC, their applicability is often limited by advanced disease stage, underlying health conditions, and organ availability.[4,5] This underscores the critical challenge of late-stage diagnosis, which restricts treatment options to palliative care.[6] Despite advancements, current therapeutic strategies often face limitations like drug resistance in advanced HCC, leading to frequent recurrences.[7] The substantial disease burden of cirrhosis and HCC underscores the urgency for the development of novel biomarkers and ultimately more effective therapeutics.[8]

Emerging research has explored miRNAs as promising diagnostic, prognostic, and therapeutic response biomarkers for various diseases. miRNAs, small noncoding RNAs, act as master regulators of gene expression by binding to 3′-untranslated region (UTR) of mRNA, affecting its stability and function.[9] Various studies have reported that chronic liver injury disrupts the balance of miRNAs, leading to increased cell proliferation and survival, inhibited apoptosis, enhanced fibrosis, and metastasis, highlighting their role as therapeutic targets.[10] For instance, Elabd et al. identified miR-21 as a promising candidate due to its high sensitivity and specificity.[11] While miR-21 takes center stage, Ratnasari et al. reported miR-21-5p and miR-155-5p as potential oncomiR biomarkers, highlighting the need for further exploration.[12] Similarly, Fang et al. emphasized the value of circulating miR-16 and miR-122 for early detection of HCC.[13] Meanwhile, Wang et al. explored miR-374a/b-5p as novel therapeutic targets, suggesting the potential for miRNA-based HCC treatment strategies.[14] However, identifying the

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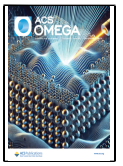

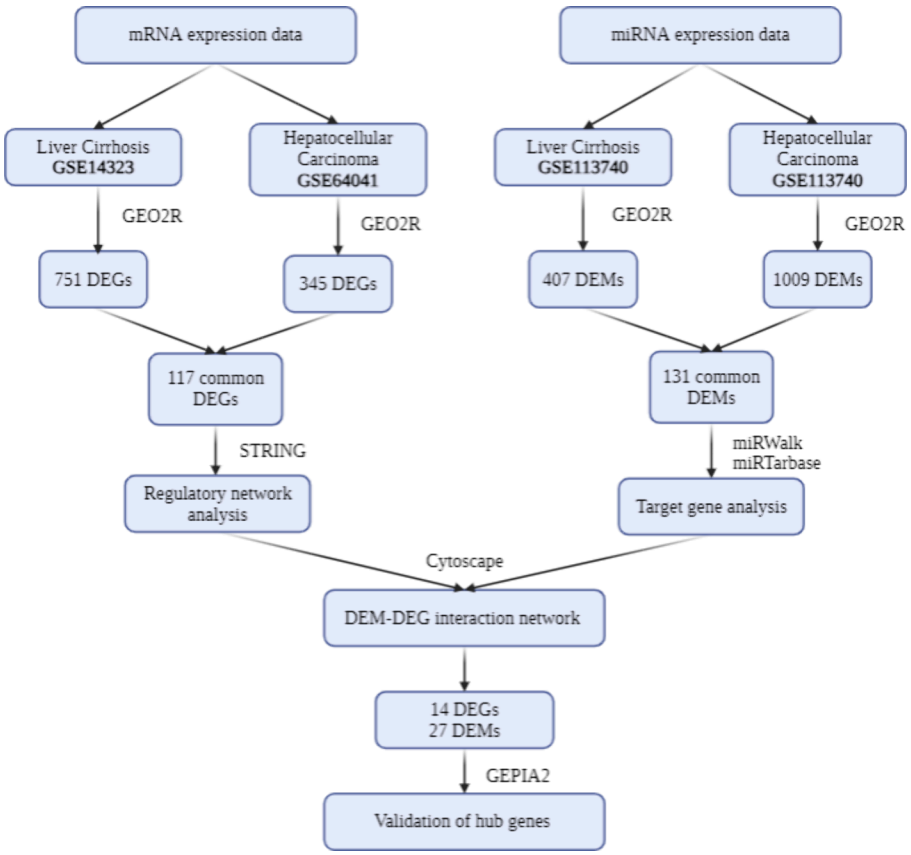

Figure 1. Schematic representation of the workflow.

Table 1. Description of the Datasets Used in the Study

S no.	disease	experiment type	GEO Ids	platform	no. of diseased	no. of control
1	liver cirrhosis	expression profiling by array	GSE14323	GPL571 - [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array	41	19
		noncoding RNA profiling by array	GSE113740	GPL21263-3D-Gene Human miRNA V21_1.0.0	93	10
2	HCC	expression profiling by array	GSE64041	GPL6244 - [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array	60	5
		noncoding RNA profiling by array	GSE113740	GPL21263−3D-Gene Human miRNA V21_1.0.0	40	10

most clinically relevant miRNAs and developing safe and effective delivery methods remain challenges for future research.

Recent advancements in high-throughput sequencing technology coupled with powerful bioinformatics analysis have revealed a burgeoning landscape of miRNA/mRNA regulatory pairs implicated in diverse cancer processes. For example, Yang et al. pinpointed a set of genes (CLEC4G, GLS2, H2AFZ, STMN1, and TUBA1B) and miRNAs (hsa-miR-326 and has-miR-331-5p) that influence patient survival prognosis, paving the way for personalized treatment plans.[15] Likewise, Han et al. identified a panel of potential biomarkers, including PABPC1, UHRF1, SLC2A9, miR-200b-3p, miR-466, miR-200c-3p, and miR-10a-5p, for tracing the progression from cirrhosis to HCC.[16] Notably, the work of Huang et al. explored crucial miRNA axes—hsa-mir-195-5p/CDK1, hsa-mir-5589-3p/CCNB1, and hsa-let-7c-3p/CKS2—potentially involved in the genesis and development of HBV-related HCC, thus holding promise as potential novel biomarkers.[17]

Investigating miRNA/mRNA pairs grants insights into the intricate regulatory networks controlling cancer processes. These interactions can serve as both diagnostic and prognostic biomarkers. Unraveling how miRNAs regulate mRNAs can reveal potential biomarkers for cancer diagnosis and prognosis, while targeting these interactions offers novel therapeutic avenues for cancer treatment. Considering this evidence, this study leverages Gene Expression Omnibus (GEO) data sets to identify potential mRNA and miRNA axes that hold promise as novel diagnostic candidates for both early- and end-stage liver diseases.

2. METHODOLOGY

2.1. Data Collection. The microarray and miRNA data sets were downloaded from the National Center for Biotechnology Information's (NCBI) GEO database for our analysis.[18] The schematic representation of the workflow is shown in Figure 1. The data sets that surfaced on the search for "liver cirrhosis" and "hepatocellular carcinoma" were retrieved. The gene expression data that fell into the criteria (i) sample size ≥5 and (ii) must contain sufficient information on the gene (≥5000) and miRNA (≥500) were considered.[15]

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Based on these criteria, two data sets (GSE14323 and GSE64041) were chosen for gene expression analysis and GSE113740 for miRNA expression analysis. The data set GSE14323 (GPL571-[HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array platform) contained 41 liver cirrhosis and 19 healthy liver samples;[19] GSE64041 (GPL6244-[HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array platform) included 60 HCC samples and 5 healthy liver biopsy samples.[20] Likewise, GSE113740 (GPL21263−3D-Gene Human miRNA V21_1.0.0 platform) contained 93 liver cirrhosis, 40 HCC, and 10 healthy samples.[21] The details of the data sets are mentioned in Table 1.

2.2. Identification of Differentially Expressed Genes (DEGs). The DEGs are a set of genes that are dysregulated in the diseased samples when compared to the healthy samples.[22] This study employed GEO2R, an online tool adept at comparing data sets under similar experimental conditions and determining differentially expressed genes.[23] A Benjamini−Hochberg false discovery rate (FDR) of ≤0.05 and a fold change threshold of ≥1 was used to identify statistically significant DEGs.[24] This indicates that these genes are upregulated in diseased tissues. Additionally, the distribution of DEGs was visualized by using Volcano plots. Following DEG identification, InteractiVenn, an online tool, was utilized to generate a Venn diagram highlighting the overlapping genes between the two data sets that were selected for further analysis.[25]

2.3. DEG−DEG Network Analysis. To investigate protein−protein interactions within DEGs, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was utilized.[26] A network was built with a confidence score of 0.4, wherein the nodes represent the proteins, and the edges show their interaction between each other.[27] This protein−protein interaction network was further maintained to facilitate the subsequent prediction of miRNA−gene interactions.

2.4. Gene Ontology and Pathway Enrichment Analysis of the DEGs. To elucidate the functions of genes at the cellular level, gene ontology is employed by analyzing their enrichment in three categories: biological process (BP), molecular function (MF), and cellular component (CC). Similarly, the Kyoto Encyclopedia of Genes and Genomes (KEGG) serves as a pathway database to assist in the prediction of molecular signaling enrichments.[28] In this present study, ShinyGO v0.80, a user-friendly web tool specifically designed for GO and pathway enrichment analysis, was utilized.[29] The analysis focused on the top 10 enriched pathways in each category, applying an FDR threshold of <0.05 to ensure statistical significance.

2.5. Identification of Differentially Expressed miRNAs (DEMs). miRNAs, a class of noncoding RNAs, play a crucial regulatory role by binding to mRNA and thereby hindering the production of corresponding proteins.[30] To identify DEMs associated with liver cirrhosis and HCC, the aforementioned gene expression data set, GSE113740, was analyzed using the GEO2R tool. Stringent criteria were applied to ensure robust results: a *p*-value threshold of ≤0.05 and a log fold change threshold of ≥1. Subsequently, a Venn diagram generated through InteractiVenn was utilized to identify the overlapping DEMs present in both liver cirrhosis and HCC samples.

2.6. Target Gene Analysis of the DEMs. miRNAs exert critical control over numerous cellular processes including development, metabolism, cell cycle progression, differentiation, and apoptosis. Disruptions in biogenesis or regulation can contribute to disease due to their vital roles. Thus, identifying and validating interactions with mRNA targets is essential to elucidate the regulatory functions of miRNAs.[31,32] Based on this knowledge, miRWalk,[33] an online tool offering a comprehensive database of predicted and validated miRNA targets, was employed to identify potential gene targets of the DEMs. Further analysis identified commonly targeted genes present in both miRWalk and miRTarbase, another established miRNA target database. Finally, to elucidate potential biological pathways modulated by these miRNAs, pathway enrichment analysis was performed for their target genes using miRPathDB 2.0.[34] This tool highlights pathways likely to be affected by specific miRNAs, offering valuable insights into their functional roles.

2.7. DEG−DEM Regulatory Network Analysis. To analyze the interactions between the miRNAs and their target genes, a gene−miRNA network was constructed by integrating the networks obtained from Sections 2.3 and 2.6 to visualize and analyze these relationships. This network integration leveraged Cytoscape 3.10.1,[35] an open-source software platform specifically designed for the visualization and analysis of interaction networks. Only miRNAs targeting the identified DEGs were considered for further investigation.

2.8. Validation of the Hub Genes Using GEPIA. Survival analysis plays a critical role in deciphering whether the identified DEGs hold any potential influence on the survival of HCC patients. The survival and gene expression analysis of the target DEGs were carried out using Gene Expression Profiling Interactive Analysis 2 (GEPIA2). GEPIA2 is a powerful online resource for exploring gene expression in cancer. By, utilizing data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx), it offers diverse analyses like differential expression and survival studies for tumor and normal samples.[36] The gene expression analysis was visualized using boxplots and the overall survival analysis using Kaplan−Meier (KM) plots with a *p*-value threshold of ≤0.05.[37,38]

2.9. Immunohistochemical (IHC) Analysis. To evaluate the protein level expression levels of the hub genes, we performed an IHC analysis using the Human Protein Atlas (HPA) database. The HPA is a publicly accessible resource providing comprehensive protein profiling images and data.[39] Protein expression levels for the hub genes were specifically assessed within liver tissue.

3. RESULTS AND DISCUSSION

3.1. Screening of DEGs in Liver Cirrhosis and HCC. This study leveraged two publicly available data sets, GSE14323 and GSE64041, to identify the DEGs associated with liver cirrhosis and HCC allowing for the exploration of DEGs between normal and diseased samples. Utilizing the robust GEO2R tool, the investigation yielded a compelling set of DEGs—751 from GSE14323 and 345 from GSE64041, as shown in Table 2. To visualize the overlapping genes between these data sets, a Venn diagram (Figure 2) was constructed, revealing a significant

Table 2. Total Number of DEGs and DEMs from Microarray and miRNA Datasets

S no.	disease	GEO Ids	total no. of DEGs and DEMs	upregulated DEGs/DEMs
1	liver cirrhosis	GSE14323	22277	751
		GSE113740	2565	407
2	HCC	GSE64041	33297	345
		GSE113740	2565	1009

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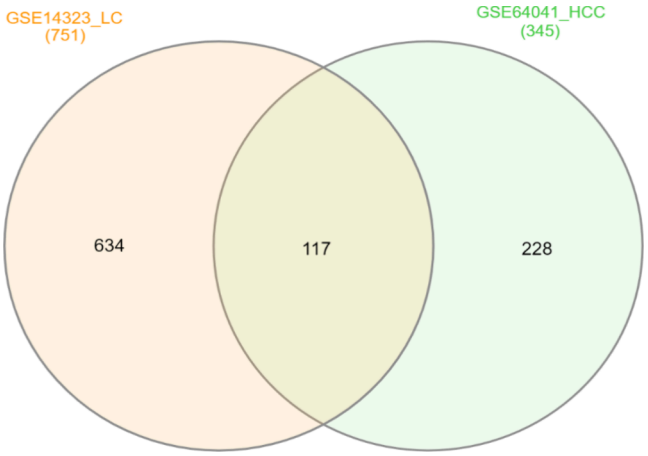

Figure 2. Venn diagram of overlapping differentially expressed genes obtained from GSE14323 and GSE64041.

intersection of 117 genes exhibiting dysregulation in both HCC and cirrhosis. Additionally, Figure 3 shows the generated volcano plots that offered a clear picture of the statistically significant DEGs within each data set, highlighting genes with strong fold changes and high confidence. The diseased samples considered are cirrhotic and tumor liver samples, whereas control refers to normal liver parenchyma. Figure 4 depicts the DEG−DEG network, providing insights into the potential functional relationships between the DEGs. Unlike traditional methods that favor only interconnected genes, in our approach, we examined all DEGs in order to facilitate the identification of overlapping DEGs with the target genes of DEMs.

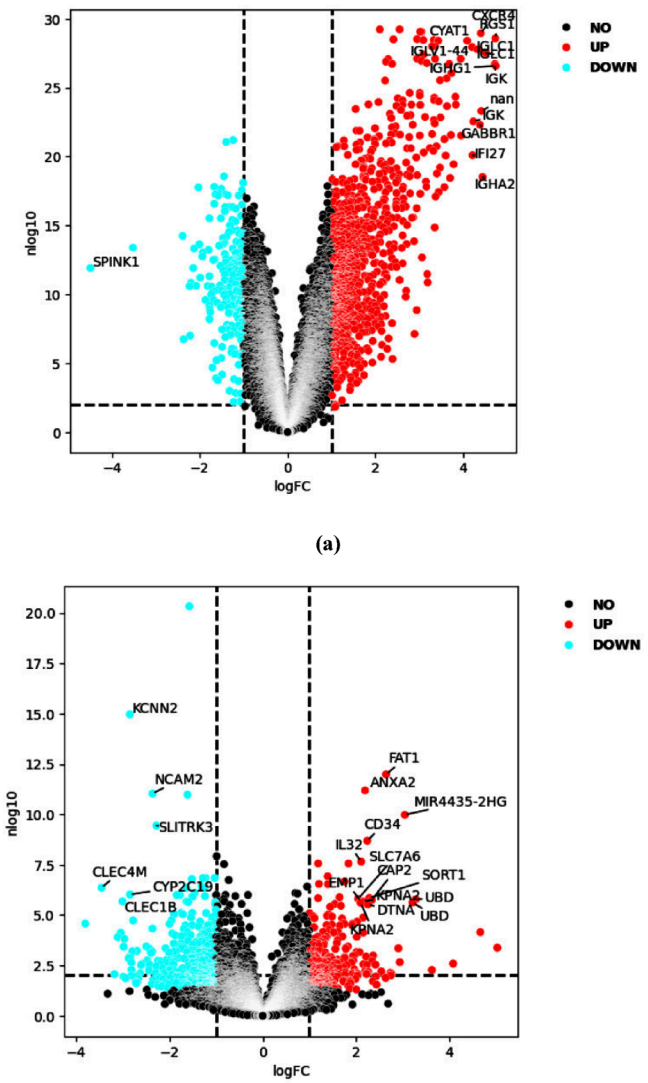

Figure 3. Volcano plot of DEGs between normal (normal liver parenchyma) and diseased tissues: (a) GSE14323 liver cirrhosis and (b) GSE64041 hepatocellular carcinoma.

3.2. Functional Enrichment Analysis of DEGs. ShinyGO was employed to gain deeper insight into DEG functions and impacted metabolic pathways, providing a more comprehensive understanding of the underlying mechanisms of liver cirrhosis and HCC.

Examining the BP terms in Table 3 from the GO analysis reveals a notable enrichment in genes associated with "Cellular Development" and "System Development". This suggests a potential dysregulation of fundamental BPs underlying the liver disease state, potentially impacting the development and function of various cell types and systems. Further enriched BP terms included cell differentiation, response to various stimuli (chemical and stress), anatomical structure morphogenesis, and regulation of biological quality, movement, localization, and response.

Delving further into the CC category, a prominent enrichment of DEGs within the "Extracellular Space" was observed. This intriguing finding suggests potential alterations in the composition and function of the cellular microenvironment, which may be associated with the condition of the diseased liver. Notably, the enriched CC terms encompassed various extracellular compartments, including the extracellular region, vesicles (both cytosolic and extracellular, including exosomes), and even extracellular organelles and membranes (Table 4). In line with Roy et al., these findings support the potential of targeting extracellular matrix (ECM) proteins in the liver tumor microenvironment (TME).[40] Liu et al. identified a potential mechanism for liver fibrogenesis. Their study suggests that a protein called β-arrestin 1 (ARRB1) increases the release of small extracellular vesicles (EVs) enriched with mannan-binding lectin serine protease 1 (MASP1) from hepatocytes. These MASP1-containing EVs then activate hepatic stellate cells (HSCs), promoting liver fibrosis through a signaling pathway involving p38 MAPK and activating transcription factor 2 (ATF2).[41]

Finally, the molecular function (MF) category revealed a noteworthy enrichment of 27 DEGs linked to "Molecular Function Regulator Activity." This intriguing finding suggests a potential dysregulation of key regulatory processes within cells, potentially impacting various cellular functions. Furthermore, as shown in Table 5, the enriched MF terms encompassed a diverse array of binding activities, including interactions with identical proteins, signaling receptors, enzymes, calcium ions, and various protein complexes. Notably, the enrichment also extended to structural components, including cytoskeletal proteins and ECM constituents, hinting at potential alterations in the cellular architecture (Figure 5).

KEGG pathway analysis provided complementary insights into the underlying cellular networks associated with the disease. A critical pathway emerged, "Focal Adhesion", harboring nearly 15 enriched DEGs (Figure 6). This compelling finding suggests a potential dysregulation of cell adhesion and migration processes, which could be central to the liver disease condition.

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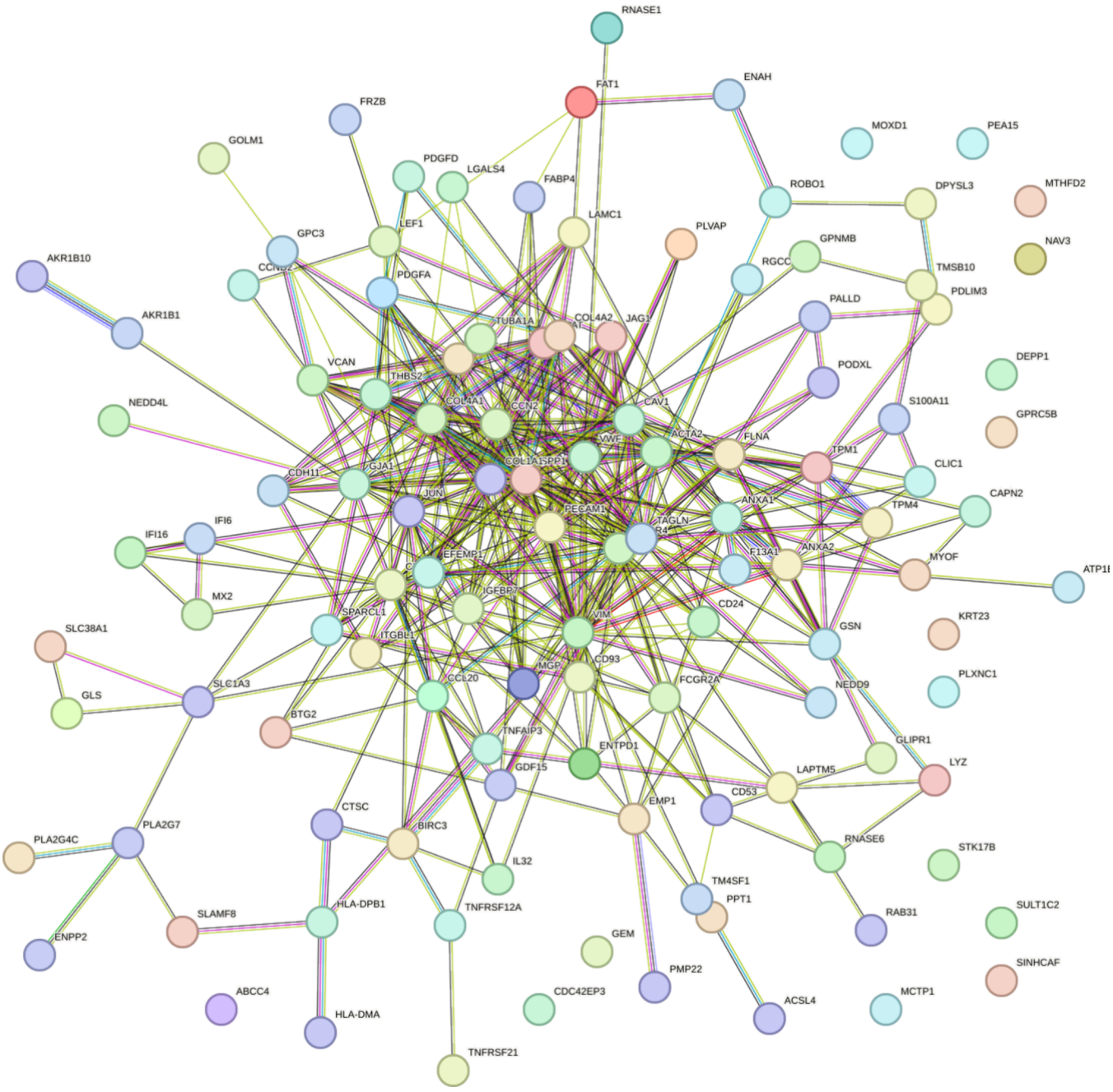

Figure 4. Network analysis of the differentially expressed genes.

The findings of this study on the role of specific focal adhesion (FA) proteins in liver diseases align with Zhang et al. who linked focal adhesion kinase (FAK) to p38-mitogen-activated protein kinase-activated protein kinase 2 (p38-MK2), actin remodeling, and fenestrae loss in liver sinusoidal endothelial cells (LSECs) under stiff conditions. Notably, FAK or p38-MK2 inhibition restored fenestrae, suggesting a potential therapeutic target for liver fibrosis.[42] Furthermore, Greuter et al. demonstrated the importance of FA proteins in mechanotransduction of other cell types, highlighting the crucial role of FA proteins in LSEC communication within the liver microenvironment in response to mechanical cues.[43] As shown in Table 6, the KEGG analysis revealed enrichment in a diverse range of additional pathways, hinting at the complex cellular processes potentially involved. These findings warrant further investigation to elucidate their precise roles in the disease state.

3.3. Identification of DEMs. The analysis of differentially expressed miRNAs revealed a shift in miRNA expression between healthy and diseased states. GEO2R analysis identified 407 and 1009 DEMs in liver cirrhosis and HCC samples, respectively. Figure 7 presents a Venn diagram analysis revealing 131 differentially expressed miRNAs (DEMs) shared by both diseases. This suggests a potential set of common miRNAs underlying the molecular basis of both conditions. Further analysis using a volcano plot (Figure 8) promises to identify the key drivers of disease progression. The control samples mentioned in the volcano plot refers to the normal liver parenchyma tissues.

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Table 3. Top Enriched Biological Process of the Hub Genes

S. no.	GO IDs	biological process	no. of genes	*p*-value
1	GO:0048869	cellular developmental process	57	1.03×10^{-11}
2	GO:0048731	system development	57	1.32×10^{-11}
3	GO:0030154	cell differentiation	56	2.75×10^{-11}
4	GO:0042221	response to chemical	53	1.04×10^{-08}
5	GO:0009653	anatomical structure morphogenesis	46	2.02×10^{-12}
6	GO:0065008	regulation of biological quality	46	1.64×10^{-07}
7	GO:0006950	response to stress	46	1.49×10^{-06}
8	GO:0048583	regulation of response to stimulus	44	6.72×10^{-06}
9	GO:0006928	movement of cell or subcellular component	43	7.20×10^{-14}
10	GO:0032879	regulation of localization	43	3.35×10^{-10}

Table 4. Top Enriched Cellular Components of the Hub Genes

S. no.	GO IDs	cellular components	no. of genes	*p*-value
1	GO:0005576	extracellular region	65	3.26×10^{-17}
2	GO:0005615	extracellular space	61	6.66×10^{-20}
3	GO:0031982	vesicle	59	5.88×10^{-14}
4	GO:0043230	extracellular organelle	41	9.79×10^{-13}
5	GO:0065010	extracellular membrane-bounded organelle	41	9.79×10^{-13}
6	GO:0070062	extracellular exosome	41	9.79×10^{-13}
7	GO:1903561	extracellular vesicle	41	9.79×10^{-13}
8	GO:0031410	cytoplasmic vesicle	36	3.07×10^{-07}
9	GO:0097708	intracellular vesicle	36	3.07×10^{-07}
10	GO:0030054	cell junction	31	8.51×10^{-07}

Table 5. Top Enriched Molecular Function of the Hub Genes

S. no.	GO IDs	molecular function	no. of genes	*p*-value
1	GO:0098772	molecular function regulator activity	27	0.000348838
2	GO:0042802	identical protein binding	24	0.005572755
3	GO:0005102	signaling receptor binding	20	0.005224743
4	GO:0019899	enzyme binding	20	0.042097345
5	GO:0005509	calcium ion binding	19	8.69×10^{-7}
6	GO:0044877	protein-containing complex binding	18	0.00549032
7	GO:0008092	cytoskeletal protein binding	17	0.000825762
8	GO:0005198	structural molecule activity	16	0.000658278
9	GO:0005201	extracellular matrix structural constituent	11	6.69×10^{-7}
10	GO:0030546	signaling receptor activator activity	11	0.004579291

The DEMs obtained in this study were consistent with previous literatures. For instance, Wu et al. focused on miR-191-5p, demonstrating its upregulation in HCC tissues. Their findings suggest that miR-191-5p functions as a tumor promoter, raising the possibility of using miR-191−5p inhibitors for targeted HCC treatment.[44] Nie et al. explored miR-382-5p, finding its expression elevated in HCC tissues alongside a decrease in a protein called farnesoid X receptor (FXR). They further revealed that miR-382-5p promotes HCC cell proliferation by inhibiting FXR expression and function. These findings position miR-382-5p as an oncogenic miRNA, potentially useful as both a biomarker and a therapeutic target for HCC.[45]

3.4. DEG−DEM Interaction Prediction. To identify the DEGs potentially regulated by DEMs, the study analyzed miRNA target prediction databases such as miRWalk and miRTarbase. This analysis revealed 27 DEMs with potential interactions with 14 DEGs. The strongest DEM−DEG interaction exhibited a remarkably low binding energy of −31.7 kcal/mol, with hsa-miR-1238-5p acting as the miRNA and GPRC5B as its target gene. The other DEM−DEG interactions are provided along with their binding energy in Table 7. To visualize this interaction between miRNAs and their targets, an interactive network was constructed using Cytoscape (Figure 9).

3.5. Enrichment Analysis of DEMs. The miRPathDB was employed to perform enrichment analysis of these DEM target genes, aiming to unravel biological pathways potentially regulated by the identified miRNAs. The results unveiled a captivating enrichment of target genes in pathways critical to liver function and disease progression. Notably, these pathways included "Signaling of Hepatocyte Growth Factor Receptor", "ncRNAs involved in Wnt signaling in hepatocellular carcinoma", "Hepatitis C and Hepatocellular Carcinoma", and "Hepatitis B" (Table 8). Singling out a particularly intriguing example, a staggering 104 genes targeted by hsa-miR-6887-5p displayed a significant enrichment (*p*-value of 4.26×10^{-4}) in the "ncRNAs involved in Wnt signaling in hepatocellular carcinoma" pathway within WikiPathways. This miRNA was reported in a study by Morales-Martínez and Vega, where they investigated potential miRNAs regulating p38 signaling. Among those miRNAs reported, miR-6887-5p was also suggested to have a potential role in p38 regulation by regulating MAPK13.[46]

The other miRNA, hsa-miR-197-5p, was found to be associated with genes relevant to hepatitis C (HCV), while hsa-miR-140-3p intersected with genes linked to hepatitis B (HBV). These findings are significant, because both HCV and HBV are major risk factors for developing cirrhosis and HCC.

3.6. Validation of Hub Genes. To validate the identified hub genes, the study leveraged the GEPIA2 Web site that focused on assessing the expression levels of 14 DEGs that were obtained as the targets for the DEMs (Figure 10). The asterisk indicates that the difference between groups is statistically significant, with a *p*-value less than 0.05. Based on this observation, it was seen that four genes, CAV1, EMP1, ENAH, and PEA15, emerged as significantly differentially expressed between the two groups, suggesting their potential importance in understanding the differences between the conditions.

Ting et al. convincingly demonstrated caveolin 1 (CAV1) promotional effect on HCC cell growth, motility, invasion, and tumor formation.[47] Following this, Yu et al. implicated CAV1 in promoting HCC cell invasion and metastasis. Their work suggests that CAV1 upregulates matrix metalloproteinase 7 (MMP-7) expression, an enzyme linked to ECM breakdown, while simultaneously decreasing E-cadherin, a protein crucial for cell adhesion. This shift promotes an epithelial-to-mesenchymal transition (EMT), a process associated with increased cell motility and metastasis. Additionally, their findings suggest ERK activation as a potential pathway underlying this gene's effects.[48] Takeda et al. further elucidated the role of CAV1 in HCC development, specifically within the context of nonalcoholic

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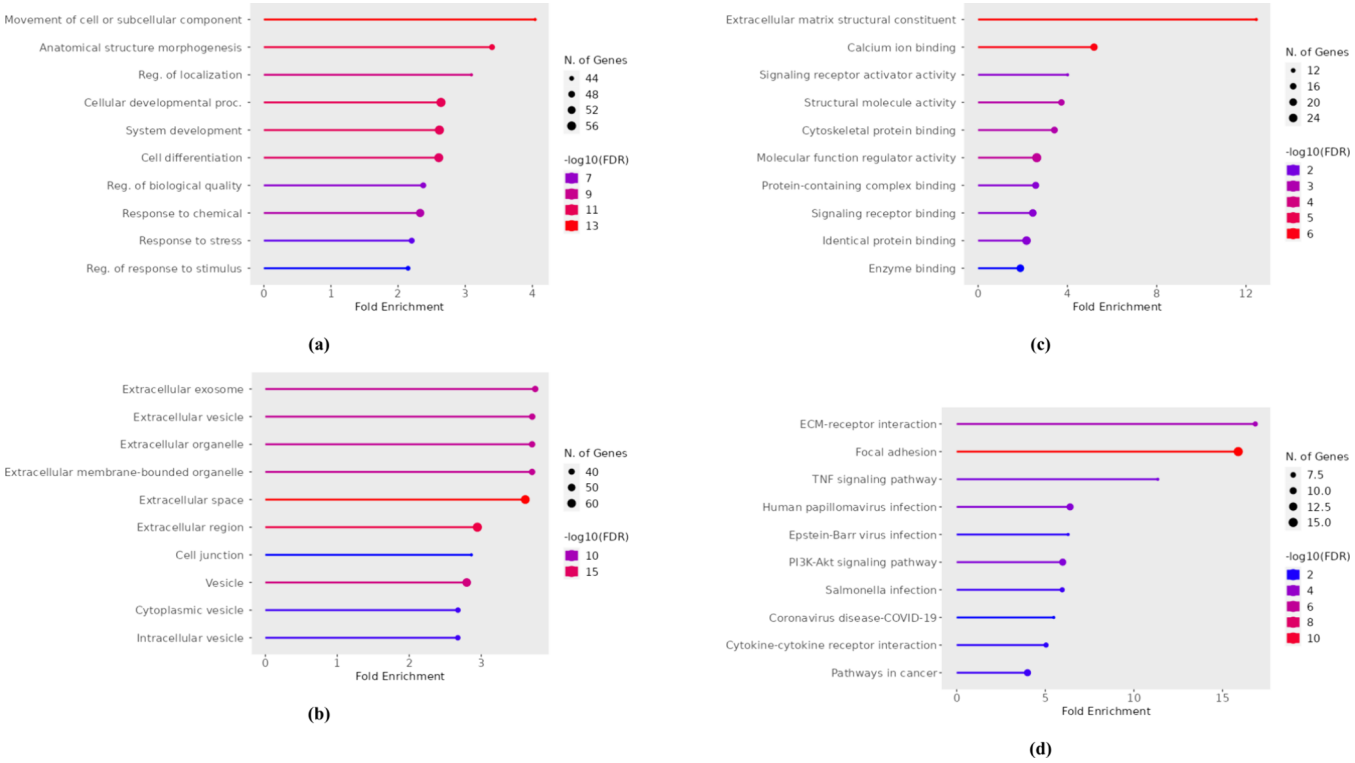

Figure 5. Functional enrichment analysis of DEGs: (a) biological process, (b) cellular component, (c) molecular function, and (d) KEGG. DEG—differentially expressed gene; KEGG—Kyoto Encyclopaedia of Genes and Genomes.

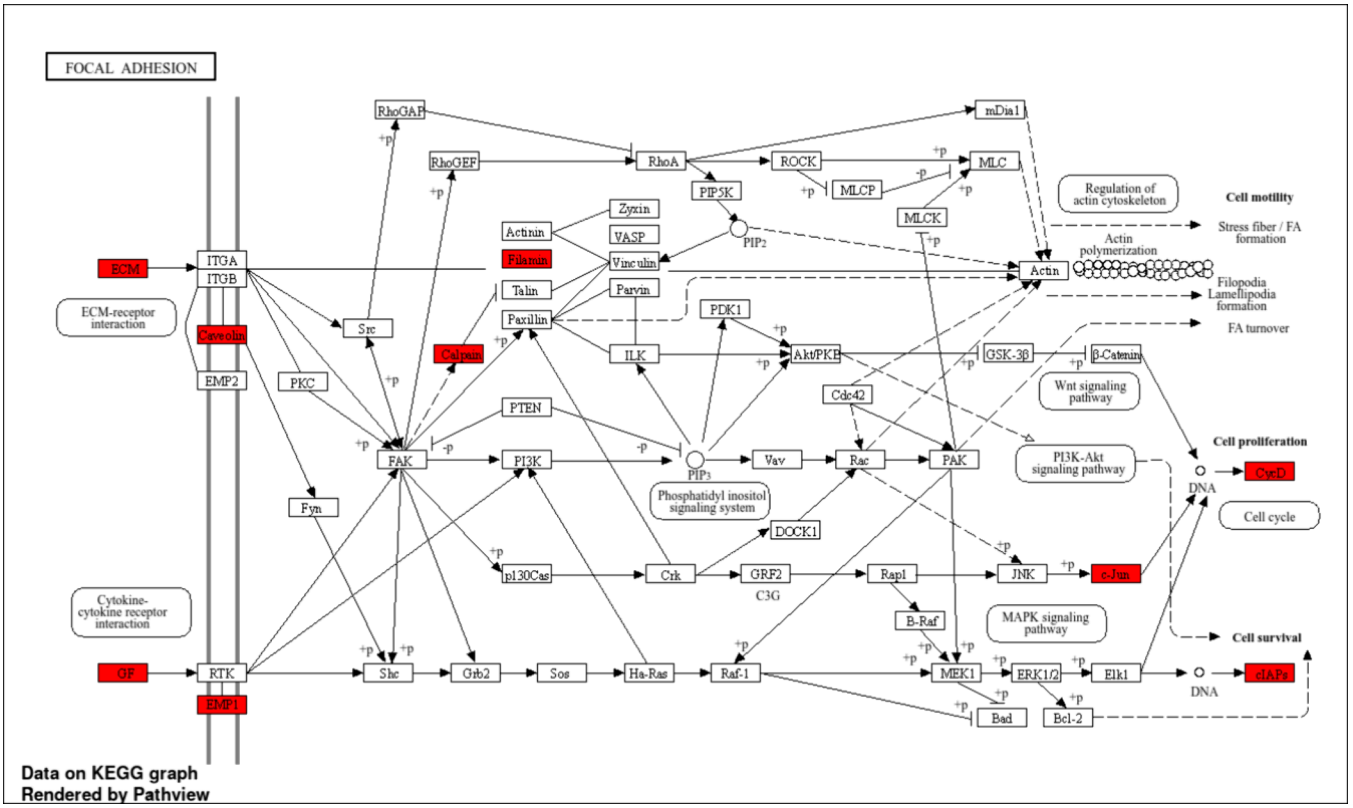

Figure 6. Schematic representation of the focal adhesion pathway. The expressed hub genes are highlighted in red color.

fatty liver disease (NAFLD) progression. Their work showed that overexpression of CAV1 protected HCC cells from apoptosis and promoted their proliferation in an environment rich in oleic acid, a fatty acid commonly found in abundance during NAFLD. This finding highlights CAV1, along with lipids

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Table 6. Top Enriched KEGG Pathways of the Hub Genes

S. no.	path IDs	KEGG pathways	no. of genes	*p*-value
1	path: hsa04510	focal adhesion	15	6.72×10^{-12}
2	path: hsa05165	human papillomavirus infection	10	0.000258286
3	path: hsa04151	PI3K-Akt signaling pathway	10	0.000349777
4	path: hsa05200	pathways in cancer	10	0.005936083
5	path: hsa04512	ECM−receptor interaction	7	1.99×10^{-5}
6	path: hsa05132	*Salmonella* infection	7	0.005936083
7	path: hsa04060	cytokine−cytokine receptor interaction	7	0.008381714
8	path: hsa04668	TNF signaling pathway	6	0.000613237
9	path: hsa05169	Epstein−Barr virus infection	6	0.008381714
10	path: hsa05171	Coronavirus disease-COVID-19	6	0.011774697

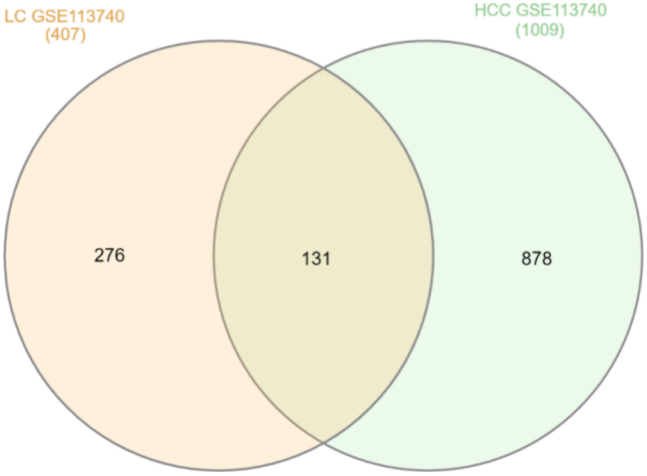

Figure 7. Venn Diagram of overlapping differentially expressed miRNAs (DEMs) obtained from GSE113740.

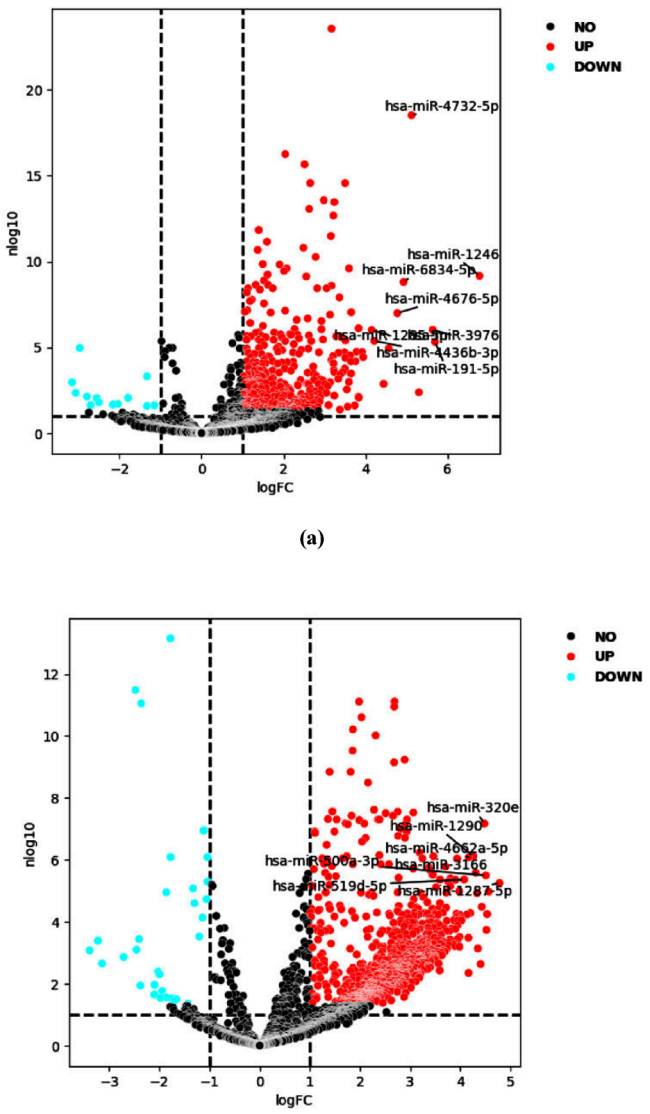

Figure 8. Volcano plot of DEGs between normal liver parenchyma and diseased tissues. (a) GSE113740 liver cirrhosis samples. (b) GSE113740 hepatocellular carcinoma samples.

and the enzymes regulating them, as potential targets for treatment of NAFLD-associated HCC.[49]

Recent research has focused on the potential of phosphoproteins enriched in astrocytes-15 (PEA15) as biomarkers for hepatocellular carcinoma (HCC). Quintavalle et al. demonstrated that high PEA15 expression correlated with poor patient survival and promoted cancer cell migration. Interestingly, their work also revealed that high PEA15 levels lessened the effectiveness of sorafenib, a common drug for HCC treatment.[50] Further strengthening the role of PEA15 as a biomarker, Wu et al. identified PEA15 phosphorylated at Ser116 as a candidate for prognosis alongside p27 phosphorylated at Thr187 residue.[51]

In another study, Chen et al. utilized bioinformatic analysis to explore potential markers for liver fibrosis. Their data pointed toward epithelial membrane protein (EMP1) as a promising candidate. The analysis revealed an association between EMP1 expression and liver fibrosis stages, suggesting its potential as a marker for disease progression.[52]

Xia et al. initially demonstrated the ability of enabled homologue (ENAH), along with other genes, to differentiate HCC tumors from surrounding healthy tissue. This suggests ENAH's potential as a diagnostic tool.[53] Building on this foundation, Deng et al. further explored ENAH's functional role in HCC. Their work revealed that ENAH promotes cancer cell proliferation, invasion, and migration. To understand the mechanism, they found that SF3B4, a protein regulating ENAH expression, promotes HCC development by activating Notch signaling. These findings not only solidify ENAH's potential as a biomarker but also highlight it as a candidate therapeutic target. By targeting ENAH or its regulatory pathways like SF3B4, researchers might be able to develop novel strategies to impede HCC progression.[54] In another study, Li et al. included PEA15 and ENAH among a panel of target genes for the long noncoding RNA (lncRNA) TUG1, with high expression of both TUG1 and its targets linked to poorer patient outcomes. While Li et al. focused on prognosis, their findings also suggest that PEA15 and ENAH, along with other identified genes, might hold promise for HCC diagnosis.[55] These findings pave the way for the further exploration of their potential role in diagnosis and therapeutic development.

Subsequent analysis using KMplot, a powerful survival analysis tool, identified two genes, EMP1 and ENAH, with

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Table 7. DEG−DEM Interaction Prediction

S. no.	miRNA	transcribed mRNA	gene symbol	binding energy (kcal/mol)
1	hsa-miR-1233-5p	NM_153321	PMP22	−25.4
2	hsa-miR-1238-5p	NM_016235	GPRC5B	−31.7
3	hsa-miR-1260a	NM_006763	BTG2	−23.2
5	hsa-miR-140-3p	NM_006868	RAB31	−27.1
4	hsa-miR-140-3p	NM_006763	BTG2	−21.5
6	hsa-miR-188-3p	NM_006763	BTG2	−26.6
7	hsa-miR-191-5p	XM_024448315	ENAH	−21.7
8	hsa-miR-197-5p	NM_003768	PEA15	−29
9	hsa-miR-2682-5p	NM_006763	BTG2	−21
10	hsa-miR-3125	NM_001759	CCND2	−21.3
11	hsa-miR-3158-3p	XM_024448315	ENAH	−25.2
12	hsa-miR-3612	NM_004226	STK17B	−19.9
13	hsa-miR-371a-5p	XM_024448315	ENAH	−26.9
14	hsa-miR-382-5p	NM_006636	MTHFD2	−20.3
15	hsa-miR-3976	NM_001759	CCND2	−17.4
16	hsa-miR-4428	NM_001759	CCND2	−21.6
17	hsa-miR-4441	NM_006763	BTG2	−17.6
18	hsa-miR-4514	NM_001367837	TPM4	−21.2
19	hsa-miR-4524b-3p	NM_004226	STK17B	−22.3
20	hsa-miR-4692	NM_001172895	CAV1	−23
21	hsa-miR-4692	NM_001367837	TPM4	−21.6
22	hsa-miR-494-5p	NM_003467	CXCR4	−22.7
23	hsa-miR-6509-5p	NM_006868	RAB31	−20.9
24	hsa-miR-654-3p	NM_001164178	ENTPD1	−24.5
27	hsa-miR-6753-5p	NM_001423	EMP1	−27.9
25	hsa-miR-6753-5p	NM_006763	BTG2	−26.9
26	hsa-miR-6753-5p	NM_001172896	CAV1	−21.9
28	hsa-miR-6760-5p	NM_006763	BTG2	−25.9
29	hsa-miR-6760-5p	NM_001759	CCND2	−25.3
30	hsa-miR-6769a-5p	NM_003768	PEA15	−26.8
31	hsa-miR-6864-3p	NM_001759	CCND2	−24.4
32	hsa-miR-6887-5p	NM_006636	MTHFD2	−28

potentially significant roles in HCC patient survival. These genes represented in Figure 11 showed a p-value ≤0.05, indicating a statistically relevant impact. By satisfying the criteria of both GEPIA and KMplot analysis, EMP1 and ENAH stand out as promising candidates for liver cirrhosis and HCC biomarkers. Patients with high EMP1 expression exhibited significantly longer overall survival compared to those with low expression (HR = 0.7, p = 0.047). Conversely, patients with low ENAH expression had significantly better survival outcomes than those with high expression (HR = 1.5, p = 0.034). We hypothesize that the variation in survival analysis results may be due to the limited availability of data on EMP1 expression in HCC patients. We believe that this finding will open new avenues for further research. In spite of these results, it is notable that among the 14 genes, these two genes significantly affect the overall survival of the patient. The present study considers only the p value; thus, we have taken both these genes into account, and further studies are required to elucidate the underlying mechanisms.

ENAH appears to be regulated by hsa-miR-191-5p, hsa-miR-3158-3p, and hsa-miR-371a-5p and EMP1 by hsa-miR-6753-5p. In a study, Wu et al. have identified miR-191-5p as overexpressed in HCC tissues. Analysis of gene expression data suggests a potential oncogenic role for miR-191-5p, targeting specific genes involved in tumor suppressor pathways, such as p75 neurotrophin receptor (p75-NTR) and liver kinase B1-mediated (LKB1) signaling. Additionally, miR-191-5p may regulate genes like early growth response 1 (EGR1) and ubiquitin conjugating enzyme E2 D3 (UBE2D3), which could contribute to HCC progression suggesting miR-191-5p as a potential target for HCC treatment.[56]

Zhang et al. in their study have reported that miR-371a-5p expression increased with the development of hepatic fibrosis, a condition often preceding liver cancer.[57] While the exact mechanisms remain unclear, Ernst et al. have shown a significant upregulation of miR-371a-5p in metastatic tumors compared to nonmetastatic ones.[58] This finding aligned with the observation in HCC, where higher miR-371a-5p levels correlated with poorer patient outcomes, suggesting a potential role for miR-371a-5p in promoting liver cancer progression and metastasis.

Studies have shown upregulation of hsa-miR-3158-3p in various diseases, including Alzheimer's disease.[59] Notably, Gupta et al. identified hsa-miR-3158-3p as a potential biomarker for cerebral malaria (CM) prognosis across all age groups suggesting hsa-miR-3158-3p as a noninvasive alternative to neuroimaging for monitoring disease progression in CM. Furthermore, if hsa-miR-3158−3p's connection to specific CM complications like brain swelling and oxygen deprivation is confirmed, it could become a valuable tool for developing future CM treatments.[60]

3.7. IHC Analysis. The protein level expression of EMP1 and ENAH specific to HCC was explored in the HPA database. A strong ENAH expression of about 75−25% was observed in HCC tissue sample (depicted in Figure 12), meaning that a significant portion of samples showed a high protein expression and a smaller portion of the samples showed low levels of protein expression. This implies that this gene could possibly play a role in the initiation and progression of HCC. The absence of EMP1 data reiterates the novelty of our study. Notably, this is the first research to highlight the overexpression of EMP1 in HCC patients. This emphasizes the need for further exploration of EMP1 in the context of HCC.

To the best of our knowledge, the involvement of hsa-miR-3158-3p and hsa-miR-6753-5p in liver diseases remains unexplored. This research unveils hsa-miR-371a-5p/ENAH and hsa-miR-191-5p/ENAH axes alongside novel regulatory

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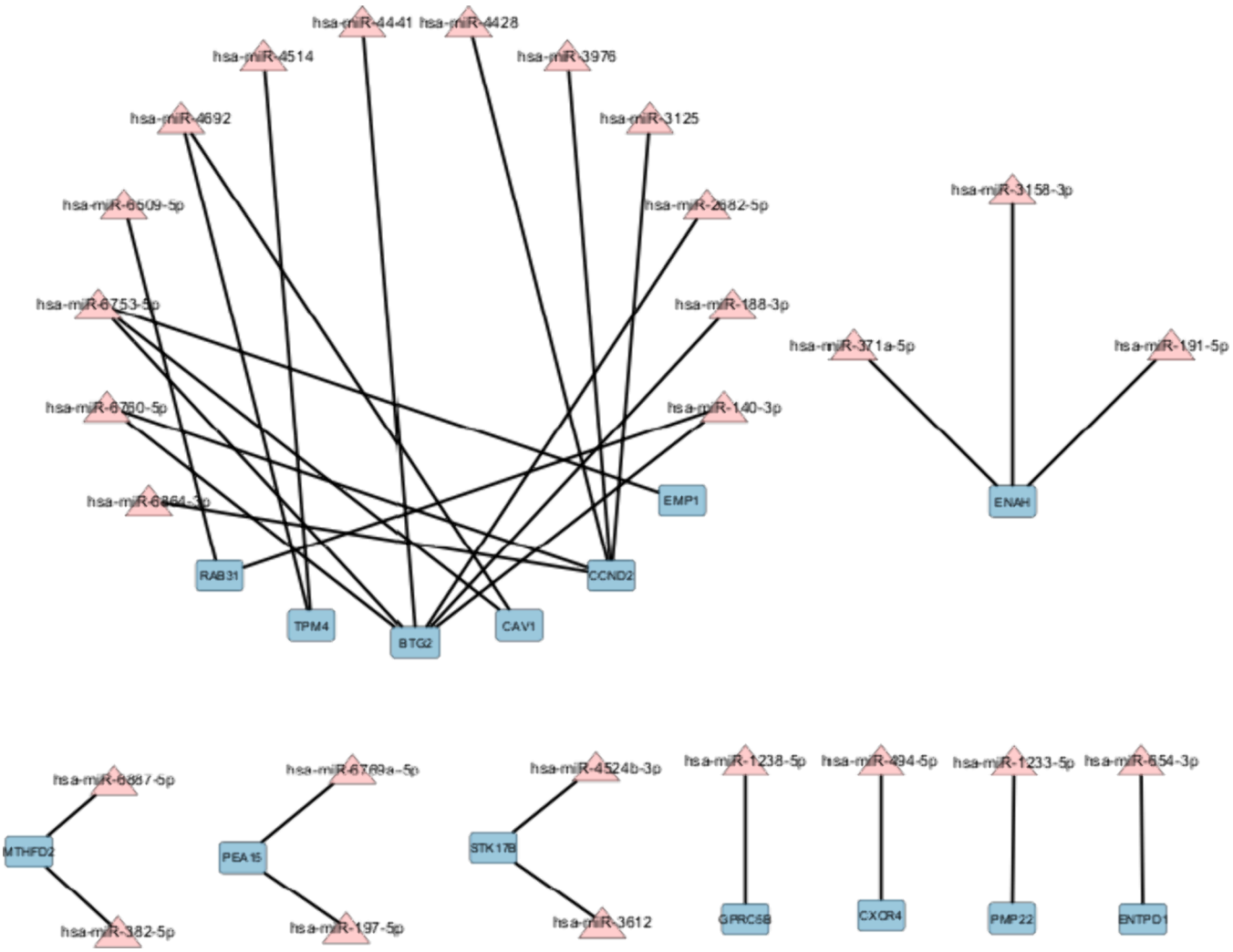

Figure 9. DEG−DEM interaction network. The miRNAs are represented in red triangles and genes in blue rectangles.

Table 8. Enrichment Analysis of DEMs by miRPathDB

S no.	miRNA	database	pathway	no. of Hits	*p*-value
1	hsa-miR-6760-5p	WikiPathways	signaling of hepatocyte growth factor receptor	28	0.02
2	hsa-miR-140-3p	WikiPathways	signaling of hepatocyte growth factor receptor	26	0.002
3	hsa-miR-4428	WikiPathways	signaling of hepatocyte growth factor receptor	19	0.018
4	hsa-miR-6887-5p	WikiPathways	ncRNAs involved in Wnt signaling in hepatocellular carcinoma	104	4.26×10^{-4}
5	hsa-miR-6769a-5p	WikiPathways	ncRNAs involved in Wnt signaling in hepatocellular carcinoma	51	0.05
6	hsa-miR-197-5p	WikiPathways	hepatitis C and hepatocellular carcinoma	2	0.049
7	hsa-miR-140-3p	KEGG	hepatitis B	42	0.048

mechanisms involving hsa-miR-3158-3p/ENAH and hsa-miR-6753-5p/EMP1, contributing to liver cirrhosis and HCC progression. Further investigations are warranted to explore the potential of these newly identified miRNAs as diagnostic biomarkers in liver diseases.

3.8. Prognostic Biomarkers in Cirrhosis to HCC. Additionally, we conducted a direct comparison of genes and miRNAs between liver cirrhosis and HCC tissues, identifying 234 genes and 1503 miRNAs that were dysregulated. A Venn diagram analysis (Figure S1) revealed that 70 genes and 1278 miRNAs were exclusively differentially expressed during the progression from liver cirrhosis to HCC. These genes hold potential as biomarkers for predicting HCC risk in liver cirrhosis patients.

Subsequently, we determined the miRNA targets of these 70 genes and focused on those miRNAs that overlapped with the dysregulated miRNAs identified in the cirrhosis to HCC progression (Table S1). To elucidate the functional implications of these genes, we performed GO and pathway enrichment analyses using ShinyGO (Figure S2 and Tables S2−S5). Survival analysis using the GEPIA2 tool indicated that AGO3, NCOA3, and TNPO1 were significantly associated with poor survival ($p < 0.05$, HR > 1), suggesting that high expression levels of these genes are linked to higher progression (Figure S3). IHC analysis done using HPA revealed decreased NCOA3 expression and increased TNPO1 expression in HCC tissues compared with normal liver tissue (Figure S4), corroborating our previous findings. Given the previously established roles of these genes in

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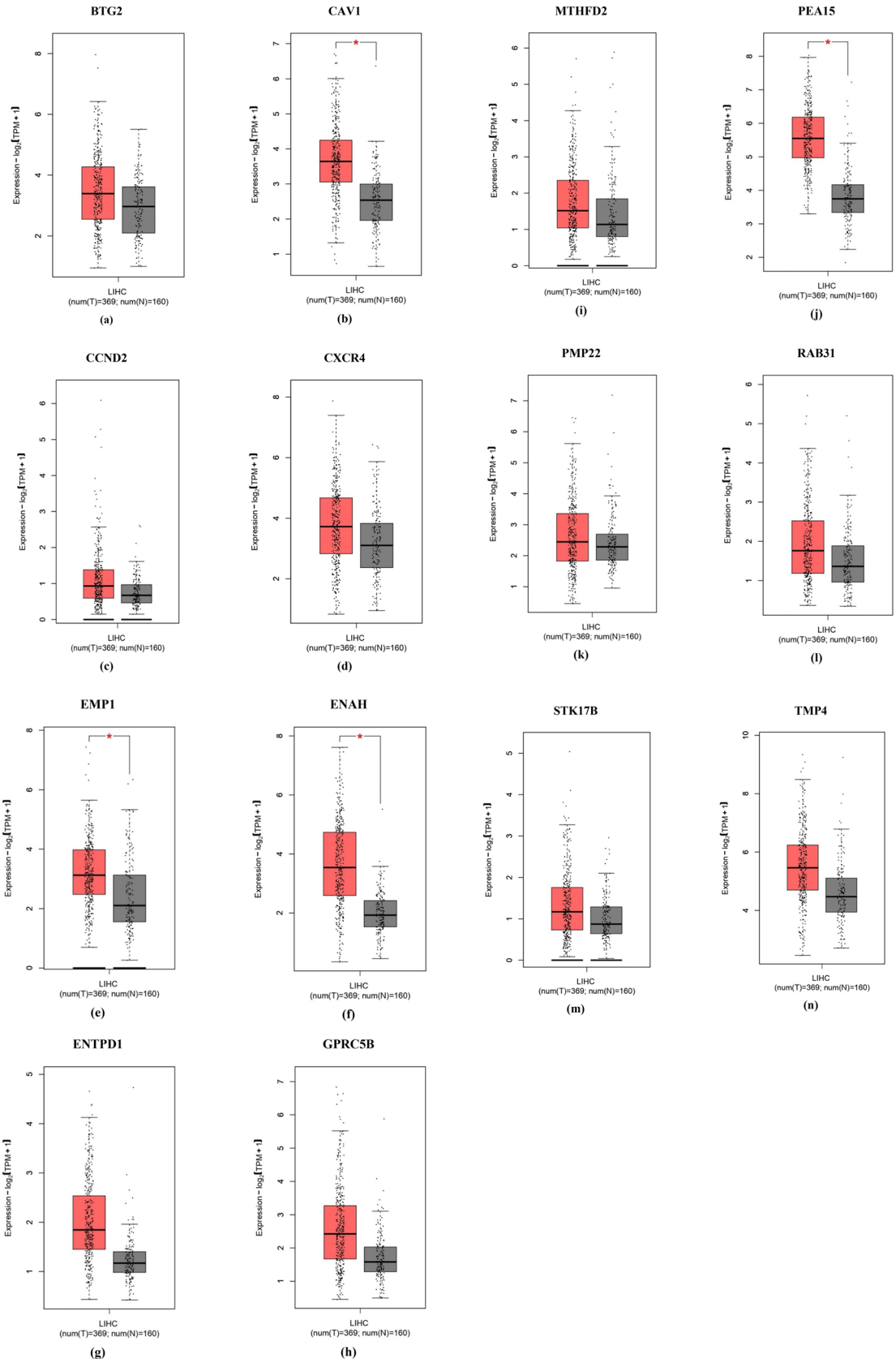

Figure 10. Validation of the hub genes by GEPIA. The symbol asterisk indicates the significance level of the comparison. The genes CAV1, EMP1, ENAH, and PEA15 were shown to have a significant difference in expression in normal and tumor samples. T—HCC samples and N—normal liver parenchyma tissue.

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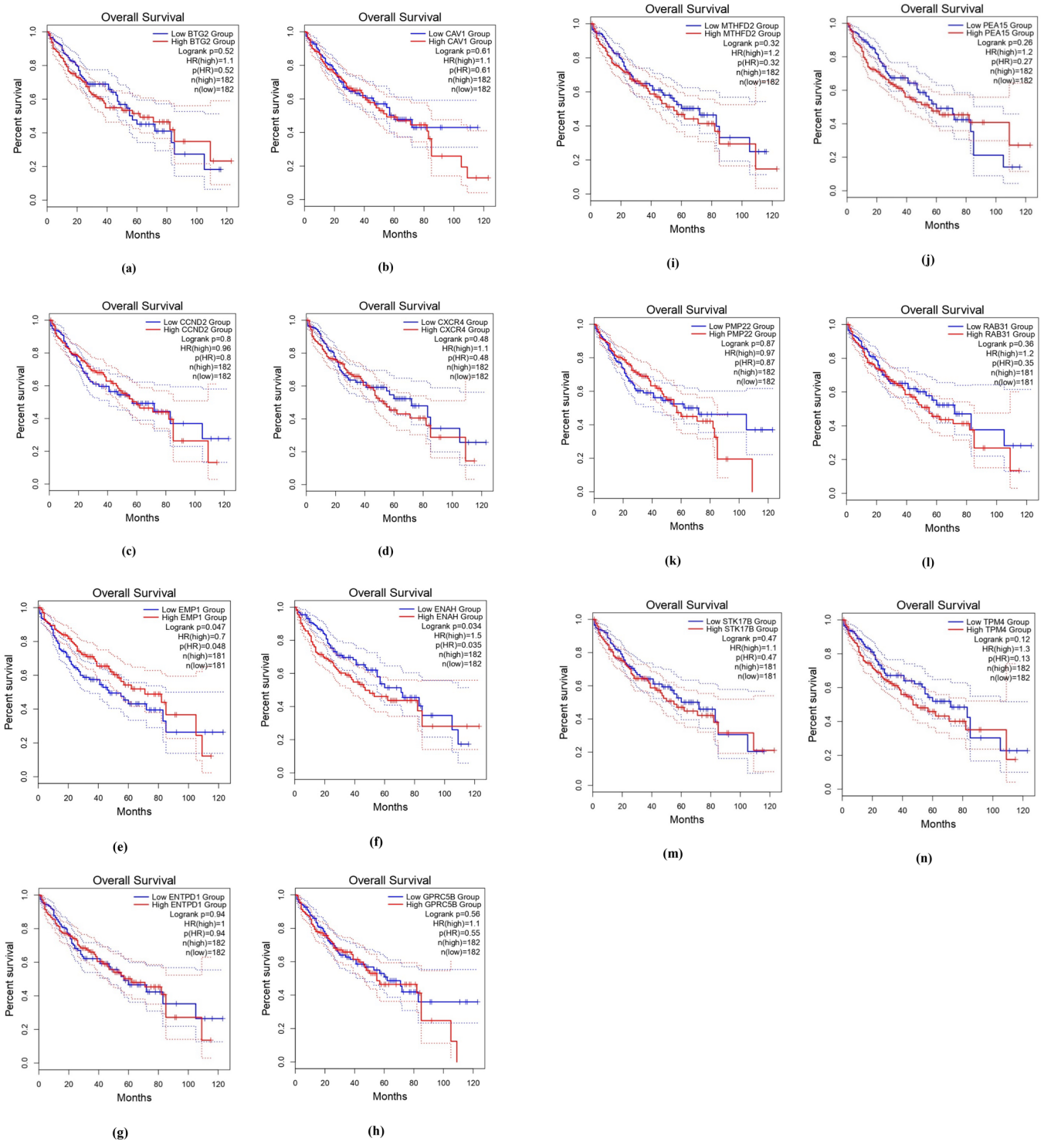

Figure 11. Overall survival plot of the hub genes. Genes EMP1 and ENAH shows significant impact in the patient survival.

HCC, they may serve as valuable biomarkers specifically for HCC development in liver cirrhosis patients.

4. CONCLUSIONS

Liver cirrhosis and HCC progression are poorly understood at the molecular level. Aiming to bridge this knowledge gap, this present study analyzes the gene expression data, identifying DEGs and DEMs. The identified DEGs suggest a potential role for the focal adhesion pathway in disease development, while the DEMs point toward the involvement of the Wnt signaling pathway specifically in HCC. To gain deeper insights, the study constructed a network of interacting DEGs and DEMs allowing for a more comprehensive understanding of the underlying molecular mechanisms. Following this, boxplots were generated, identifying four genes, CAV1, PEA15, EMP1, and ENAH with significant expression differences between healthy and tumor tissues. Interestingly, survival analysis identified ENAH and EMP1 as key genes that significantly impact the overall survival of patients. These key genes are regulated by a group of four miRNAs (hsa-miR-191-5p, hsa-miR-3158-3p, hsa-miR-371a-5p,

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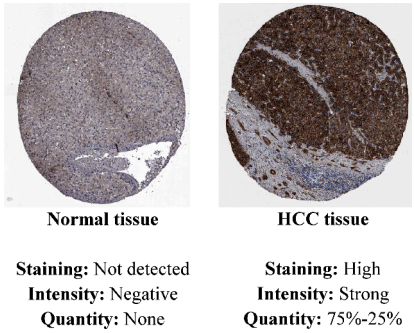

Figure 12. Protein level expression of ENAH in normal liver and HCC tissues.

and hsa-miR-6753-5p). The role of ENAH was validated through an IHC analysis. Additionally, the lack of existing data on EMP1 expression in HCC reiterates the novelty of our findings, marking a significant contribution to further research. This gene−miRNA regulatory axis not only aids in pinpointing disease-related genes but also refines the biomarker selection for improved specificity. However, this study presents the first identification of the ENAH/hsa-miR-3158-3p and EMP1/hsa-miR-6753-5p regulatory axes in liver cirrhosis and HCC widening the understanding of the molecular basis of the disease. Direct comparison of genes between liver cirrhosis and HCC unravels AGO3, NCOA3, and TNPO1 genes along with their regulatory miRNAs, which could potentially serve as diagnostic and prognostic biomarkers in liver cirrhosis patients. These findings underscore the importance of these genes in advancing diagnostic and therapeutic strategies. Further investigation is needed to explore these newly identified miRNAs as potential diagnostic biomarkers for liver diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06551.

miRNA targets for the dysregulated genes, top enriched Gene Ontology and KEGG pathways of the DEGs between cirrhosis and HCC, Venn diagram of genes between healthy, cirrhotic and tumor samples, survival analysis, and protein level expression of NCOA3 and TNPO1 in normal liver and HCC tissues (PDF)

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Author Contributions

V.P. performed the computational work, analyzed the data, and prepared tables and figures. S.V. conceived this study and is responsible for the overall design, interpretation, manuscript preparation, and communication. All authors have reviewed and approved the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HCC - hepatocellular carcinoma
miRNA - microRNA
mRNA - mRNA
DEGs - differentially expressed genes
DEMs - differentially expressed miRNAs
UTR - untranslated region
GEO - Gene Expression Omnibus
FDR - false discovery rate
STRING - Search Tool for the Retrieval of Interacting Genes/Proteins
GO - Gene Ontology
BP - biological process
CC - cellular components
MF - molecular function
KEGG - Kyoto Encyclopedia of Genes and Genomes
GEPIA 2 - Gene Expression Profiling Interactive Analysis 2
TCGA - The Cancer Genome Atlas
GTEx - genotype-tissue expression
KM - Kaplan−Meier
ECM - extracellular matrix
TME - tumor microenvironment
HSCs - hepatic stellate cells
EVs - extracellular vesicles
FA - focal adhesion
FAK - focal adhesion kinase
LSECs - liver sinusoidal endothelial cells
HCV - hepatitis C virus
HBV - hepatitis B virus
NAFLD - nonalcoholic fatty liver diseases
CAV1 - caveolin 1
PEA15 - phosphoprotein enriched in astrocytes-15
EMP1 - epithelial membrane protein
ENAH - enabled homologue

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