RESEARCH ARTICLE

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Biomarker Panels Associated with Diagnosis and Overall Survival in Hepatocellular Carcinoma Revealed from Protein-Protein and mRNA-miRNA Interaction Networks

Reyhaneh Farrokhi Yekta[1], Mostafa Rezaei-Tavirani[1], Afsaneh Arefi Oskouie[2]*, Nasrin Amiri-Dashatan[3]

Abstract

Background: Hepatocellular carcinoma (HCC), the most common form of liver cancer, has a significant mortality rate, largely due to late diagnosis. Recent advances in medical research have demonstrated the potential of biomarkers for early detection. Moreover, the discovery and use of prognostic biomarkers offer a ray of hope in the fight against liver cancer. **Methods:** Three gene transcript collections (*GSE57957, GSE76427*, and *GSE84402*) were retrieved from the GEO database, and significantly expressed genes were identified through a comprehensive screening process. Subsequently, key potential biomarkers were identified using various methods, including functional pathway enrichment, protein-protein interaction network analysis, mRNA-miR interaction study, and ROC curve and survival analysis. **Results:** After analyzing the expression of hub proteins and miRs, 12 proteins were found to have AUC values greater than 0.9 and log-rank KM-plot p values less than 0.05. Therefore, these proteins can be considered as potential diagnostic and prognostic biomarkers. Among these proteins, the top 5 were CDC6, PTTG1, CDCA5, RACGAP1, and RAD51AP1. The microRNAs with the highest diagnostic significance (AUC≥0.8) were hsa-mir-101-3p, hsa-mir-195-5p, hsa-mir-130a-3p, hsa-mir-26b-5p, hsa-mir-29c-3p, hsa-mir-26a-5p, and hsa-mir-34a-5p. Notably, hsa-mir-34a-5p, hsa-mir-195-5p, and hsa-mir-130a-3p also showed prognostic potential as predictors of overall survival in HCC patients. **Conclusion:** Harnessing the potential of these biomarkers will enable healthcare professionals to make informed decisions, leading to improved care and more favorable outcomes in the fight against HCC. However, the next step is to thoroughly validate these potential markers in large cohorts.

Keywords: Hepatocellular carcinoma- biomarkers- diagnostic- prognostic- network

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Introduction

When it comes to hepatocellular carcinoma, the most common form of liver cancer, the importance of timely identification and accurate prognosis to improve patient outcomes cannot be overemphasized. Hepatocellular carcinoma, also known as primary liver cancer, develops in the hepatocytes, the main liver cell type. Risk factors such as persistent hepatitis B or C infection, liver cirrhosis, excessive alcohol consumption, and certain inherited liver disorders increase the likelihood of developing HCC [1]. Early detection of HCC is essential to improving patient outcomes. Traditional diagnostic methods, such as imaging and biopsy, are often unable to detect HCC in its early stages. Biomarkers are measurable biological entities that offer a non-invasive alternative that can identify the disease at an earlier, more treatable stage. As symptoms often go unnoticed until later stages, HCC prognosis becomes even more crucial in determining appropriate interventions. The stage of the tumor upon diagnosis is correlated with the reception of curative treatment and the overall survival rate. This includes a 5-year survival rate below 5% in patients with advanced-stage disease, in contrast to a rate exceeding 70% for those with early-stage [2]. In recent years, the identification of prognostic biomarkers has emerged as a promising avenue for predicting HCC progression and tailoring personalized treatment strategies. In the context of hepatocellular carcinoma, researchers have identified several biomarkers that can assist in predicting tumor behavior, treatment response, and overall survival rates. Alpha-fetoprotein (AFP) has long been considered the gold standard biomarker for

*[1]Proteomics Research Center, School of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [2]Department of Basic Sciences, School of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [3]Zanjan Metabolic Diseases Research Center, Zanjan University of Medical Sciences, Zanjan, Iran. *For Correspondence: dr.afsaneh.arefi@gmail.com*

HCC [3]. However, its effectiveness is limited, as AFP levels can also be elevated in other liver conditions, such as cirrhosis, which leads to false positive results [4]. AFP lacks the necessary accuracy and specificity to be used as a standalone diagnostic tool. Therefore, combining AFP with other biomarkers enhances its predictive value [5]. AFP-L3, also known as lens culinaris agglutinin-reactive AFP, is a glycoform variant of AFP that has been extensively investigated for its utility as a biomarker in identifying early-stage hepatocellular carcinoma [6]. Although AFP-L3 exhibits a superior level of specificity in comparison to AFP, its sensitivity is considerably lower (about 50%–60%) in the context of early HCC detection [7]. Further validation of AFP-L3 in phase III biomarker evaluation is required in order to ascertain if it possesses additional value when compared to or used in conjunction with AFP. Des-gamma-carboxy prothrombin (DCP), also known as PIVKA-II or protein induced by vitamin K absence or antagonist-II, shows promise as a prognostic biomarker for HCC [8]. Studies have shown that elevated DCP levels are strongly associated with HCC, particularly in patients with normal AFP levels. It is also indicated that elevated levels of DCP are associated with the aggressiveness of tumors and an unfavorable prognosis. Combining AFP and DCP tests can significantly improve the sensitivity and specificity of HCC diagnosis [9]. There are also some other biomarkers such as osteopontin, midkine, glypican-3, dikkopf-1, alpha-1 fucosidase, golgi protein-73, and Squamous cell carcinoma antigen that exhibit promising outcomes during the phase II assessment; however, they still necessitate phase III authentication [10, 11]. Another category of biomarkers being developed for HCC are genetic markers. This includes non-coding RNAs, DNA methylation, and other epigenetic modifications [12]. Several investigations have assessed the potential of circulating miRs as diagnostic biomarkers for hepatocellular carcinoma due to their inherent stability and involvement in tumor proliferation [13-15]. While the aforementioned biomarkers have shown promise in predicting HCC prognosis, ongoing research aims to identify additional markers with enhanced accuracy and reliability. By harnessing the power of the validated biomarkers in the future, healthcare professionals can make informed decisions, leading to improved patient care and better outcomes in the fight against hepatocellular carcinoma. The utilization of multi-omics strategies and systems biology in generating complex datasets has become critical to accelerate the identification and validation of diagnostic, prognostic, or therapeutic biomarker panels. Genes/proteins or miRs, apart from their presence in various samples such as tissues, blood samples, and urine, can be identified by novel methods within the tumor microenvironment (TME). One of the novel highthroughput strategies for identifying biomarkers involves exploring them within the TME at the level of individual cells using the single-cell RNA sequencing (scRNA-seq) technique, which also takes into account the interactions between cells in the TME. In this investigation, high-throughput transcriptomic data of HCC patients were obtained from the GEO database. Combining the datasets with protein-protein and mRNA-miRNA interaction networks revealed several proteins and microRNAs that may be useful as potential diagnostic and prognostic biomarkers for hepatocellular carcinoma.

Materials and Methods

Microarray datasets and data analysis

After performing a search on the NCBI/GEO series platform [16], using the specified keywords ["mRNA" OR "gene" OR "transcript"] AND ["liver cancer" OR "hepatocellular carcinoma"], a total of 812 data series results were acquired. The obtained results were then narrowed down to include only "human" and "tissue", leaving 546 datasets. Among them, 7 datasets with a large number of participants were selected and analyzed using the GEO2R platform. Finally, three datasets with the largest number of significant DEGs were selected. The three gene transcriptome datasets used in this study were *GSE57957*, *GSE76427*, and *GSE84402* [17-19]. The expression datasets were normalized using the GEO2R tool. Differentially expressed genes (DEGs) were identified based on the cutoff criteria, adj.p-value<0.05 and $\left|\text{Fold-change}\right|>2$. For more details on each dataset, including sample number and microarray platform type, see Table 1. Volcano plots of significant genes and normalized datasets are also shown in Figure 1.

Integration of datasets results

Differentially expressed genes were analyzed using the Venny online platform [20] to identify the overlapping genes among the datasets. Consequently, a collection of 143 genes that were shared between the three microarrays were acquired.

Construction of protein-protein and mRNA-miRNA interaction networks

The common genes were searched in the String database [21] to find all the possible interactions of the DEGs and construct the protein-protein interaction (PPI) network. The resulting network was further analyzed using Cytoscape software [22]. The common genes were searched in "mirnet" [23] by selecting the "miRDB" and "Tarbase" databases to find all possible interactions between DEGs and miRs associated with liver tissue.

Gene ontology (GO) enrichment and Pathway analysis

The ClueGO plugin [24] in Cytoscape was used to find the significantly enriched biological processes (BP), molecular functions (MF), cellular components (CC), and KEGG pathways in the PPI network. Bonferroni-corrected p-values less than 0.05 were considered as the threshold for statistical significance. KEGG pathway enrichment analysis was also performed on the key miRs of the mRNA-miRNA network using the NcPath database (http://ncpath.pianlab.cn/), a tool for visualization and enrichment analysis of non-coding RNAs and KEGG signaling pathways in humans.

Hub selection

The networks created in Cytoscape were analyzed and the nodes with the highest interaction levels were identified

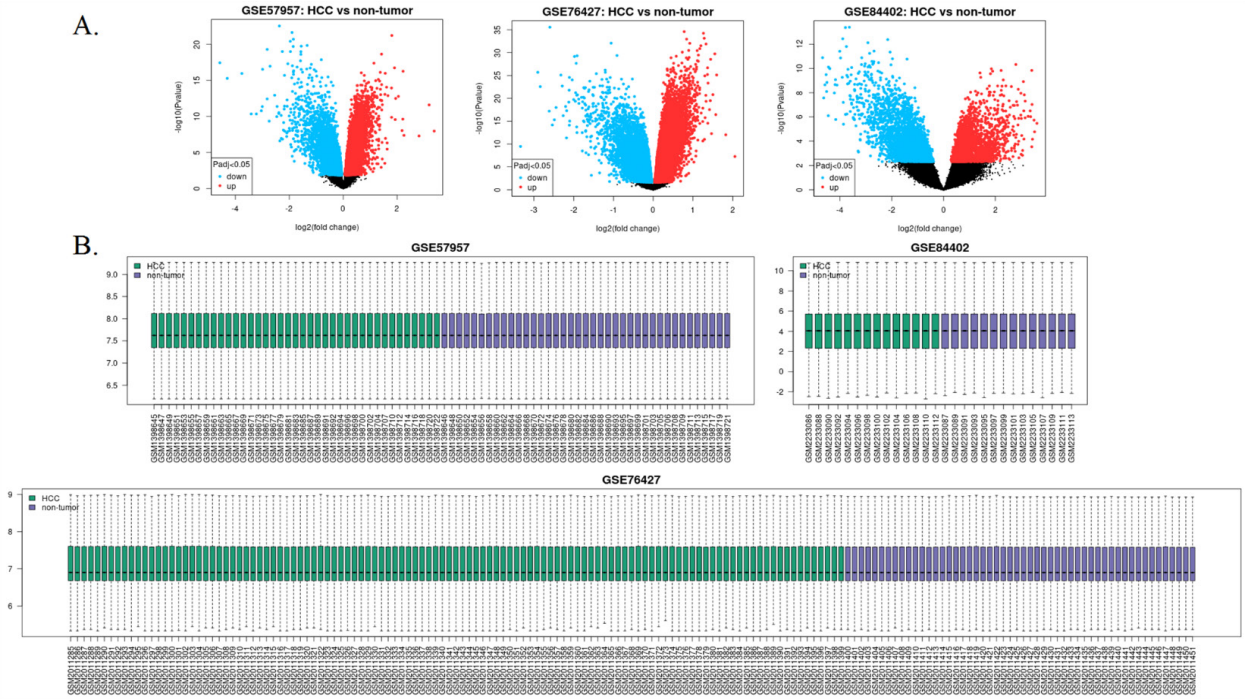

Figure 1. (A) The volcano plots and (B) the normalized GEO datasets, showing the significant up/down regulated genes in each dataset.

as the hub genes and hub miRs. Hub nodes usually perform important functions of monitoring the paths connected in the network. Furthermore, these nodes could potentially serve as biomarkers for various diseases. A total of 20 top hub nodes were selected for additional investigation from both the PPI and mRNA-miRNA networks.

ROC analysis

After identifying hub genes that play a role in the diagnosis and survival of liver cancer patients, Graphpad prism 8.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) was used to construct the ROC curves of the DEGs based on the nodes normalized expression data. To determine potential biomarkers, a p-value of less than 0.05 and an area under the curve (AUC) greater than 0.80 were set as the threshold. The ROC curves associated with miRs were obtained from the CancerMIRNome database [25].

Survival analysis

Survival analysis was exclusively conducted for the hub genes and miRs with an AUC greater than 0.80. To explore possible relationships between the DEGs and overall survival, both the Human Protein Atlas database [26] and the UALCAN databases [27] were used. The Human Protein Atlas, an open-source online platform, facilitates the mapping of human proteins in various tissues, cells, and organs by using data obtained from omics studies and antibody-based imaging techniques. In the "pathology" section of the Human Protein Atlas, we examined the impact of the expression levels of the DEGs on the survival of liver cancer patients. Proteins showing significant correlation with overall survival were identified using Kaplan-Meier survival curves. The predetermined cutoff p-value for significance was set at 0.05. For miRs, survival data were extracted from the CancerMIRNome database.

Results

Differentially expressed genes

To find the differentially expressed genes, the intersection between datasets was extracted from the Venn diagram. In the "*GSE57957*" dataset, a total of 428 DEGs were identified, while in the "*GSE76427*" and "*GSE84402*" datasets, 312 and 2118 DEGs were identified, respectively. The intersection between these datasets resulted in 143 common genes (Figure 2). The DEG names are provided in Supplementary Table 1. The common DEGs were used for further bioinformatic analyses.

Protein-protein interaction network analysis and hub genes

The PPI network consisted of a total of 118 nodes and 552 edges after removing disconnected nodes, as shown in Figure 3. The network was constructed with high confidence interval of 0.7. The network was then analyzed using Cytoscape software. The hub nodes which exhibited the greatest degree of connectivity were then identified and designated as potential biomarkers for HCC. The top 20 hub/bottleneck DEGs included *CDC6, AURKA, MCM2, RAD51, PRC1, NCAPG, PTTG1, MCM5, NCAPH, RFC4, GINS2, CDKN3, ASPM, KIF23, RAD51AP1, MELK, PCLAF, RACGAP1, CDCA5,* and *SERPINF2* (Table 2).

Gene ontology results

Based on the ClueGO Cytoscape plugin, the meaningful enriched gene ontology terms and KEGG pathways were determined for the DEGs (Table 3). The

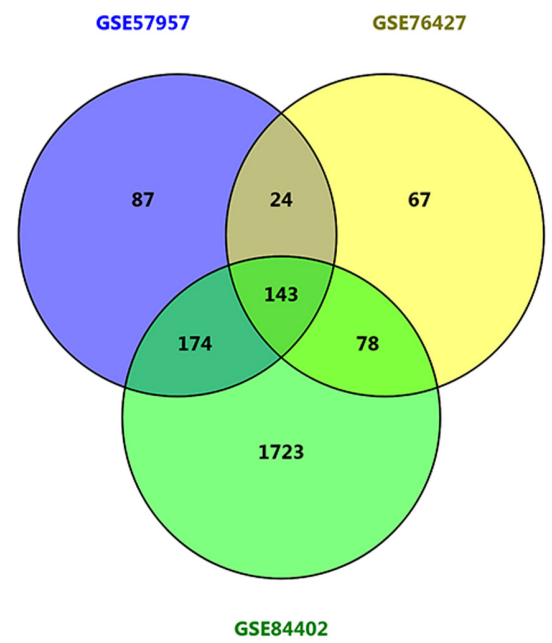

Figure 2. Venn Diagram Showing Common Genes between the Selected GEO Datasets.

most significant biological processes included cellular hormone metabolic process (adj.p-val=7.8E-06), protein homotetramerization (adj.p-val=0.00048), androgen metabolic process (adj.p-val=0.00016), response to phenylpropanoid (adj.p-val=0.0026), and cellular amino acid catabolic process (adj.p-val=3E-07). The top enriched molecular functions included fatty acid ligase activity (adj.p-val=0.0037), polysaccharide binding (adj.p-val=0.0063), oxidoreductase activity acting on the CH-CH group of donors (adj.p-val=0.0018), oxidoreductase activity acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor (adj.p-val=0.0041), monooxygenase activity (adj.p-val=4.3E-06), and insulin-like growth factor binding (adj.p-val=0.0077). The most significant cellular components were the mitotic spindle (adj.p-val=0.0017) and the plasma lipoprotein particles (adj.p-val=0.0033). The enrichment results from the KEGG pathways also indicated that the most noteworthy pathways related to liver cancer were the Pentose and glucuronate interconversions (adj.p-val=0.0069), Tryptophan metabolism (adj.p-val=0.000019), Complement and coagulation cascades (adj.p-val=0.0036), and Fatty acid degradation (adj.p-val=0.0051). Figure 4 shows the resulting network of gene ontology and pathway enrichment, with larger circles indicating more significant terms. The key miRs in the mRNA-miRNA network were also subjected to KEGG pathway enrichment analysis. The results showed that Rap1 signaling pathway (adj.p-value=0.001) and Apelin signaling pathway (adj.p-value=0.001) were the most important enriched signaling pathways that could be involved in the development of HCC. The Supplementary Table 2 contains the enrichment results of miRs.

The mRNA-miRNA network and hub miRs

The miRNA-mRNA network created in Cytoscape contained 214 nodes and 830 edges (Figure 5). Then, analysis of the network was performed, and hub nodes with the highest connectivity were identified as potential biomarkers for further investigation by ROC and survival curve analysis. Specifically, the top 20 hub miRs were identified as hsa-mir-1-3p, hsa-mir-124-3p, hsa-mir-129-

Table 1. Details of the Selected Microarray Platforms

GEO series	Platform	#Tumor samples	#control samples	Ref.
GSE57957	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	39	39	[17]
GSE76427	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	115	52	[18]
GSE84402	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	14	14	[19]

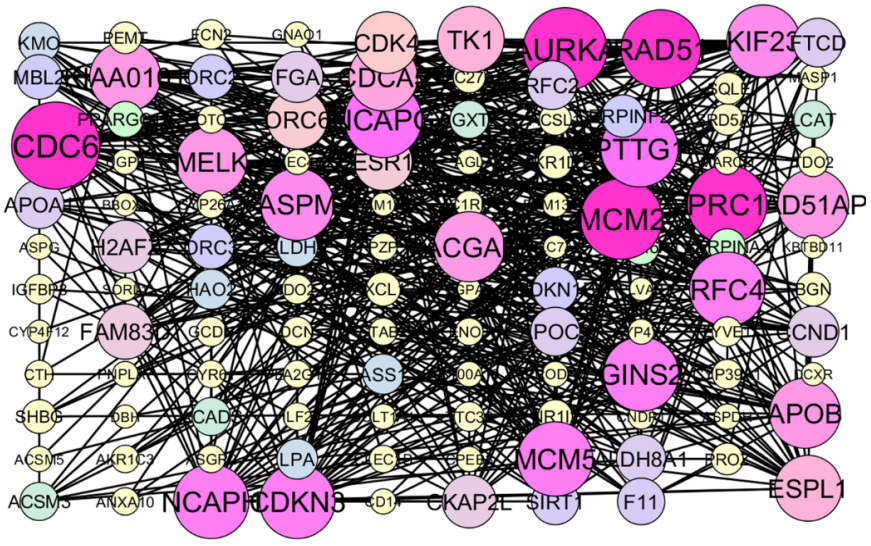

Figure 3. The Protein-Protein Interaction Ntwork, Constructed in Cytoscape Based on All the Possible Interactions of DEGs. Larger circles denote higher degree hub genes.

Table 2. Top 20 hub/bottleneck proteins/genes in the Protein-Protein Interaction Network. (↑: increased in tumor group, ↓: decreased in tumor group)

Gene symbol	Degree	Betweenness centrality	Adj.P-value	Fold change
CDC6	31	0.02014	0.0000377	4.21 ↑
AURKA	29	0.04503	1.73E-10	2.68 ↑
MCM2	28	0.02966	2.86E-19	2.82 ↑
RAD51	27	0.02401	0.000171	4.88 ↑
PRC1	27	0.00363	5.54E-12	3.05 ↑
NCAPG	26	0.02008	0.0242	2.19 ↑
PTTG1	26	0.00492	2.90E-12	2.68 ↑
MCM5	25	0.00338	0.000334	2.52 ↑
NCAPH	25	0.00439	0.00127	5.86 ↑
RFC4	25	0.01272	8.73E-10	2.1 ↑
GINS2	25	0.00378	0.00238	2.88 ↑
CDKN3	25	0.02069	5.61E-11	3.15 ↑
ASPM	24	0.00161	1.03E-09	2.54 ↑
KIF23	24	0.00171	0.0201	2.85 ↑
RAD51AP1	23	0.00283	0.000171	4.88 ↑
MELK	23	0.00142	0.000115	4.59 ↑
PCLAF	23	0.00226	2.13E-11	3.53 ↑
RACGAP1	23	0.00142	9.93E-13	2.3 ↑
CDCA5	22	0.00122	2.25E-11	2.23 ↑
SERPINF2	10	0.15982	1.78E-17	2.6 ↓

2-3p, hsa-mir-34a-5p, hsa-let-7b-5p, hsa-mir-155-5p, hsa-mir-195-5p, hsa-mir-200b-3p, hsa-mir-26a-5p, hsa-mir-126-3p, hsa-mir-26b-5p, hsa-mir-101-3p, hsa-mir-130a-3p, hsa-mir-429, hsa-mir-192-5p, hsa-mir-194-5p, hsa-mir-92a-3p, hsa-mir-212-3p, hsa-mir-29c-3p, and hsa-mir-24-3p (Table 4).

Roc curve analysis

ROC curves were constructed using the Graphpad prism software. The ROC (Receiver-Operating Characteristic) analysis is a valuable tool utilized for evaluating the performance of a diagnostic test or the precision of a statistical model. It consists of a graph that represents the sensitivity (true positive rate) for different cutoff values of a variable as a function of 100-fold specificity (false positive rate). AUC is used to measure how effectively a variable can distinguish between diagnostic groups, such as liver cancer, and control samples. The closer the curve is to the upper left corner, the higher the overall test accuracy. ROC curve analysis was performed on the top 20 hub genes in the network. The threshold for selection of potential biomarkers was set at an AUC value

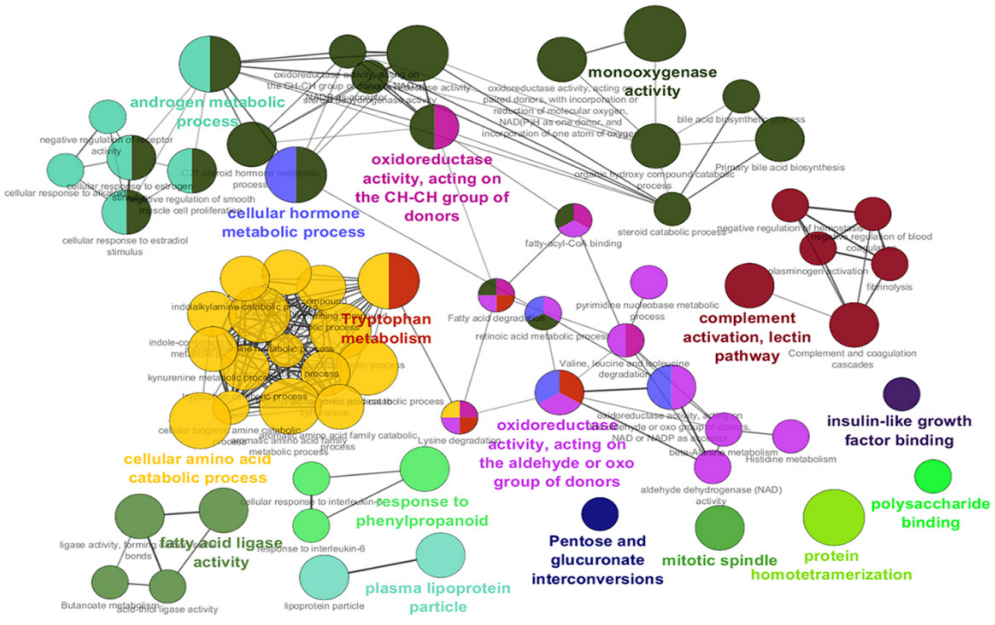

Figure 4. The Network of Gene Ontology and Pathway Enrichment Results.

Table 3. Gene ontology and KEGG Pathway Enrichment Results for the Genes with Altered Expression. (CC: Cellular Component)

Ontology	GOID	GOTerm	Corrected P-Value	% Associated Genes	Associated Genes Found
Biological Process	GO:0034754	cellular hormone metabolic process	7.80E-06	7.826	*[AKR1C3, AKR1D1, ALDH8A1, CYP26A1, ESR1, PPARGC1A, SRD5A2, STARD5, SULT1A1]*
	GO:0051289	protein homotetramerization	0.00048	8.571	*[ACADS, CTH, DCXR, GLYAT, TK1, TRPM8]*
	GO:0008209	androgen metabolic process	0.00016	15.151	*[AKR1C3, AKR1D1, ESR1, PPARGC1A, SRD5A2]*
	GO:0080184	response to phenylpropanoid	0.0026	23.076	*[FGA, GLYAT, PPARGC1A]*
	GO:0009063	cellular amino acid catabolic process	3.00E-07	7.913	*[AGXT2, ALDH6A1, BBOX1, CTH, FTCD, GCDH, GSTZ1, IDO2, KMO, PRODH2, TDO2]*
CC	GO:0072686	mitotic spindle	0.0017	8.62	*[ASPM, AURKA, FAM83D, NCAPG, RACGAP1]*
	GO:0034358	plasma lipoprotein particle	0.0033	7.317	*[LCAT, LPA, SORL1]*
Molecular Function	GO:0015645	fatty acid ligase activity	0.0037	20	*[ACSL1, ACSM3, SLC27A5]*
	GO:0030247	polysaccharide binding	0.0063	12	*[AGL, CLEC4G, FCN2]*
	GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	0.0018	8.771	*[ACADS, AKR1C3, AKR1D1, GCDH, SRD5A2]*
	GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	0.0041	10	*[AKR1C3, ALDH2, ALDH6A1, ALDH8A1]*
	GO:0004497	monooxygenase activity	4.30E-06	8.411	*[AKR1C3, CYP26A1, CYP39A1, CYP4F12, CYP4V2, DBH, KMO, SQLE, TDO2]*
	GO:0005520	insulin-like growth factor binding	0.0077	10.714	*[CYR61, ESM1, IGFBP3]*
KEGG pathway	GO:0000040	Pentose and glucuronate interconversions	0.0069	8.333	*[ALDH2, DCXR, UGP2]*
	GO:0000380	Tryptophan metabolism	0.000019	15	*[ALDH2, GCDH, IDO2, KMO, OGDHL, TDO2]*
	GO:0004610	Complement and coagulation cascades	0.0036	7.246	*[F11, FGA, MASP1, MBL2, SERPINF2]*
	GO:0000071	Fatty acid degradation	0.0051	9.09	*[ACADS, ACSL1, ALDH2, GCDH]*

of 0.80. Among the results, *CDC6, PTTG1, CDCA5, RACGAP1, RAD51AP1, NCAPH, ASPM, AURKA*, and *MELK* showed the highest accuracy with AUC values above 90 percent. These specific entities are recommended as potential diagnostic markers for liver cancer (Table 5 and Supplementary Figure 1). Moreover, they may play

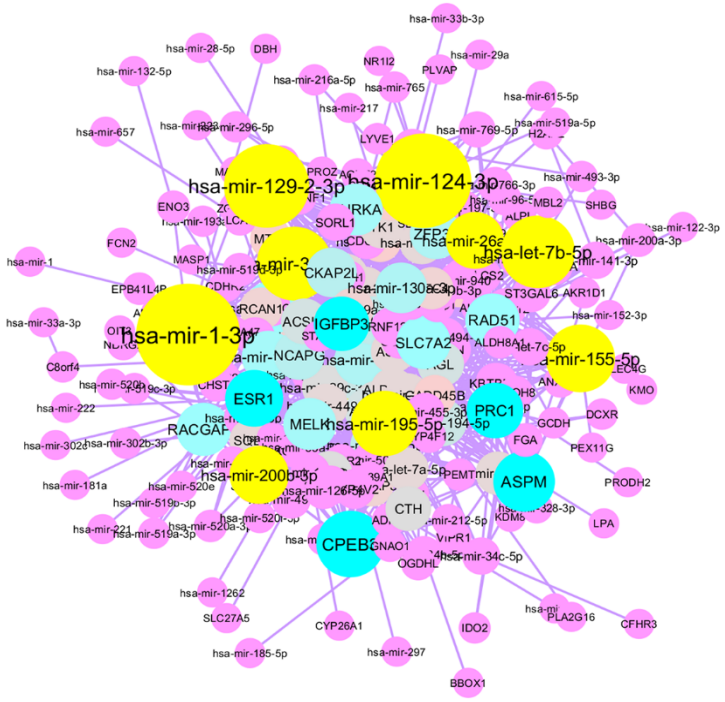

Figure 5. The DEG-miR Interaction Network Constructed in Cytoscape based on All Possible Interactions of DEGs with Their Targets in TarBase and miRDB. Larger circles denote highest degree hub miRs.

Table 4. ROC and kaplan-Meier Survival Curves Analysis Results for the top 20 hub miRs in the mRNA-miRNA Interaction Network, According to the CancerMIRNome Database.

miR Name	Degree	Betweenness centrality	ROC-curve AUC	KM-plot p-val	Hazard ratio
hsa-mir-1-3p	60	0.183	0.61	0.017	0.66
hsa-mir-124-3p	54	0.143	0.54	0.025	1.75
hsa-mir-129-2-3p	45	0.095	0.56	0.18	0.71
hsa-mir-34a-5p	37	0.061	0.81	8.55E-03	0.63
hsa-let-7b-5p	36	0.059	0.8	0.34	0.84
hsa-mir-155-5p	32	0.05	0.51	0.51	1.12
hsa-mir-195-5p	30	0.036	0.91	0.03	0.68
hsa-mir-200b-3p	24	0.04	0.79	0.67	1.08
hsa-mir-26a-5p	23	0.037	0.87	0.94	1.01
hsa-mir-126-3p	21	0.021	0.76	5.08E-03	0.61
hsa-mir-26b-5p	20	0.038	0.89	0.74	0.94
hsa-mir-101-3p	19	0.035	0.93	0.059	0.72
hsa-mir-130a-3p	18	0.026	0.89	0.044	0.7
hsa-mir-429	18	0.017	0.71	0.54	1.11
hsa-mir-192-5p	16	0.018	0.66	0.3	0.83
hsa-mir-194-5p	15	0.015	0.54	1.50E-03	0.57
hsa-mir-92a-3p	15	0.013	0.59	0.43	0.87
hsa-mir-212-3p	13	0.01	0.56	7.93E-03	1.61
hsa-mir-29c-3p	13	0.007	0.88	0.16	0.78
hsa-mir-24-3p	12	0.023	0.62	0.3	1.2

a fundamental biological role in the pathogenesis of HCC. Supplementary Figure 1 shows only ROC curves with AUC values exceeding 0.9. For miRs, those with ROC curves with AUC values greater than 0.8 were selected as potential diagnostic and/or prognostic markers for hepatocellular carcinoma. Among the top 20 miRs identified in the network, eight showed AUC≥0.8, including has-mir-34a-5p, hsa-let-7b-5p, has-mir-195-5p, has-mir-26a-5p, has-mir-26b-5p, has-mir-101-3p, has-mir-130a-3p, and has-mir-29c-3p. Detailed results are shown in Table 4. Supplementary Figure 2 shows the results of ROC curves and miR expression boxplots. The boxplots showed that the expression levels of eight miRs were lower in the HCC group compared with normal samples, except for hsa-mir-34a-5p. Among these miRs, hsa-mir-101-3p and hsa-mir-195-5p showed the highest AUC values of 0.93 and 0.91, respectively.

Table 5. ROC and Kaplan-Meier Survival Curve Analysis Results for the top 20 hub/bottleneck Genes in the Protein-Protein Interaction Network. It is also shown that if the expression of the gene is favorable or unfavorable with the survival according to the HPA database.

Gene symbol	KM-plot log-rank p	ROC curve p-value	AUC	Correlation with survival
CDC6	6.00E-08	0.0001	0.94	unfavorable
AURKA	0.00015	<0.0001	0.91	unfavorable
NCAPG	3.10E-06	0.0067	0.8	unfavorable
PTTG1	1.60E-06	<0.0001	0.94	unfavorable
MCM5	0.00018	0.0005	0.88	unfavorable
NCAPH	9.50E-06	0.0002	0.91	unfavorable
RFC4	6.80E-07	<0.0001	0.9	unfavorable
ASPM	0.00013	<0.0001	0.91	unfavorable
KIF23	0.000032	0.0016	0.85	unfavorable
RAD51AP1	0.00035	0.0001	0.92	unfavorable
MELK	2.90E-07	0.0002	0.91	unfavorable
PCLAF	0.000033	<0.0001	0.88	unfavorable
RACGAP1	1.90E-06	<0.0001	0.93	unfavorable
CDCA5	8.20E-06	<0.0001	0.93	unfavorable
SERPINF2	0.000023	<0.0001	0.9	favorable

Table 6. A Panel of the Top 5 Potential Protein Biomarkers for Liver Cancer Diagnosis and Prognosis, Sorted based on the Area under the ROC Curves. Kaplan-Meier survival curves log-rank p-values are extracted from the UALCAN database.

Gene Name	AUC	KM-plot log-rank p-value	Diagnostic	Prognostic
CDC6	0.94	6.00E-08	+	+
PTTG1	0.94	1.60E-06	+	+
CDCA5	0.93	8.20E-06	+	+
RACGAP1	0.93	1.90E-06	+	+
RAD51AP1	0.92	0.00035	+	+

Table 7. A Panel of the Top Ranked Potential Diagnostic and Prognostic miR Biomarkers in Liver Cancer, Sorted based on the Area under the ROC Curves.

miR Name	AUC	KM-plot log-rank p-value	Diagnostic	Prognostic
hsa-mir-101-3p	0.93	0.059	+	-
hsa-mir-195-5p	0.91	0.03	+	+
hsa-mir-130a-3p	0.89	0.044	+	+
hsa-mir-26b-5p	0.89	0.74	+	-
hsa-mir-29c-3p	0.88	0.16	+	-
hsa-mir-26a-5p	0.87	0.94	+	-
hsa-mir-34a-5p	0.81	8.55E-03	+	+

Survival analysis results

To evaluate the association between the proposed biomarkers and the overall survival of liver cancer patients, gene and miR survival data were retrieved from primary databases, including Human Protein Atlas, UALCAN, and CancerMIRNome. The Kaplan-Meier survival curve represents the probability of survival over a certain time interval, taking into account time in many smaller sections [28]. Typically, the horizontal axis represents time in months or years, while the vertical axis represents the estimated cumulative survival probability. A steeper increase means an increase in the number of deaths, and therefore a less optimistic survival prognosis. Conversely, a gentler slope indicates a lower frequency of accidents and an improved chance of survival. To construct the Kaplan-Meier curve, patients are divided into two groups based on the expression of the target gene or miR, and the survival time of each patient is recorded. Normally, there should be hundreds or more patients in each group. In such cases, the log-rank t-test is usually used to compare the high and low expression groups. The Kaplan-Meier survival curve of the genes with an AUC of 0.9 or higher and a p-value of less than 0.05 from the UALCAN database was used to explore the effect of the expression levels of these genes on liver cancer survival. A log-rank p-value of less than 0.05 was set as the cutoff value. All the putative gene/protein markers with AUC values greater than 0.9 showed unfavorable correlation with overall survival. However, SERPINF2 showed favorable correlation according to both the Human Protein Atlas and UALCAN (Table 5, Supplementary Figure 3). This suggests that higher levels of these genes or proteins are associated with poorer survival in HCC patients, while increased levels of SERPINF2 are associated with better survival outcomes. Therefore, we proposed a theoretical group of diagnostic and prognostic markers associated with liver cancer survival, which consisted of a set of five most significant gene/protein markers and seven microRNAs, listed in Table 6 and 7. The *CDC6, PTTG1, CDCA5, RACGAP1*, and *RAD51AP1* were the five top protein markers. The top-ranked microRNAs with diagnostic significance (AUC≥0.8) include hsa-mir-101-3p, hsa-mir-195-5p, hsa-mir-130a-3p, hsa-mir-26b-5p, hsa-mir-29c-3p, hsa-mir-26a-5p and hsa-mir-34a-5p (among which hsa-mir-34a-5p, hsa-mir-195-5p and hsa-mir-130a-3p can also function as prognostic markers) used to predict overall survival of HCC patients. The Kaplan-Meier survival curves in Supplementary Figure 4 show that increased levels of hsa-mir-34a-5p (p-val=0.03), hsa-mir-195-5p (p-val=0.044), and hsa-mir-130a-3p (p-val=8.55E-03) are associated with improved survival in HCC patients. The hazard ratios (HRs) shown in this figure are used to quantitatively evaluate the degree of difference between the two curves shown in the Kaplan-Meier plot, and the P-value determines the statistical significance of this difference. A hazard ratio of 1 means equal risk. A number greater than 1 indicates increased risk, and a number less than 1 indicates decreased risk. The hazard ratios for the above miRs ranged from 0.6 to 0.7, indicating that increased expression was associated with a 30-40-fold increase in mortality. The protein levels of *CDC6, PTTG1, CDCA5*, and *RACGAP1* that were analyzed in the HPA database by immunohistochemistry are shown in Supplementary Figure 5.

Discussion

Hepatocellular carcinoma ranks as the fourth highest cause of cancer-related deaths globally [29]. Although many targeted therapies have been developed, there is still a requirement for more effective biomarkers to help early detection of HCC. In the modern era of precision medicine,

a variety of bioinformatics techniques are being used to enhance researchers' understanding of molecular abnormalities in cancer and make it more widely available worldwide [30]. Recently, non-coding RNAs, mainly miRNAs, in conjunction with genes and transcripts, have emerged as molecular markers for clinical treatment due to their altered expression patterns in cancer [31]. The aim of this study was to identify potential biomarkers for HCC detection and overall survival prognosis through omics and bioinformatics analysis, and to determine the final hub genes and miRNAs through network analysis. Insights from PPI network analysis, ROC analysis, and Kaplan-Meier curve analysis were combined to generate a selection of the top five proteins that may potentially serve as both diagnostic and prognostic markers for HCC. These proteins include *CDC6, PTTG1, CDCA5, RACGAP1*, and *RAD51AP1*. The replication process is controlled in part by replication licensing elements under careful surveillance [32]. Among all these factors, *CDC6* has been highlighted as an essential molecule. *CDC6* or Cell division cycle 6, is a protein responsible for monitoring DNA replication and participating in control checkpoints [33]. In the case of liver cancer, Kong et al. conducted a study showing a correlation between *CDC6* expression levels and poor prognosis as well as the progression of hepatocellular carcinoma [34]. A recent study by Jia et al. also reported that *CDC6, RACGAP1, ASPM, AURKA*, and a type of NCAP protein were the most highly expressed proteins in live cancer cells, all of which were included in the DEG results [35]. Furthermore, data obtained from comparative toxicogenomic analysis revealed that these basic genes were related to cell death, immune response, hepatocellular carcinoma, liver cirrhosis, and adenoid cystic carcinoma [35]. *CDCA5* is the other cell division cycle-associated gene, according to our results. The cellular division cycle-associated gene family is composed of notable regulators of cell proliferation that are known to be critically involved in various malignancies [36, 37]. Several studies have demonstrated that *CDCA5* and *CDCA8* are important factors in the development of HCC [38, 39]. To illustrate, investigations have demonstrated that *CDCA5* is overexpressed in HCC, and this phenomenon exhibits a substantial correlation with tumor advancement and an unfavorable prognosis [36]. Based on our findings, *PTTG1* (Pituitary tumor-transforming gene 1) has emerged as another promising biomarker candidate for HCC. *PTTG1* expression has been significantly increased in most human tumors, promoting tumor cell proliferation, migration, invasion, and angiogenesis [40]. A suggested mechanism of action of *PTTG1* in HCC is that it promotes the transcription of asparagine synthetase by binding to its promoter. As a result, the levels of asparagine (Asn) increase accordingly. Elevated Asn levels then activate the mTOR signaling pathway, promoting the progression of HCC [41]. The *RACGAP1* gene (RacGTPase-activating protein 1) is another top marker in the PPI network. The *RACGAP1* gene plays a role in many cellular processes, including the regulation of cytokinesis, proliferation, migration, transformation, invasion, and metastasis [42]. Overexpression of *RACGAP1* correlates with poor overall survival and disease-free survival in HCC patients [43]. Several studies have elucidated the underlying mechanisms of RACGAP1 upregulation in hepatocellular carcinoma [43]. ECT2 interacts and colocalizes with *RACGAP1*, thereby protecting it from degradation. Additionally, *RACGAP1* facilitates ECT2-mediated activation of RhoA and metastasis in HCC cells [44]. The initial documentation of ECT2 (Epithelial Cell Transforming 2) identified it as an oncogene that plays a role in various forms of human cancer [45]. Results of previous studies have also shown that aberrant expression and abnormal distribution of RACGAP1 in the cytoplasm and nucleus may correspond to the development and progression of HCC [46]. The next notable protein was RAD51-associated protein 1 (*RAD51AP1*). Overexpression of *RAD51AP1* plays an important role in both cell cycle and repair processes. Moreover, it holds significant diagnostic and prognostic value in hepatocellular carcinoma [47]. A study conducted by Chai et al. investigated the regulatory mechanism of the competing endogenous RNA (ceRNA) networks. They hypothesized that the long noncoding RNA MSC-AS1 exerts its influence on hsa-miR-23c, thereby modulating the DNA damage repair process in HCC through interaction with *RAD51AP1* [48]. In this study, we selected the top 20 candidate miRNAs as useful biomarkers (with significant degree and betweenness centrality) from the mRNA-miRNA network study in HCC. Of these, three miRNAs, namely hsa-mir-1-3p, hsa-mir-124-3p, and hsa-mir-129-2-3p, showed the highest node degree. Hubs are actively involved in a large number of interactions, which makes them more likely to play the role of master regulators in both signaling and transcriptional processes [49]. According to the literature, miR-1-3p is dysregulated in various tumors and is closely related to tumor initiation and progression and drug resistance [50]. For example, Chen et al. conducted a study showing that miR-1-3p is downregulated in HCC tissues and cells. Conversely, upregulation of miR-1-3p may impede the proliferation, migration, and invasion of HCC cells, thereby activating the apoptotic signaling pathway [51]. Hsa-mir-124-3p was identified as another hub miR in our study. Studies have shown that decreased expression of miR-124-3p is associated with poor survival outcomes in HCC patients [52]. Recently, Zhao et al. demonstrated that miR-124-3p inhibited HCC proliferation and epithelial-mesenchymal transition (EMT) by binding to the 3′UTR of arrestin domain-containing 1 (ARRDC1) [53]. Another study found that miR-124-3p functions as a key miR in suppressing HCC development by targeting CRKL [54]. These findings suggest that the miR-124-3p/ARRDC1 and miR-124-3p/CRKL regulatory pathways may serve as novel diagnostic and therapeutic targets to inhibit HCC proliferation and metastasis. Hsa-mir-129-2-3p was identified as another top hub in this study. The results of previously conducted studies confirmed the idea that miR-129-2 plays a role in tumor suppression in various human malignancies [55]. In this context, Liu et al. showed that miR-129-2 levels were significantly decreased in HCC tissues and cell lines. Furthermore, they found that DNA methylation was involved in the downregulation of miR-129-2. However, demethylation of miR-129-2 increased

its expression in HCC cells, resulting in a remarkable inhibitory effect on cell migration and invasion [56]. In conclusion, miR-129-2 exerts a tumor-suppressive function and therefore can be considered as a prognostic biomarker for HCC patients. Based on the results of ROC curve analysis and the AUC values, as well as the findings from Kaplan-Meier plots, we selected a set of top ranked microRNAs that possess diagnostic and prognostic significance in the context of liver cancer. The seven microRNAs of diagnostic relevance are hsa-mir-101-3p, hsa-mir-195-5p, hsa-mir-130a-3p, hsa-mir-26b-5p, hsa-mir-29c-3p, hsa-mir-26a-5p and hsa-mir-34a-5p. In particular, hsa-mir-34a-5p, hsa-mir-195-5p and hsa-mir-130a-3p may also serve as prognostic markers for predicting the overall survival of patients with hepatocellular carcinoma. In this regard, many studies have been conducted that proved the primary importance of the above miRs in HCC [57, 58]. The expression of miR-34a-5p was observed to be reduced in hepatocellular carcinoma cells and tumor tissue. Overexpression of miR-34a-5p led to a reduced invasive ability of HCC cells [59]. Increasing studies suggest that aberrant downregulation of miR-101-3p and miR-26a-5p in both tumor tissues and cell lines is associated with the occurrence and development of hepatocellular carcinoma, as well as a poor prognosis in HCC [60, 61]. Our findings ranked hsa-mir-195-5p as the top biomarker for both diagnosis and prognosis of HCC. Chen et al. conducted a study to determine the diagnostic significance of serum miR-195 in hepatocellular carcinoma [62]. The results showed that the expression of miR-195 was decreased in both HCC cells and the serum of patients compared to the controls. Thus, they concluded that miR-195 may serve as a noninvasive diagnostic biomarker for patients with HCC. Another miR of high significance was hsa-mir-130a-3p. HOXD-AS1, a long noncoding RNA, has been reported to bind to miR-130a-3p and prevent miR-induced degradation of SOX4 protein. This process leads to the activation of EZH2 and MMP2 which facilitates metastasis of HCC tumors. Hsa-mir-34a-5p is another miR in our results with both diagnostic and prognostic potential, which showed a favorable correlation with the survival of tumor samples compared with adjacent normal tissues. Research demonstrated that miR-34a enhances the sensitivity of HCC tumor cells to targeted therapy by attacking Bcl-2. It has also been suggested that miR-34a-5p may hinder the metastasis of liver cancer cells by inhibiting MYCT1 transcription [63]. In this study, investigation of KEGG pathway enrichment of significantly altered expressed genes revealed that the foremost pathways of notable importance involved interconversions of pentose and glucuronate, metabolism of tryptophan, cascades of complement and coagulation, and fatty acid degradation. The conversion of pentose to glucuronate in the pentose phosphate pathway, a metabolic pathway that occurs in parallel with glycolysis, provides cancer cells with another way to utilize glucose and also produces ribose-5-phosphate. Ribose-5-phosphate is an essential precursor of nucleic acids which is crucial for promoting rapid cell division and growth in hepatocellular carcinoma. A previous serum metabolomics study performed on HCC patients found 757 distinct metabolites. These metabolites were found to be abundant in the pathways of pentose and glucuronate interconversions, as well as tryptophan biosynthesis, which were identified as the most enriched pathways in the development of HCC [64]. Based on our findings, previous bioinformatics studies have often highlighted the complement and coagulation cascade signaling pathways as enriched functional pathways for understanding the mechanisms of hepatocellular carcinoma. Moreover, the involvement of C8B in the complement and coagulation cascade signaling pathways has been proposed as a prognostic biomarker for the survival of HBV-related HCC patients [65]. It is noteworthy that the liver is the primary source of the biosynthesis of more than 80% of complement components and also expresses various complement receptors. Recent studies have shown that the complement system is involved in liver inflammation, fibrosis, abnormal regenerative responses, carcinogenesis and the development of hepatocellular carcinoma [66]. Another KEGG pathway that showed enrichment in HCC was related to tryptophan metabolism, whose complex mechanisms are important in controlling cancer growth and metastasis. The liver is the primary site of tryptophan catabolism. However, the exact involvement of tryptophan metabolism in hepatocellular carcinoma development remains unclear. Clinical investigations have provided evidence suggesting that the metabolic breakdown of tryptophan promotes the advancement of tumors by exerting an influence on the immunosuppressive microenvironment through various mechanisms [67]. Previous studies have demonstrated the excellent predictive ability of genes related to tryptophan metabolism in determining survival outcomes in HCC cohorts. In addition, genetic signatures related to tryptophan metabolism were found to be significantly associated with specific immune infiltration patterns and drug sensitivity [68]. Fatty acid degradation was another important pathway in HCC. Metabolic reprogramming, mainly of lipid metabolism, is crucial in cancer development and progression, mainly providing energy source and micro-environmental adaptation and producing signaling molecules. In the case of HCC, various studies have suggested that fatty acid degradation pathways, such as alpha, beta, and omega oxidation, are key features of HCC progression. In a recent study, Li et al. utilized the fatty acid degradation pathway to distinguish different subtypes of HCC patients at similar stages and develop and improve targeted therapy [69]. Apelin and Rap1 signaling pathways were the most abundant pathways associated with miRs in the mRNA-miRNA network. Apelin, a 77-amino acid peptide, acts as a natural ligand for the angiotensin-like receptor 1 (APJ), a G protein-coupled receptor that regulates various physiological processes such as angiogenesis [70]. Previous studies have shown that apelin stimulates arteriogenesis in HCC by increasing smooth muscle cell proliferation and dilating blood vessels [71]. Rap1 signaling is another pathway that was enriched in our results. Previous studies have also demonstrated the role of the Rap1 signaling pathway in HCC [72]. It triggers MAPK signaling to

control migration and proliferation, thereby affecting cancer development. The MAPK-Rap1A signaling pathway plays a role in the tumor microenvironment and improves the prognosis of HCC [73]. Various research studies have shown a correlation between the expression and polymorphisms of Rap genes and tumorigenicity in HCC cells [74].

Challenges and limitations

Tumor gene expression profiles are associated with clinical and pathological characteristics, and patient prognosis. Many studies have shown the feasibility of conducting tissue biomarker studies in patients with hepatocellular carcinoma. Genetic biomarkers associated with HCC prognosis need to be further validated in clinical trials. A limitation of the present study concerns the limited size of the sample cohort. Subsequent research efforts should aim to increase the sample size to investigate the prognostic validity of the proposed biomarker panel and formulate a predictive model for predicting HCC prognosis. There are also some limitations such as individual or instrumental errors during the experiments. There are a number of other potential obstacles and considerations that must be taken into account before implementing a biomarker-based screening approach for hepatocellular carcinoma. First, the performance of the biomarker must be at least comparable to that of alternative screening methods, such as ultrasound or alpha-fetoprotein, in terms of early detection of HCC. Biomarker-based strategies have the potential to streamline the results reporting process, thereby accelerating diagnostic evaluation and addressing deficiencies in the subsequent screening workflow. Nevertheless, it is essential that the sensitivity of the biomarker is sufficient for consistent detection in different patient subgroups while ensuring specificity to minimize false-positive cases. In addition to incomplete cohort data and limited sample sizes, another obstacle in biomarker studies is the presence of selection bias in sampling. Furthermore, it is important to consider the detection technology and patient characteristics in clinical biomarker studies. An essential limitation of this study lies in the inability to establish a link between our results and the various subcategories within the histological cancer classification. The downloaded dataset lacked histological information, complicating the ability to make such correlations. These subtypes are known to be associated with aggressiveness, metastasis, and unfavorable outcomes such as poor prognosis in patients diagnosed with HCC. Tumor heterogeneity denotes the presence of varied cellular subpopulations within a tumor or across tumors of the same histopathological category. These specific groups of cells exhibit different genetic and physical characteristics, leading to unique biological behaviors. The presence of genetic differences within a tumor is crucial for the emergence of treatment resistance and recurrence of HCC. Therefore, it is crucial to acquire a thorough knowledge of the molecular processes responsible for the development of tumor heterogeneity. The development of new translational research frameworks that more accurately reflect the characteristics of native tumor cells will improve our knowledge of the different mechanisms underlying HCC heterogeneity and their impact on the emergence of drug resistance and treatment ineffectiveness. Enhanced exploration employing sophisticated translational platforms in large patient cohorts with diverse etiological and genetic profiles may accelerate the discovery of key biomarkers essential for accurate early detection of HCC, ultimately paving the way for more effective therapeutic interventions.

In conclusion, although hepatocellular carcinoma remains a challenging disease, the discovery and use of diagnostic and prognostic biomarkers offer hope in the fight against liver cancer. While the existing biomarkers have shown promise in predicting HCC prognosis, ongoing studies are aimed at identifying additional markers that may further improve accuracy and reliability. Incorporating these biomarkers into clinical practice may further refine prediction of overall survival and individualize treatment approaches. In the current study, the integration of GEO datasets with protein-protein and mRNA-miRNA interaction networks identified several proteins and microRNAs that may be promising biomarkers for the diagnosis and prognosis of HCC. The proteins *CDC6, PTTG1, CDCA5, RACGAP1*, and *RAD51AP1* performed best in terms of diagnostic and prognostic value. Similarly, the microRNAs hsa-mir-101-3p, hsa-mir-195-5p, hsa-mir-130a-3p, hsa-mir-26b-5p, hsa-mir-29c-3p, hsa-mir-26a-5p, and hsa-mir-34a-5p were highly ranked with diagnostic value, while hsa-mir-34a-5p, hsa-mir-195-5p, and hsa-mir-130a-3p were additionally found to serve as prognostic markers for predicting overall survival in individuals with HCC. Future studies should mainly focus on exploring the mechanisms by which these proteins and miRs promote HCC progression and evaluating the therapeutic potential of these biomolecules in HCC patients. Furthermore, it will be essential to validate these findings in larger cohorts and by using different analytical methods.

Author Contribution Statement

R.FY, A.AO, and N.AD contributed to the conception and design of the study. All the authors contributed to data collection, statistical analysis, and interpretation of the data. R.FY and A.AO wrote the paper draft. All the authors reviewed and approved the final manuscript.

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Ethical issue

No humans or animals were included in this study.

Availability of data

The datasets analyzed during the current study are available in the NCBI GEO database. Other data are available as Supplementary files.

Conflict of interest

The authors have no conflicts of interest to declare.

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