Received: 14 October 2025 | Revised: 23 January 2026 | Accepted: 29 January 2026

DOI: https://doi.org/10.69547/NS.41.02



NATURE'S SYMPHONY

ISSN-E: 3007-2034

ISSN-P: 3007-2026

Website link: www.tsfns.com

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To cite: Baig, EA., Ashraf, MR., Noreen, S. and Nawaz, S. 2026. Bioinformatics-driven Discovery of Key Biomarkers and Therapeutic Targets in Hepatocellular Carcinoma. *Nature's Symphony*, 4(1):21-46.

Article QR

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Published online: 16 February 2026

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Research Article

Bioinformatics-driven Discovery of Key Biomarkers and Therapeutic Targets in Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) ranks as the sixth most common cancer globally and is also the third leading cause of deaths caused by cancer. Although the current biomarkers of HCC such as alpha-fetoprotein (AFP) and des-γ-carboxyprothrombin (DCP) have proven clinical utility in HCC management, their limited sensitivity and specificity necessitate the discovery of novel prognostic and therapeutic markers with improved sensitivity. This study aimed to identify novel prognostic biomarkers and therapeutic targets for HCC by integrated bioinformatics and systems biology approaches. Expression profiling in two HCC datasets from NCBI-GEO (GSE121248 and GSE102079) led to the discovery of 205 common DEGs, 137 of which were up-regulated and 68 were down-regulated genes. Biological pathway enrichment analysis showed important roles of metabolic dysfunction, cell cycle regulation, complement activation, xenobiotic metabolism, and Jak-STAT signaling pathways in the process of hepatocarcinoma formation. Protein–protein interaction network and Cytoscape analyses identified six key seed genes: BIRC5, RDH16, ADH1B, TTC36, ROBO1, and NQO1, all exhibiting differential expression between tumor tissues and their normal counterparts. The seed genes identified in this study have a much wider biological role as compared to the traditional HCC biomarkers, which include inhibition of apoptosis, oxidative stress response, metabolic equilibrium, suppression of tumors, and metastasis-related signaling. It is worth mentioning that the increased expression of BIRC5 in advanced HCC patients can indicate its utility as a potential prognostic and therapeutic target, making Survivin-based therapy a potentially useful approach for managing HCC.

Keywords:
Cancer, Biomarkers, Hepatocellular Carcinoma, Liver cancer, Bioinformatics, Gene Expression Analysis, Seed Genes, Survival Analysis

1. INTRODUCTION

Hepatocellular carcinoma (HCC), also called Liver cancer comprises ~90% of primary liver malignancies (Asafo-Agyei and Samant, 2026). Liver cancer (LC) stands out as the only cancer where survival rates have not shown improvement in the past few decades (Ladd *et al.*, 2024). HCC has a very high mortality rate with the survival of most of the patients measured in months after diagnosis rather than in years. In 2022, there were over three-quarters

of a million liver cancer deaths globally, ranking LC third in cancer related deaths after lung and colorectal cancers (Bray *et al.*, 2024).

HCC cases have increased throughout the country due to high incidence of HBV and HCV in Pakistan (Konyn *et al.*, 2021; Ullah Khan *et al.*, 2024). Limited access to screening, vaccination, and treatment of HBV are also contributing factors to new cases of HCC (Sayiner *et al.*, 2019). Depending on the disease's stage, liver cancer is currently treated with chemotherapy, radiation and surgery (Mahdiannasser and Karami, 2018). Determining the entire value of HCC screening requires an understanding of its benefits, considering potential financial, psychological, and physical risks (Narasimman *et al.*, 2024).

Currently, early diagnosis is crucial for better therapeutic management of HCC, including surgery (liver transplantation), and liver resection by monitoring high-risk patients using daily ultrasound and tumor markers measurement or not (Singh *et al.*, 2020). Alpha-fetoprotein (AFP) is a key biomarker used for HCC in recent years (Goossens *et al.*, 2015). Recently, *PON1*, an enzyme made of glycoproteins, was proposed as a new marker of HCC using iTRAQ quantitative proteomics (isobaric tags for relative and absolute quantification) (Zhang *et al.*, 2020).

Although Alpha-fetoprotein is the most common marker for liver cancer diagnosis since 1964 (Ius, 1964) but not fully optimal with respect to its sensitivity and specificity (Juárez-Hernández *et al.*, 2017). The tumors that are 3 cm or less are 25% less sensitive while differential expression of AFP is almost undetected in 80% of small hepatic carcinoma (Saffroy *et al.*, 2007). AFP-L3 being expressed only by HCC cells serves as an early diagnostic indicator of HCC (Wang and Wei, 2020).

Des-γ-carboxyprothrombin (DCP), alternatively known as prothrombin raised by lack of vitamin K (PIVKA-II), is considered for early stages HCC detection. DCP is an inactive prothrombin generated by HCC (Beudeker and Boonstra, 2020). The lack of screening markers and bad prognosis regarding HCC emphasize the importance of new biomarkers in the development of human hepatocarcinogenesis. Modern gene expression analysis allows researchers to pinpoint specific genetic patterns and the biochemical processes linked to HCC (Okabe *et al.*, 2001; Mas *et al.*, 2009). The use of microarray and bioinformatics technologies is valuable in identifying disease biomarkers. These tools play a significant role in studying disease-related genes and the molecular mechanisms underlying disease progression. Microarray technology has become increasingly popular in investigating alterations in cancer gene expression (Tao *et al.*, 2017).

The study aimed to use bioinformatics tools and systems biology approach to analyze Differentially Expressed Genes (DEGs) from NCBI GEO datasets, followed by GO and KEGG pathway enrichment analyses to investigate the biological functions regulating these DEGs. Additionally, Protein-Protein Interactions (PPI) and survival analyses were conducted to identify key genes and pathways.

2. MATERIALS AND METHODS

2.1 Gene Expression Datasets

A manual search in the GEO public repository, available at (https://www.ncbi.nlm.nih.gov/geo) (Davis and Meltzer, 2007), was performed in the initial phase. Two microarray gene expression datasets i.e., GSE121248 (Wang *et al.*, 2007) and GSE102079 (Chiyonobu *et al.*, 2018) were retrieved from the GEO website and selected for this study. The platform used for analysis for both datasets was GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array. The two datasets were different from each other in terms of sample

size with GSE102079 including 243 HCC and 14 adjacent normal tissue samples, whereas GSE121248 included 37 normal tissues and 70 tumor samples.

2.2 Differentially Expressed Genes (DEGs)

GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used for the analysis of Differentially Expressed Genes (DEGs) in tumor and healthy tissues. To lessen the false positive outcomes, using the Benjamini-Hochberg approach, adjusted p-values were calculated with cutoff threshold fixed at ≤ 0.05. The log2FC cut offs used for DEGs both up and down regulated was±1.5.

2.3 Ontology and Pathway Enrichment of Common DEGs

The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/tools.jsp; v6.8) and Metascape was utilized for ontology enrichment of the common DEGs (Zhou *et al.*, 2019; Lei *et al.*, 2021). The enrichment was performed in terms of molecular function (MF), biological processes (BP), KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, GAD_Diseases, GAD_Diseases_Class and DISGENET.

2.4 Protein-Protein Interaction (PPI) Network

Using information from the GeneMANIA, a PPI network was built (http://genemania.org/), while Cytoscape app was used to visualize it (http://www.cytoscape.org, v3.8) (Franz *et al.*, 2018). The gene identifiers of common DEGs identified in the study were submitted to Gene MANIA with default parameters and resulting interactions were saved in a TSV format (Shannon *et al.*, 2003; Smoot *et al.*, 2011), examined with Cytoscape Network Analyzer plugin (Assenov *et al.*, 2008). Furthermore, the Molecular Complex Detection (MCODE) module (Bader and Hogue, 2003) was utilized for analyzing densely packed clusters or modules in the PPI network.

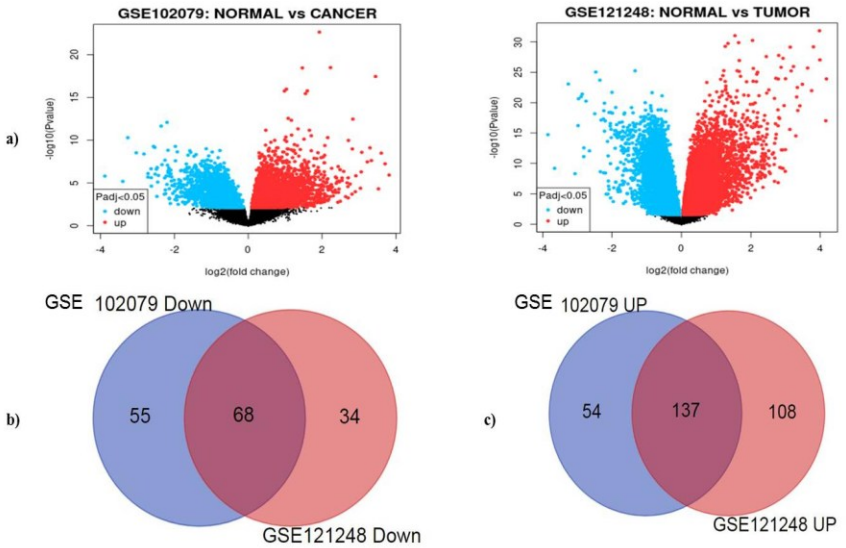


Figure 1. (a) Volcano plots of DEGs as identified in GEO Datasets (b) 68 Common down-regulated DEGs and (c) 137 Common up-regulated DEGs

Table 1. Common up and down-regulated DEGs Identified in HCC datasets

DEGs	Gene Symbols
Up Regulated (137)	*MT1G, CYP4A22///CYP4A11, GREM2, BBOX1, CYP26A1, CYP2A6, PLG, SDS, ABCA8, SERPINE1, PGLYRP2, SOCS2, LINC01093, CYP2C8, CXCL14, SLC22A1, IGF1 SULT1E1, CYP39A1, MBNL2, HAO2, LINC01554, FAM134B, MT1F, SLC25A47, MFSD2A, EGR2, FLJ22763, HHIP, APOA5, ADH1B, KCND3, KCNN2, CFHR4, SLCO1B3, SORL1, PRG4, ASPA, RCAN1, CYP1A2, INS-IGF2///IGF2, MT1E, CNDP1, BCO2, FCN3, ACSM3, FOLH1B///FOLH1, GBA3, CYP2C19, ANXA10, TTC36, LOC100287413///GLYATL1, CLEC4G, C3P1, CYP2B6, GYS2, FXYD1, KBTBD11, FOLH1B, KMO, LPA, GHR, CLEC1B, MIR675///H19, CXCL2, TAT, FOSB, LIFR, CYP2C9, CLRN3, CFHR3, CYP2A7, CYP2E1, MT1H, LCAT, CTH, ATF5, CLEC4M, VNN1, LYVE1, ESR1, FOS, LOC101928916///NNMT, PLAC8, HAMP, ALDOB, DNASE1L3, DCN, NAT2, RND3, BCHE, IL1RAP, C8ORF4, RDH16, AKR1D1, CXCL12, GNMT, TMEM27, AFM, HPGD, CRHBP, MME, AVPR1A, WDR72, F9, CYP4A11, HGFAC, IGFALS, ADGRG7, ACADL, HAL, PZP, FBP1, AADAT, ADH4, GPM6A, OIT3, HGF, MT1M, CYP3A4, GLYAT, CYP2B7P///CYP2B6, GLS2, SRD5A2, ADRA1A, ECM1, C6 SLC3A1, PBLD, APOF, PLGLB1///PLGLB2, C9 FCN2, OAT, LINC00844*
Down Regulated (68)	*DNAJC6, CDK1, FAM72A///FAM72D///FAM72B///FAM72C, SPINK1, UBD///GABBR1, COL4A1, CENPW, CAP2, PEG10, DTL, RACGAP1, CDC20, FAM83D, CTHRC1, UHRF1, EDIL3, RRM2, ZWINT, CCNB1, NDC80, TOP2A, KIAA0101, ASPM, HELLS, REG3A, HMMR, CD24, KIF4A, TTK, LYZ, CDKN3, AKR1B10, PBK, NCAPG, GINS1, GPC3, SULT1C2, COL1A2, CCL20, ROBO1, CENPU, SPP1, PRR11, ZIC2, MAP2, PTTG1, NEK2, ANLN, ACSL4, BIRC5, KIF20A, SQLE, UBE2T, DUXAP10, CRNDE, CENPF, NUSAP1, NQO1, BUB1B, MAD2L1, COL15A1, DLGAP5, ECT2, CCNB2, RBM24, RAD51AP1, PRC1*

2.5 Seed Genes Expression Validation

To validate the study findings, the Gene Expression Profiling Interactive Analysis (GEPIA) server was utilized. By combining data both from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases, this server enables comparative gene expression analysis in tumor and normal tissues with a statistical significance threshold of q-value ≤ 0.01 and Log_2FC cutoff of 1. UALCAN data analysis portal, a comprehensive cancer database containing genomic, transcriptomic, epigenetic, and clinical datasets, was employed to further examine differential expression profile of seed genes across HCC stages and to gain insight into their potential clinical relevance in HCC. To explore the relation between methylation and gene expression of HCC DEGs, the cBioPortal platform was utilized (Cerami *et al.*, 2012).

2.6 Survival Analysis of Seed Genes

Survival analysis of the seed genes was accomplished via GEPIA online tool to check the clinical relevance of the identified seed genes. The survival plots were made with Log Rank P- value and the hazard ratio (HR) at a 95% confidence interval. In survival analysis, a hazard ratio (HR) more than 1 shows that higher gene expression is linked with higher risk of HCC, while an HR less than 1 indicates higher gene expression association with lower risk. An HR of 1 suggests no change in risk between the groups (De Paula Souza *et al.*, 2023).

3. RESULTS

3.1 HCC DEGs Identification

Differential HCC genes were analyzed from the two datasets used in this study with the assistance of the NCBI GEO2R tool. A total of 317 and 240 up-regulated differentially expressed genes from the GSE121248 and GSE102079 datasets were selected ($|log2FC| \geq 1.5$ and adjusted P-value ≤ 0.05). Additionally, 115 and 140 down-regulated DEGs were recognized from GSE121248 and GSE102079 ($|log2FC| \leq -1.5$ and adjusted P-value ≤ 0.05) (Fig. 1a). Furthermore, a Venn diagram was used to find the common DEGs across the two datasets, revealing a set of 205 shared DEGs, which included 137 up-regulated ($log2FC \geq 1.5$) and 68 down-regulated DEGs ($log2FC \leq -1.5$) in HCC (Table 1, Fig. 1b & 1c).

3.2 Enrichment Analysis

To further investigate the functions of the identified DEGs, functional and pathway enrichment analyses were conducted by DAVID. The genes identified with high levels in HCC primarily engaged in biological processes related to oxidation-reduction, proteolysis, and the JAK-STAT cascade, sister chromatid segregation and mitotic nuclear division, the cell cycle etc. (Table 2). HCC DEGs demonstrated significant enrichment in genetically associated diseases (GAD) linked to cancer development, including liver cancer (*CYP1A2, CYP2E1, CYP3A4*; Table 3). KEGG pathways (P-value ≤ 0.05) associated with HCC DEGs were caffeine metabolism, cysteine and methionine metabolism and glycolysis/gluconeogenesis representing metabolic reprogramming a hall mark for HCC progression.

DisGeNET analysis revealed association of HCC DEGs with diseases like spontaneous hepatitis, bacterial infection, pancreatic neoplasm, fatty liver disease, hyperlipidemia, and cholestasis (Fig. 2a). Additionally, PaGenBase analysis revealed the presence of DEGs across various tissues, including liver, kidney, brain cells, smooth muscles, placenta, and breast cells (Fig. 2b). Protein-protein interaction network of HCC DEGs subjected to ontology enrichment identified 20 enriched clusters most of which were related to complement activation, metabolic reprograming, response to metal ions etc. (Fig. 3a & b).

Table 2. GO enrichment of DEGs

Expression	GO Category	GO Terms	%	*P-Value*
Up-Regulated	GOTERM_BP_ALL	Proteolysis	7.31	0.0038
		Xenobiotic metabolic process	12.19	0.0005
		Aging	4.06	0.0112
		JAK-STAT cascade	2.43	0.0272
		Aflatoxin metabolic process	1.62	0.0295
		Biosynthetic process	2.43	0.0138
		Complement activation	4.06	0.0000
	GOTERM_MF_ALL	Monooxygenase activity	8.9	0.0000
		Arachidonic acid epoxygenase activity	6.5	0.0000
		Aromatase activity	7.3	0.0000
		Iron ion binding	10.6	0.0000
		Caffeine oxidase activity	3.3	0.0000

Expression	GO Category	GO Terms	%	*P-Value*
		Estrogen 2-hydroxylase activity	2.4	0.0003
		Steroid binding	3.3	0.0008
		Signaling receptor binding	6.5	0.006
	GOTERM_CC_ALL	Extracellular region	27.6	0.0000
		Extracellular space	26	0.0000
		Neuronal cell body	5.7	0.02
		Mitochondrial matrix	5.7	0.03
		Membrane attack complex	1.6	0.04
Down-regulated	GOTERM_BP_ALL	Cell cycle process	48.4	0.0000
		Cell cycle	53.1	0.0000
		Sister chromatid segregation	25	0.0000
		Mitotic division	23.4	0.0000
		Spindle organization	21.9	0.0000
		Positive cell cycle regulation	23.4	0.0000
		Cytoskeleton organization	32.8	0.0000
	GOTERM_MF_ALL	Microtubule binding	17.2	0.0000
		Tubulin binding	17.2	0.0000
		Binding with Protein	90.6	0.0000
		Cytoskeletal protein binding	20.3	0.0000
		Extracellular matrix structural constituent	7.8	0.002
		Identical protein binding	25	0.003
	GOTERM_CC_ALL	Collagen network	3.1	0.04
		Supramolecular fiber	18.8	0.003
		Membrane-bounded organelle	87.5	0.002

Table 3. Enrichment of HCC DEGs in terms of GAD_Disease, GAD_Disease Class and KEGG pathways

Category	Terms	Genes	%	P-Value
GAD_ DISEASE	Smoking	*NAT2, CYP1A2, CYP2A6, CYP2B6, ESR1*	4.1	0.0004
	Alcohol consumption	*FOS, ADRA1A, ADH1B, ADH4, AVPR1A, CRHBP, CYP2E1*	5.7	0.0007
	Liver cancer; liver disease	*CYP1A2, CYP2E1, CYP3A4*	2.4	0.0006
	HIV Infections	*CXCL12, CLEC4M, CYP2A6, CYP2B6, CYP2E1, CYP3A4*	4.9	0.0002
	Hepatoxicity, drug-induced	*CYP2C19, CYP2C9*	1.6	0.01
GAD_ DISEASE_ CLASS	Metabolic	*HPGD, ABCA8, CXCL12, CXCL14, CLEC1B, FOS, FOSB, HGFAC, LIFR, NAT2, PZP, RND3, WDR72, ACADL, ACSM3, ADRA1A, AFM, ADH1B, ALDOB, AADAT, APOA5, APOF,*	65.9	0.0000

Category	Terms	Genes	%	P-Value
		AVPR1A, ASPA, BCHE, CNDP1, F9, C9, CFHR3, CFHR4, CRHBP, CTH, CYP1A2, CYP2A6, CYP2A7, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2E1, CYP26A1, CYP3A4, CYP4A11, DCN, EGR2, ESR1, FBP1, BBOX1, GBA3, GLS2, GNMT, GYS2, GPM6A, GREM2, GHR, HHI, HGF, HAMP, IGF1, IGFALS, IL1RAP, LCAT, LPA, MT1E, MBNL2, OAT, PLG, KCNN2, KCND3, PRG4, SDS, SERPINE1, SLC22A1, SLC3A1, SLCO1B3, SORL1, SRD5A2, SULT1E1, SOCS2, TAT, VNN1		
	Reproduction	*HPGD, CXCL12, NAT2, ADH1B, APOA5, CRHBP, CTH, CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP2E1, CYP3A4, ESR1, GHR, HAMP, IGF1, IL1RAP, LCAT, LPA, PLG, SERPINE1, SRD5A2*	18.7	0.0000
	Developmental	*NAT2, NQO1, ADH1B, ADH4, APOA5, ASPM, CTH, CYP1A2, CYP2A6, CYP2E1, CYP3A4, DCN, ESR1, GNMT, GHR, IGF1, IGFALS, IL1RAP, LCAT, MME, SPP1, SERPINE1, SLCO1B3, SRD5A2, SOCS2, PLG, MT1F, NCAPG, LYZ, HHIP, FBP1, CYP26A1, CAP2, COL1A2, CENPW, ASPA, RND3, WDR72, ZIC2, ACSL4*	24.4	0.0000
	Cancer	*HPGD, BUB1B, CCL20, CXCL12, CXCL14, CD24, TOP2A, FOS, LIFR, MFSD2A, NAT2, NQO1, NDC80, NEK2, PTTG1, TTK, ZWINT, ADRA1A, ADH1B, ADH4, ALDOB, APOA5, ASPM, BIRC5, BCHE, CDC20, CENPF, CTHRC1, C6, C9, CCNB2, CDK1, CTH, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2E1, CYP3A4, CYP39A1, DCN, DTL, EGR2, ESR1, GNMT, GHR, HELLS, HGF, HAL, HMMR, HAO2, IGF1, IGFALS, IL1RAP, LCAT, MME, MAD2LI, PGLYRP2, RCAN1, SPP1, SPINK1, SERPINE1, SLC22A1, SLCO1B3, SRD5A2, SULT1C2, SULT1E1, SOCS2*	37.4	0.0000
	Chemdependency	*CXCL14, FOS, NAT2, PZP, WDR72, ATF5, ACSM3, ADRA1A, ADH1B, ADH4, AKR1D1, AVPR1A, ASPA, BCHE, CNDP1, CLRN3, CRHBP, CTH, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2E1, CYP3A4, DCN, DNASE1L3, ESR1, BBOX1, GYS2, GPM6A, GREM2, HHIP, IGF1, IL1RAP, LPA, MBNL2, KCND3, RCAN1, SLC22A1, SORL1, SULT1E1*	34.1	0.01
KEGG_ PATHWAY	Caffeine metabolism	*NAT2, CYP1A2, CYP2A6, CYP2A7*	3.3	0.0000

Category	Terms	Genes	%	P-Value
	Cysteine and methionine metabolism	*CTH, GNMT, SDS, TAT*	3.3	0.01
	Glycolysis/ Gluconeogenesis	*ADH1B, ADH4, ALDOB, FBP1*	3.3	0.02

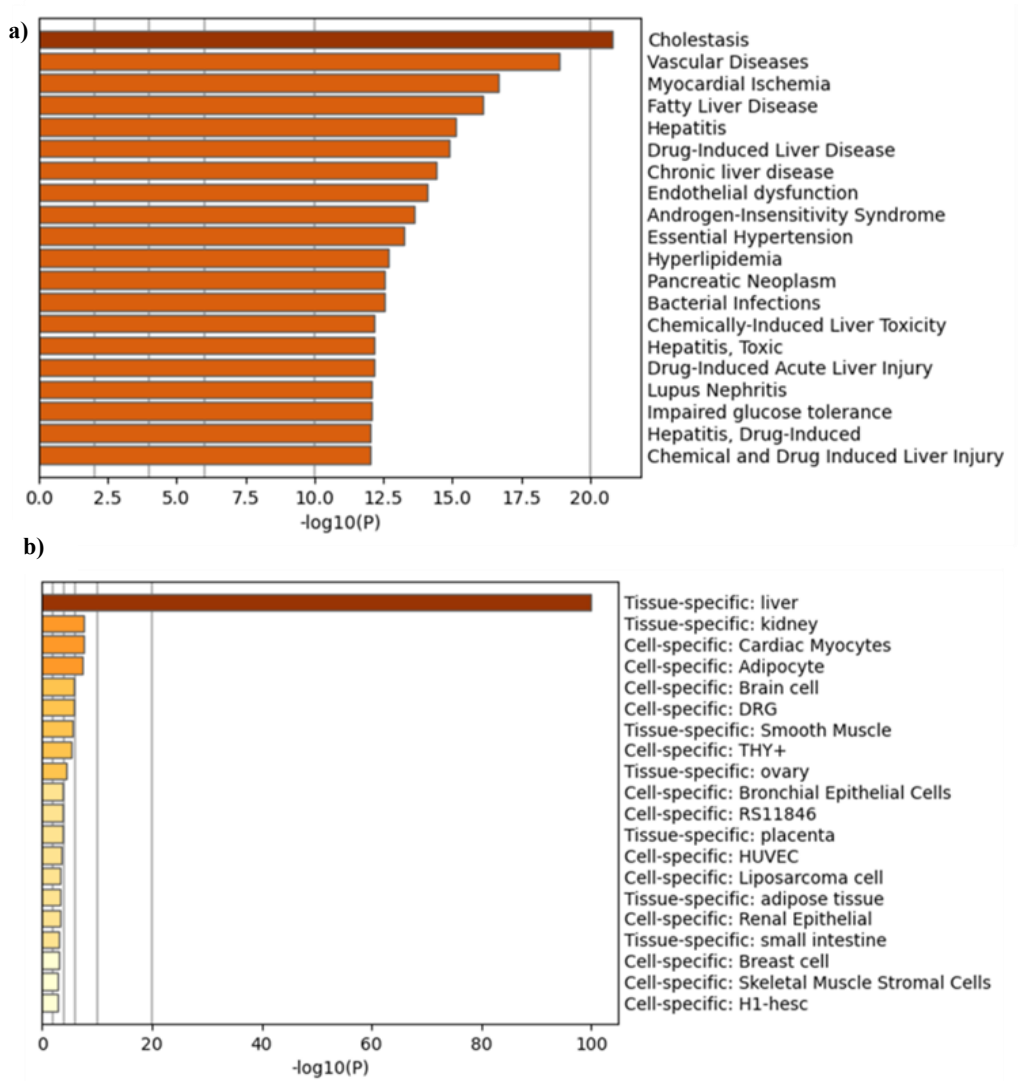


Figure 2. GAD-Disease enrichment (a) and cell/tissue enrichment of HCC DEGs (b)

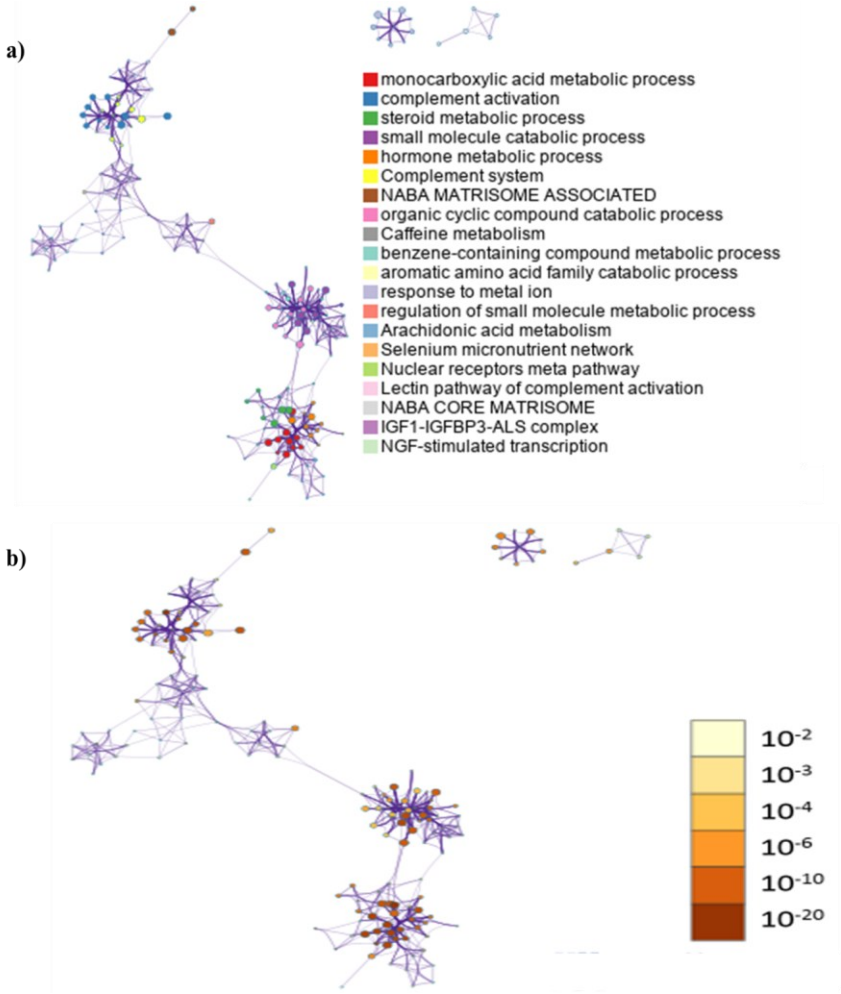


Figure 3. (a) Enriched ontology clusters of DEGs with individual nodes colored by the enrichment terms (b) Enriched ontology clusters of DEGs colored by significance value

Table 4. Topologies of PPI network analyzed by Network Analyzer tool

Topology	Values
Network diameter	3
Network radius	2
Clustering coefficient	0.243
Network density	0.132
Network heterogeneity	0.468
Network centralization	0.192
Average number of neighbors	46.672
Characteristic path length	1.916

3.3 PPI Network Analysis

Analyzing PPI is essential as genes and proteins with similar functions often form interconnected in biological systems. The network revealed 6,632 interactions, excluding any self-loops. The network data was exported in TSV format for further exploration and visualization using Cytoscape software. Diverse topologies identified in the PPI network with the Cytoscape Network Analyzer tool (Smoot *et al.*, 2011), included several important metrics i.e. Network radius (2), Network diameter (3), Network density (0.269), Clustering coefficient (0.520), Network centralization (0.211), Network heterogeneity (0.353), Characteristic path length (1.747), and usual number of neighbors (53.480), all of which are detailed in Table 4.

The reconstructed network was analyzed for densely connected modules with MCODE (Bader and Hogue, 2003) with default values (degree cutoff = 2, node score cutoff = 0.2, haircut = true, fluff = false, k-core = 2, max depth from seed = 100). A total of six clusters were analyzed in the HCC network, each containing a seed gene (Fig. 4). The identified seed genes in these clusters included *BIRC5* (score: 44.857), *RDH16* (score: 38.95), *ADH1B* (score: 9.545), *TTC36* (score: 4.25), *ROBO1* (score: 3.74), and *NQO1* (score: 3.5), all denoted by yellow-colored nodes (Fig. 5).

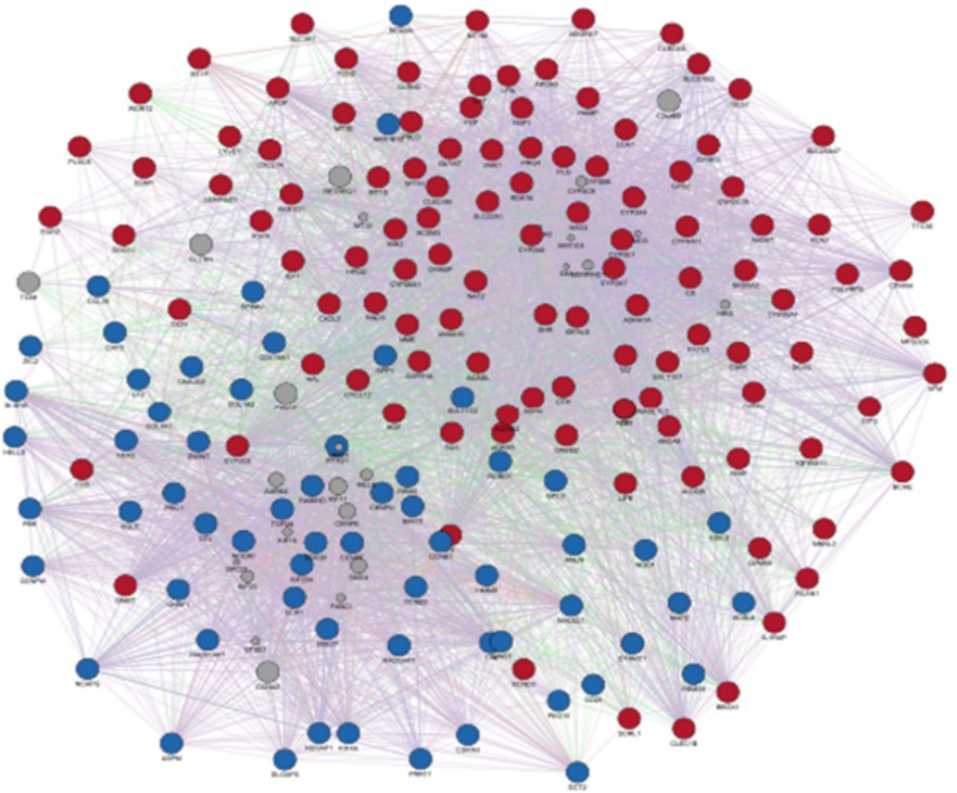


Figure 4. PPI network of HCC DEGs. The network was created using GeneMANIA displayed with Cytoscape software, and evaluated with the Network Analyzer tool

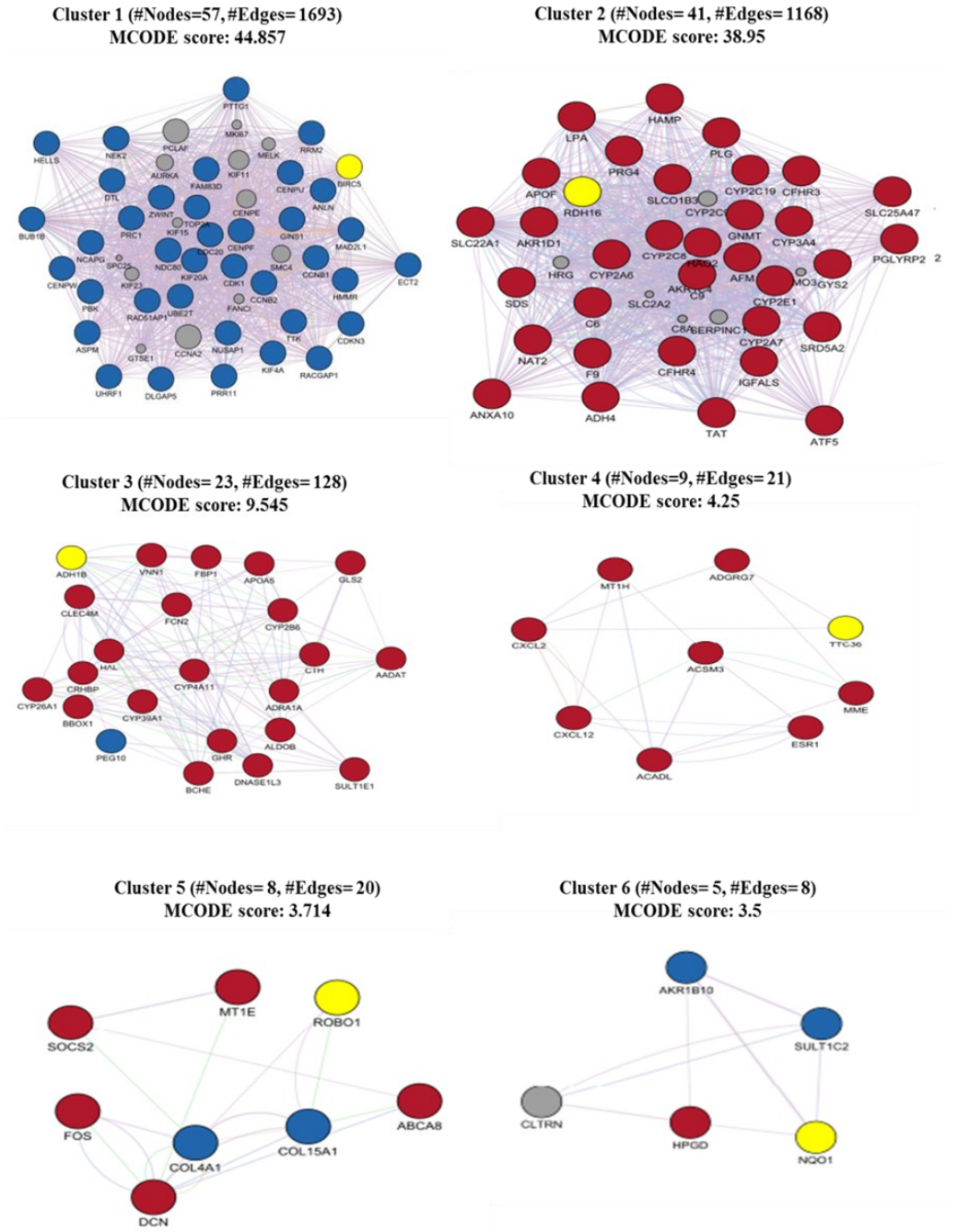


Figure 5. Modules in the HCC network, the yellow nodes signify seed genes

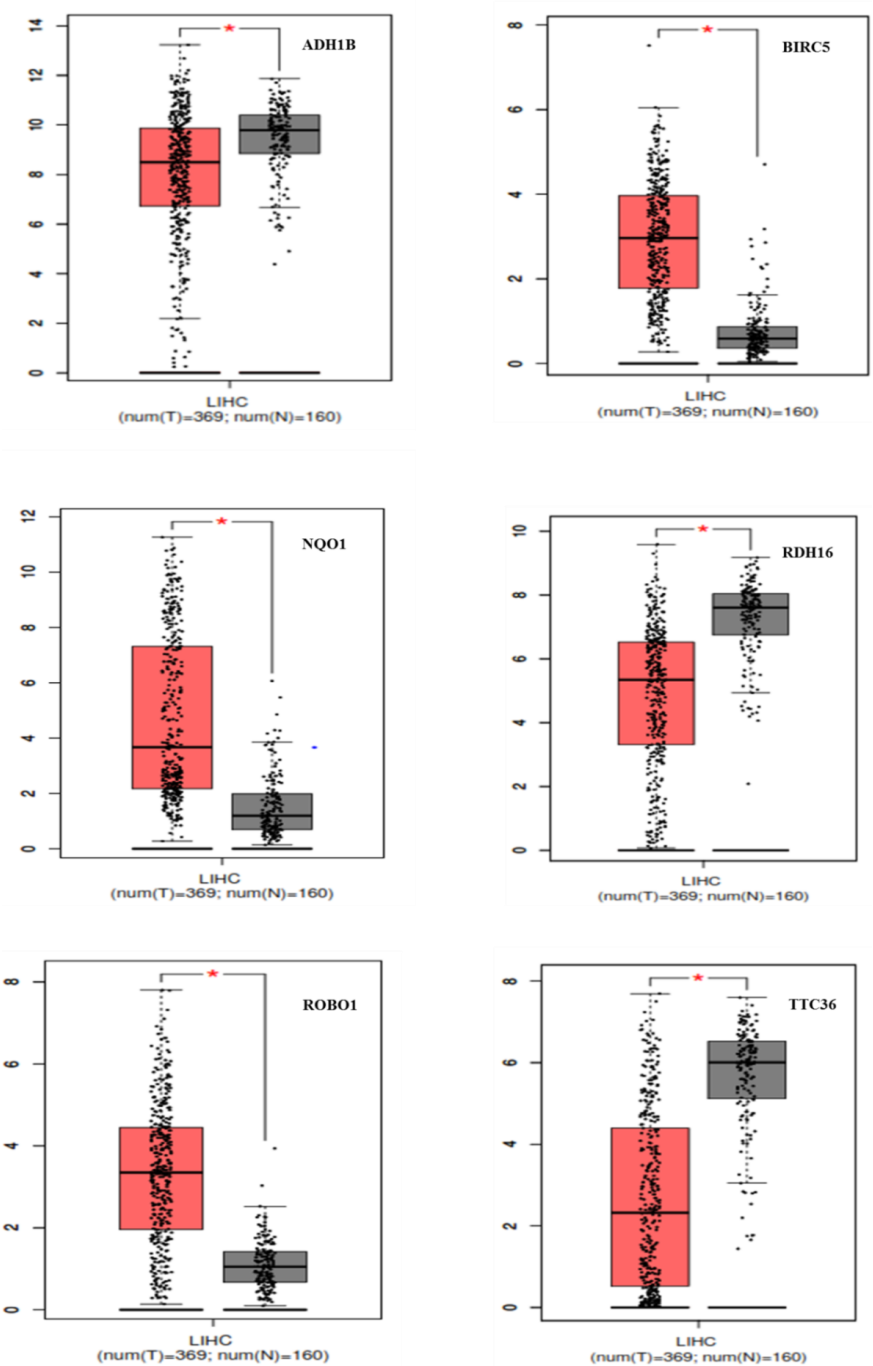


Figure 6. Validation of seed genes expression profile in HCC (Red bar) and normal liver tissues (Grey bar)

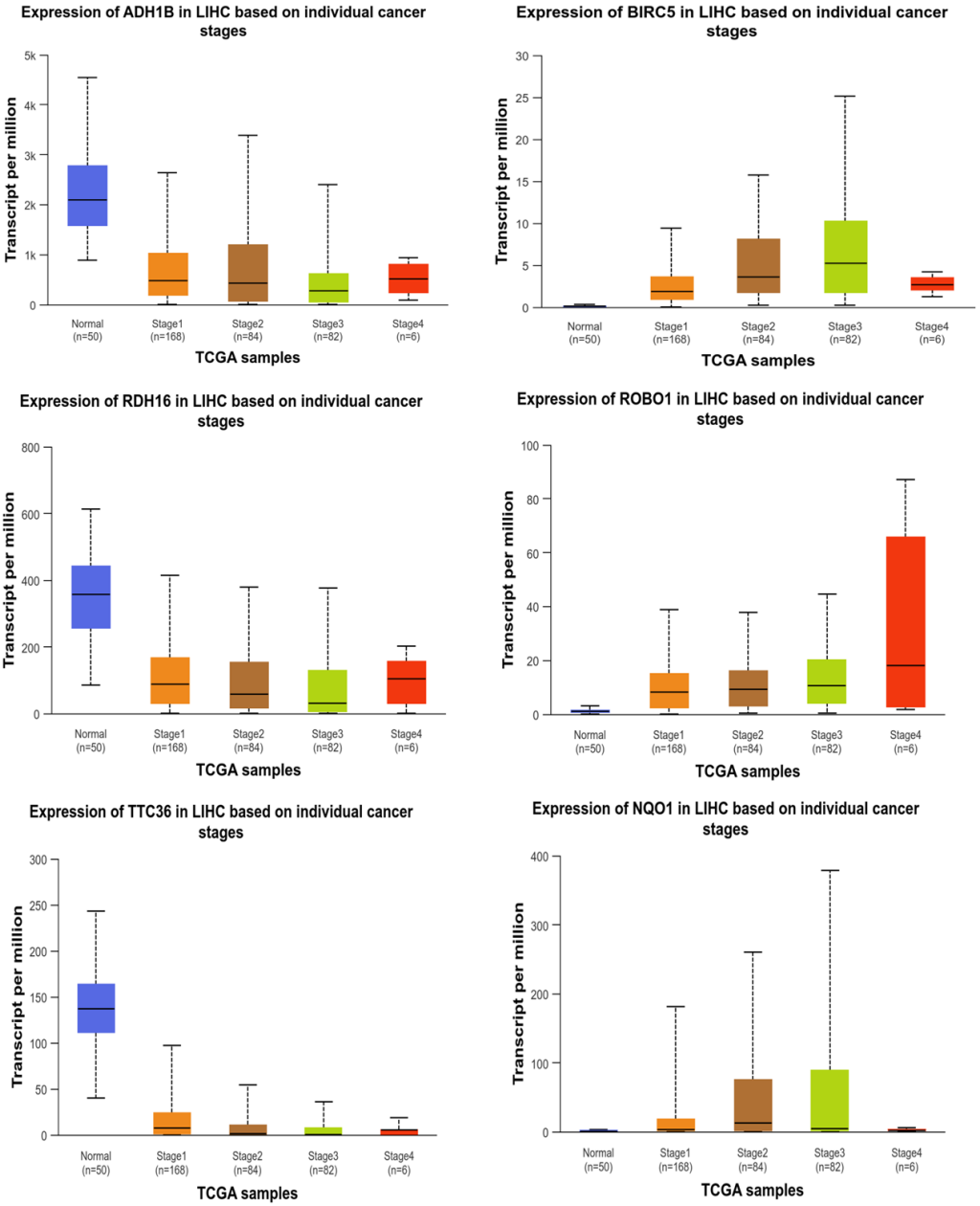


Figure 7. Stagewise expression profile of seed genes in TCGA-LIHC patients and normal GTEx samples

3.4 Validation of Seed Genes

The seed genes were further examined to validate their differential expression profiles in TCGA LIHC dataset and expression patterns across different stages using the UALCAN tool (Chandrashekar *et al.*, 2017), and the GEPIA tools. The GEPIA comparative expression profiling of seed genes revealed a significant overrepresentation of three seed genes *i.e., ROBO1, NQO1*, and *BIRC5* in tumor samples comparative to the normal liver tissues. However, the rest of the three seed genes (*TTC36, ADH1B, and RDH16*) were comparatively down-regulated in tumor samples shown in Fig. 6 & 7.

Methylation profiling of seed genes showed hypermethylation of *BIRC5, NQO1,* and *RDH16* (Beta value ≥ 0.5), while *ADH1B, TTC36,* and *ROBO1* were found hypomethylated in tumor samples (Fig. 8). Correlation analysis between gene levels and methylation exposed negative correlation between ADH1B levels and methylation at several cg islands, ROBO1 expression and methylation at two cg islands, *BIRC5* expression and methylation at cg 25986469 island and NQO1 levels and methylation at cg08836861 island. For *RDH16*, no significant association was noticed (Table 5).

Table 5. Correlation between DNA Methylation and mRNA levels

Genes	Correlation between Methylation and mRNA levels
RDH16	cg 02629586 [Spearman: -0.08 (p-0.122)] cg 07933493 [Spearman: -0.03 (p-0.604)]
ADH1B	cg05641529 [Spearman: -0.57 (p < 0.0001)] cg01151642 [Spearman: -0.77 (p < 0.0001)] cg05641529 [Spearman: -0.57 (p < 0.0001)] cg15395354 [Spearman: -0.35 (p < 0.0001)] cg24368912 [Spearman: -0.36 (p < 0.0001)]
BIRC5	cg 25986469 [Spearman: -0.15 (p < 0.0001)]
ROBO1	cg27221334 [Spearman: -0.27 (p < 0.0001)] cg2478794 [Spearman: -0.26 (p < 0.0001)]
NQO1	cg08836861 [Spearman: -0.34 (p < 0.0001)]

3.5 Survival Analysis of Seed Genes

Out of six seed genes, two seed genes *RDH16*, (HR = 0.63), and *TTC36* (HR = 0.67) returned with significant outcomes impacting HCC patients' overall survival. This means that high levels of both *RDH16* and *TTC36* are associated with good OS of liver cancer patients. Hence *RDH16*, and *TTC36* were characterized as potential protective genes. On the other hand, three seed genes, *ROBO1*, *NQO1*, and *BIRC5*, with HR values greater than 1, revealed negative association of their up regulation with the OS of HCC patients. These genes are thus characterized as risk genes in HCC (Fig. 8).

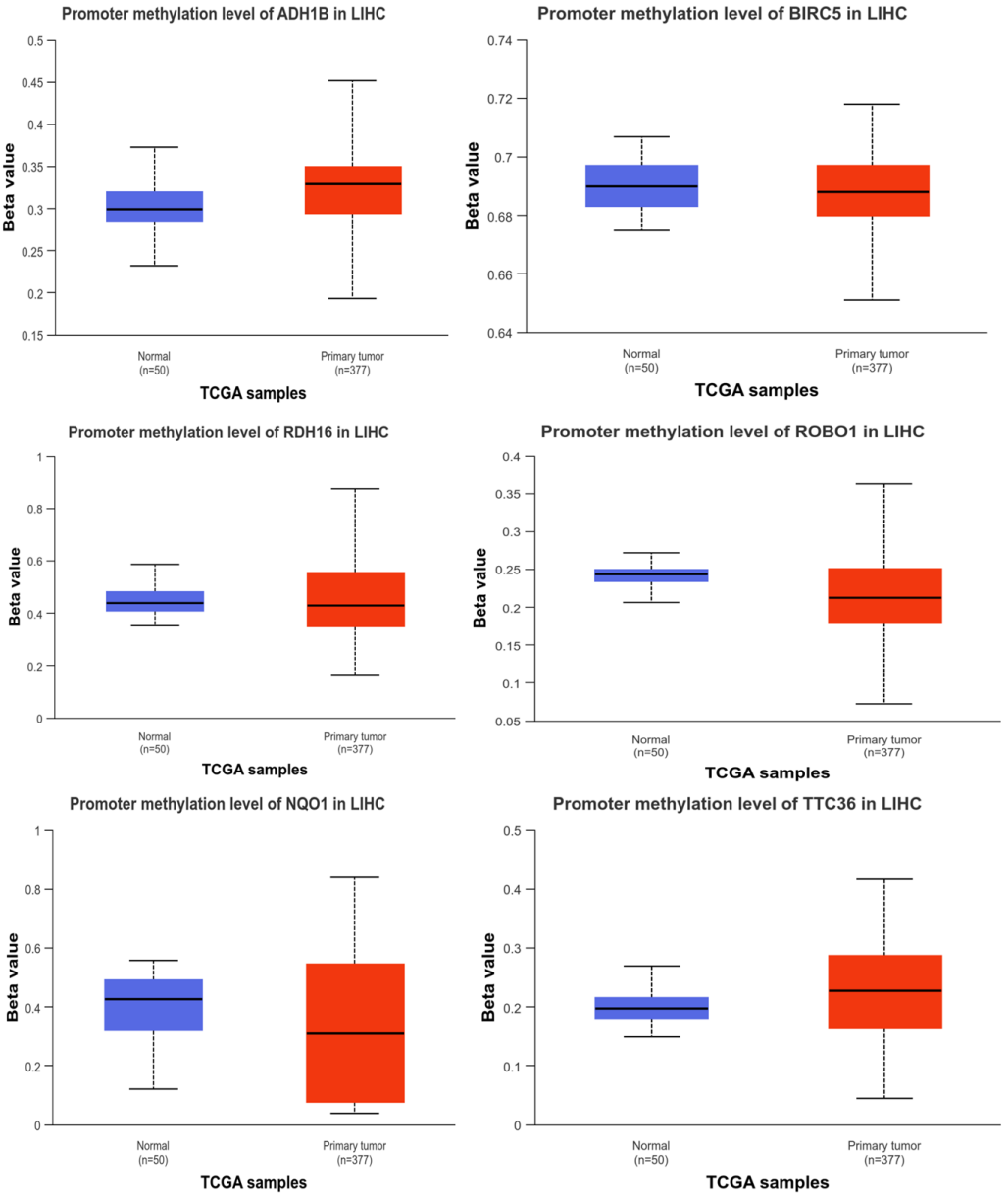


Figure 8. Methylation profiling of seed genes by UALCAN

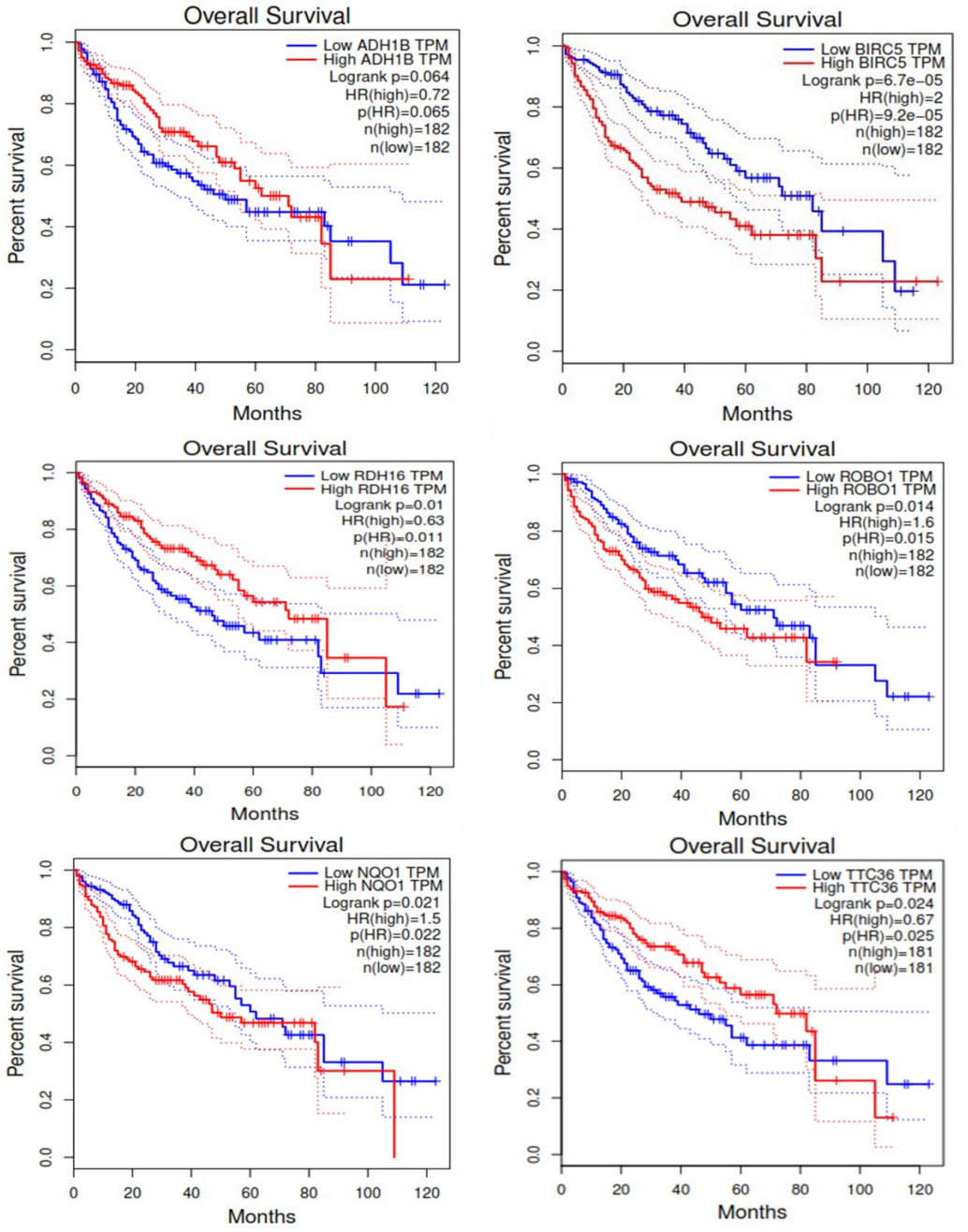


Figure 8. KM survival plots of seed genes

4. DISCUSSION

HCC being among the most common malignancies, poses a considerable risk to worldwide public health. Considering incidence, HCC ranks as the sixth most common cancer, while in terms of mortality, it ranks third globally (Bray *et al.*, 2024). Various modifiable risk factors contribute to liver cancer, including infectious agents (HBV & HCV), behavioral influences, and metabolic conditions, all of which exhibit significant geographical variability (Marengo *et al.*, 2016; Singal *et al.*, 2023). The liver is involved in vital body roles like detoxification, metabolism, and excretion (Ledda *et al.*, 2017). Thus, HCC can result from the harmful effects of exposure to both organic compounds (Rapisarda *et al.*, 2016), and inorganic chemical toxicants like arsenic (Choiniere and Wang, 2016).

Biomarkers have been invaluable tools for cancer care including early diagnosis and therapeutics. Several biomarkers have already been identified with demonstrated clinical utility in HCC. Higher AFP levels are related to advanced stage HCC tumors and poor patient outcomes. Further, the AFP-L3/AFP ratio guides the diagnosis tumors with low AFP expression (Omata *et al.*, 2017). Likewise, Des-γ-carboxyprothrombin (DCP), an inactive prothrombin, is considered for early-stage HCC detection (Beudeker and Boonstra, 2020). Studies indicate that combining DCP and AFP improves diagnostic sensitivity from 47.5% to 94.0% (Yu *et al.*, 2015). However, despite their clinical relevance, current biomarkers of HCC including AFP and DCP have poor sensitivity and specificity, mainly in early diagnosis. This necessitates the identification of novel biomarkers for the disease with improved prognostic and therapeutic value. High-throughput transcriptomic and bioinformatics analyses have emerged as powerful tools for the identification of candidate biomarkers and molecular drivers of various diseases including cancer (Shen *et al.*, 2019; Yang *et al.*, 2019; Noreen *et al.*, 2021; Akhtar *et al.*, 2026; Noreen *et al.*, 2026).

Here, we performed differential gene expression analysis in HCC using two independent GEO datasets, GSE121248 and GSE102079, respectively. This resulted in the identification of 205 DEGs with a potential role in HCC pathogenesis. Functional enrichment analysis exposed significant association of upregulated DEGs with proteolysis, complement activation, xenobiotic metabolism, biosynthetic processes, and the JAK–STAT signaling cascade. Downregulated DEGs, on the other hand, were found to be enriched in cell-cycle regulation, mitotic nuclear division, and segregation of sister chromatids (Table 2). Previously Zhang *et al.* (2021) also described the involvement of HCC DEGs in cell-cycle dysregulation and tumor proliferation. In this study, not only have we recognized previously known proliferative genes like BUB1B, TOP2A, CDC20, CDK1, and CCNB2, but have discovered another set of six seed genes viz. BIRC5, RDH16, ADH1B, TTC36, ROBO1, and NQO1 with diverse roles as oncogenes and tumor suppressor signatures.

The GAD enrichment analysis revealed significant involvement of various gene targets with metabolic reprogramming and carcinogenesis, among which are HPGD, BUB1B, CCL20, CXCL12, CXCL14, TOP2A, NQO1, ADH1B, BIRC5, CDC20, CDK1, and members of the cytochrome P450 family. These findings highlight the contribution of metabolic reprogramming and oncogenic signals to the process of hepatocarcinogenesis, which has been confirmed previously by the high prevalence of metabolic and cell-cycle regulation disturbances in HCC. Pathway analysis with the KEGG database indicated the presence of various pathways involved in fatty acid degradation, metabolism, chemical

carcinogenesis, cell cycle regulation, as well as various biosynthesis pathways. Fatty acid metabolism disorders have a substantial impact on HCC development because they facilitate tumor cell survival, tumor metastasis, as well as chemoresistance (Cheng *et al.*, 2024). Disorders associated with abnormal lipogenesis in HCC patients consist of upregulated lipogenesis, increased cholesterol esterification, accelerated endogenous synthesis of lipids, and abnormal lipid oxidation. Mitochondrial dysfunction of fatty acid β-oxidation and over-expression of long-chain acyl-CoA synthetase 4 (ACSL4) have been reported as mechanisms that help tumors grow (Ndiaye *et al.*, 2020). Over-expression of low-density lipoprotein receptor (LDLR) promotes increased cholesterol accumulation in cancer tissues (Lin *et al.*, 2024). The findings on the metabolic disruption seen in this study fit well with our knowledge of HCC biology today, as tumors exhibit metabolic reprogramming in order to survive and proliferate amidst the challenging environment. Metabolic adaptations can be targeted as a strategy in treating liver tumors (Satriano *et al.*, 2019; Tenen *et al.*, 2021).

In order to elucidate the clinical importance of the DEGs identified in our study, Metascape analysis was used to explore enriched pathways and interaction networks involved in HCC. It showed an extensive interaction network of genes and their functional groups that provide insight into the molecular mechanisms that operate within the HCC. Six clusters of proteins showing high connectivity among each other were revealed through the MCODE analysis, followed by the Gene Ontology (GO) enrichment analysis of these clusters (Fig. 2a). PaGenBase analysis revealed the enrichment of these genes in liver, kidney, brain, smooth muscle, placenta, and breast tissues (Fig. 2b). The DisGeNET analysis, on the other hand, revealed association of these DEGs with several diseases, such as hepatitis, bacterial infections, pancreatic neoplasms, fatty liver disease, hyperlipidemia, and cholestasis (Figure 2c).

Notably, the comparison between the seed genes identified and the known biomarkers of HCC further highlights the practical utility of our findings. Traditional biomarkers like AFP, AFP-L3, and DCP have proven to be helpful in clinics, although their performance is usually hindered by poor specificity and sensitivity, especially for early-stage tumors. According to recent findings from transcriptomic studies and bioinformatics research, cell cycle and proliferation-related genes like CDK1, CCNB1, TOP2A, CDC20, and AURKA have been suggested as promising prognostic factors for HCC. Compared to them, the key genes found in this study are advantageous in terms of a wider range of biological functions, encompassing apoptosis suppression (BIRC5), oxidation resistance (NQO1), metabolomic disruption (ADH1B and RDH16), cell signaling and migration (ROBO1), and tumor suppression (TTC36). The diversity of roles performed by the identified genes indicates that they might be effective in molecular classification of HCC and complement the current established biomarkers used for predicting the disease outcomes.

RDH16 is a member of short-chain dehydrogenase/reductase family of proteins and encodes a microsomal membrane-bound protein implicated in retinoid metabolism (Zhu *et al.*, 2020). RDH16 levels were found to be higher in normal liver tissue than to tumor tissue (Fig. 6). Low RDH16 expression showed significantly favorable survival rates among HCC patients (HR: 0.63, $p = 0.01$). It was found previously that low levels of RDH16 were associated with increased levels of AFP, progression, vascular invasion, and poor prognosis among HCC patients (Zhang *et al.*, 2025). The role of RDH16 in inhibition of fat synthesis, promotion of retinoic acid production, and prevention of propagation and

spread of HCC cells was established (Chen *et al.*, 2025). All these results support the roles of RDH16 in tumor suppression in HCC that can be utilized as a promising target in HCC treatment by metabolic reprogramming and epigenetic regulation.

Expression of tetratricopeptide repeat domain 36 (TTC36) gene has been described to be highly restricted to the liver and kidneys, with TTC36 playing role in cell cycle control, protein transport, and complex assembly (Zhou *et al.*, 2016). TTC36 gene has also been implicated in regulation of tyrosine metabolism via the interaction with 4-hydroxyphenylpyruvic acid dioxygenase (HPD) and prevents aberrant hepatocytes metabolic conditions (Xie *et al.*, 2019). This study revealed significantly low TTC36 expression in malignant tissues compared to healthy liver tissues and it was significantly related with favorable prognosis (HR = 0.67; Log-rank p-value = 0.024). In HCC, TTC36 has been stated previously to work as a tumor suppressor by increasing apoptosis and reducing cancer development (Jing *et al.*, 2022). Therefore, reduced expression of TTC36 in this study reinforces the tumor suppressor roles of TTC36 and its significance in prognosis prediction.

ADH1B is a protein of the alcohol dehydrogenase family which is abundant in liver and fat tissue, maintaining metabolic homeostasis (Gautheron *et al.*, 2024). ADH1B showed significant reductions in its expression levels in HCC tissues and a correlation with good prognosis. These results are similar to those observed in colorectal cancer, esophageal squamous cell carcinoma, ovarian cancer, and HCC patients (Xu *et al.*, 2022; Zhou *et al.*, 2023). Several studies showed that higher expression of ADH1B has been linked with improved survival in HCC and suppression of carcinogenic signaling pathways (Liu *et al.*, 2020). Thus, our results provide further support for ADH1B having a potential role as a defensive metabolic factor in HCC and a targeted metabolic intervention biomarker in precision medicine.

Survivin encoded by *BIRC5* gene is a multipurpose protein responsible for cell division and programmed cell death inhibition and acts as an essential molecule involved in tumorigenesis (Zhao *et al.*, 2022; Yesupatham *et al.*, 2023). Survivin protein exerts its action through inhibition of caspase-3 and caspase-9 leading to lack of apoptosis (Li *et al.*, 2021). As reported previously, *BIRC5* gene expression was upregulated in liver cancers, especially at an advanced tumor stage, predicting poor prognosis (Ayoub *et al.*, 2025). It should be noted that *BIRC5* has proven itself a potential therapeutic target in cancer therapeutics. There are several Survivin-targeting strategies such as small-molecule inhibition with YM155 (sepantronium bromide), Survivin-targeted immunotherapy and RNA interference that have already been applied to many different cancers (Rouse *et al.*, 2025). Therefore, our results, demonstrating elevated expression of BIRC5 gene in HCC, especially in advanced stage tumors, add another argument favoring BIRC5 application in HCC. According to our data, HCC patients showing *BIRC5* expression elevation may derive additional advantages from Survivin-targeted therapies either as a monotherapy or combined with other anticancer approaches.

5. CONCLUSION

In conclusion, this study identified 205 differentially expressed genes and six key seed genes (BIRC5, RDH16, ADH1B, TTC36, ROBO1, and NQO1) significantly associated with HCC progression, prognosis, and molecular pathogenesis. These findings expand beyond conventional biomarkers such as AFP and DCP by highlighting genes involved in apoptosis, metabolism, oxidative stress, and tumor suppression. Importantly, the

therapeutic relevance of targets such as BIRC5 emphasizes their potential utility in improving HCC diagnosis, prognostic stratification, and precision-based therapeutic interventions.

Conflict of Interest
The Authors declare that there is no conflict of interest.

Author Contributions
EAB: Investigation, Formal analysis, Writing – original draft. **MRA:** Conceptualization, Methodology, Formal analysis, Writing – original draft, review & editing. **SN:** Conceptualization, Methodology, Formal analysis, Writing – original draft, review & editing. **SN:** Writing – original draft, review & editing.

Ethics Approval
No ethical approval was required for this study as it did not involve human or animal subjects.

Generative AI Disclosure
The authors confirm that no generative artificial intelligence tools were used in this manuscript.

Availability of Data
Data will be furnished upon request.

Funding
No funding was received to conduct this study.

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