



Identification of Potential Prognostic Markers and Key Therapeutic Targets in Hepatocellular Carcinoma Using Weighted Gene Co-Expression Network Analysis: A Systems Biology Approach

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Background: As the most prevalent form of liver cancer, hepatocellular carcinoma (HCC) ranks the fifth highest cause of cancer-related death worldwide. Despite recent advancements in diagnostic and therapeutic techniques, the prognosis for HCC is still unknown.

Objectives: This study aimed to identify potential genes contributing to HCC pathogenicity.

Materials and Methods: To this end, we examined the GSE39791 microarray dataset, which included 72 HCC samples and 72 normal samples. An investigation of co-expression networks using WGCNA found a highly conserved blue module with 665 genes that were strongly linked to HCC.

Results: *APOE*, *NAT2*, *LCAT*, *TTC36*, *IGFALS*, *ASPDH*, and *VIPRI* were the blue module's top 7 hub genes. According to the results of hub gene enrichment, the most related issues in the biological process and KEGG were peroxisome organization and metabolic pathways, respectively. In addition, using the drug-target network, we discovered 19 FDA-approved medication candidates for different reasons that might potentially be employed to treat HCC patients through the modulation of 3 hub genes of the co-expression network (*LCAT*, *NAT2*, and *VIPRI*). Our findings also demonstrated that the 3 scientifically validated miRNAs regulated the co-expression network by the *VIPRI* hub gene.

Conclusion: We found co-expressed gene modules and hub genes linked with HCC advancement, offering insights into the mechanisms underlying HCC progression as well as some potential HCC treatments.

Keywords: Drug Repositioning, Hepatocellular Carcinoma, MicroRNAs, Systems Biology, WGCNA.

1. Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer death worldwide (1). The disease is estimated to have a global annual incidence rate of 1,000,000 cases, with an approximately 4:1 male to female ratio (2). HCC is a complex disease; however, the most probable risk factors for its progression include cirrhosis, hepatitis virus infections, alcohol use, smoking, diabetes, age,

and gender (3). Studies showed that several biomarkers could identify HCC, including AFP-L3 (4), Des- π -carboxyprothrombin (5), Glypican-3 (6), Golgi Protein 73, and Midkine (7). Among them, serum alpha-fetoprotein (AFP) is used as the primary and common diagnostic test for HCC patients in the clinic. The AFP protein is used as a tumor marker associated with malignancies in early screening as well as pre- and post-treatment of HCC patients (8). For HCC diagnosis

and treatment, AFP is also advised along with standard imaging techniques such ultrasonography (US), magnetic resonance imaging (MRI), and computer scanning (CT) (9). However, AFP as a diagnostic biomarker lacks specificity and sensitivity in HCC and liver cirrhosis differentiation (10).

Furthermore, it lacks the discriminatory capacity to detect damages caused by cirrhosis and chronic hepatitis triggered by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, as well as HCC and intrahepatic (11). Due to the lack of certain specific and sensitive biomarkers to confirm HCC patients, it was difficult to diagnose and treat HCC as early as possible; therefore, some patients will be missed from therapeutic and optimal prognosis (10). The limitations mentioned above highlight the necessity and urgency of finding other biomarkers to be used alone or incorporated with AFP in HCC diagnosis. Since HCC is a heterogeneous disease with progressive accumulation of gene alterations and

complex multi-step processes. We need to discover the vast molecular tumor landscapes at several levels, including gene expression, transcriptome, and epigenetic alterations (9). The strategy that can bring us closer to this goal is the use of tumor and normal samples for analyzing high-throughput gene microarray from patients and healthy individuals, respectively.

2. Objectives

We propose to utilize bioinformatics approaches in this research based on weighted gene co-expression network analysis (WGCNA) to identify the differentially expressed genes and microRNA (miRNAs) as well as drug-gene interaction between tumor and matched non-tumor surrounding tissues of HCC patients who underwent surgical resection as the treatment initiation for evaluation of late recurrences. The overall workflow of the present study is presented in **Figure 1**.

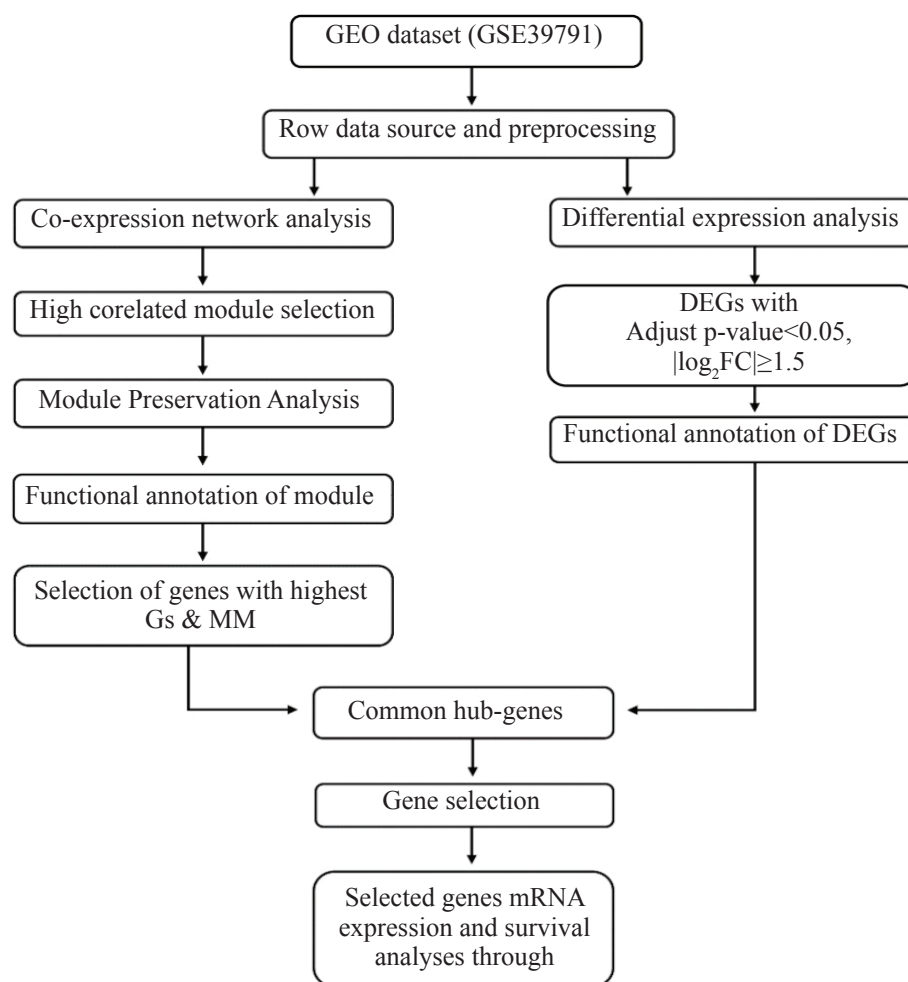


Figure 1. The flowchart of the study.

3. Materials and Methods

3.1. Microarray Data Study

The GSE39791 microarray dataset was obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). This dataset was based on the Agilent GPL10558 platform (Illumina HumanHT-12 V4.0 expression bead chip) and contained a total of 144 samples, including 72 HCC samples and 72 normal liver tissues. The raw data were normalized, quantile-normalized, and gene symbols were used instead of probe IDs. In all samples, gene symbols were filtered according to their variance; only genes with variances ranking in the top 4000 were chosen for additional studies.

3.2. Identification of Differentially Expressed Genes (DEGs)

DEG analysis was conducted using GEO2R (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) and limma package of R (version 3.48.0) among HCC and normal liver samples. A gene was deemed a DEG if it matched the following criteria: Adjust p -value < 0.05, and $|\log_2FC| \geq 1.5$. The \log_2 (fold-change) is the log ratio of a gene's expression values in two different conditions. The common genes between the two approaches (limma and GEO2R) were used for downstream analysis.

3.3. Construct Co-Expression Modules of HCC

The WGCNA package was used to create a co-expression network based on gene expression data from patients and a control group (12). First, the “good Samples Genes” function in the WGCNA package was used to check for missing values, and hierarchical cluster analysis was used to detect outliers. Following the scale-free topological algorithm, when the β value was set to 10, the adjacency matrix met the scale-free topology criteria. The topological overlap matrix (TOM) and dissimilarity TOM (dissTOM) were generated based on the adjacency matrix. Ultimately, modules were described as a group of strongly linked genes with a cut height of 0.1 and a minimum module size of 30 genes.

3.4. Construct Module-Trait Relationships of HCC

In order to determine modules that were connected with the examined clinical characteristic, the module eigengene (ME) was used as the first principal component of the expression matrix to summarize

each module's expression profile. To investigate the connection between particular genes and HCC, the gene significance (GS) values were used. Module membership (MM) was also identified as the relationship between the ME and the gene expression profile of each module. The modules' most significant (central) parts were likewise directly connected with the trait if the GS and MM were significantly correlated (13). As a result, they may be utilized to build a network and pinpoint the genes that act as hubs. Genes with both GS and MM0.8 were chosen as hub-genes as the final stage if they were differentially expressed relative to control samples.

3.5. Module Preservation Analysis

To verify the reliability of the identified modules with significant correlation to HCC, we conducted the module preservation analysis by datasets such as GSE121248 and GSE82177. The GSE121248 is a microarray dataset containing 107 chronic hepatitis B induced HCC and adjacent-normal tissues. On the other hand, GSE82177 contains 27 samples of human liver RNA-seq data corresponding to un-infected non-malignant, HCV infected non-malignant, and HCV+ HCC tissue. The first 4000 genes with the highest coefficient of variation were utilized as an input to determine the extent of module preservation in each dataset. The extent of module preservation was quantified by Z_{summary} statistics, in which $Z_{\text{summary}} < 2$ shows no preservation, $2 < Z_{\text{summary}} < 10$ indicates weak-moderate preservation, $Z_{\text{summary}} > 10$ suggested strong evidence for preservation.

3.6. Functional Enrichment Analysis of Significant Modules

Functional enrichment was investigated using the DAVID (Database for Annotation Visualization and Integrated Discovery) (<https://david.ncifcrf.gov/tools.jsp>), GO (Gene Ontology) (<http://geneontology.org/>), and KEGG (<https://www.genome.jp/kegg/>) databases. We picked enriched ontological words and pathways with a Benjamin-adjusted p -value threshold of 0.05. If there were more than five records, the top five were considered. GeneMANIA (<https://genemania.org/>) and Cytoscape v 3.0 software created functional networks (14). The Venn diagram was created using the free “Venny” v 2.1 software (<http://bioinfogp.cnb.csic.es/tools/venny/>).

3.7. Validation of the Hub Genes

To validate differential expression hub genes among HCC and normal samples, the Gene Expression Profiling Interactive Analysis database (GEPIA; <http://gepia.cancer-pku.cn>) was used. Moreover, the differences in the protein level of hub genes were examined between HCC and normal samples by immunohistochemistry (IHC), using the Human Protein Atlas database (HPA; <https://www.proteinatlas.org/>). Survival analyses of hub genes were carried out by log-rank tests and were plotted using Kaplan–Meier survival curves. Samples were divided into two groups (high-risk and low-risk) by GEPIA online database. After that, risk hazard ratio (HRs), relative confidence intervals (CIs), and *p*-values were generated.

3.8. Identification of Candidate Regulatory MiRNAs and Drugs

Using the miRWalk database (http://mirwalk.umm.uni-heidelberg.de/search_genes/) the miRNA regulatory

network was constructed for recognized hub genes. miRWalk stores predicted data obtained with a machine learning algorithm, including experimentally verified miRNA-target interactions. The focus lies on accuracy, simplicity, user-friendly design and most up-to-date information. In addition, the well-firmly established Drug-Gene Interaction Database (DGIDB) (<http://www.dgidb.org/>) was utilized to predict functional and drug-able hub-genes. Drugs in this database are used clinically, or they are presently used in clinical trials (15).

4. Results

4.1. Identification of DEGs

A total of 50 genes, including 4 up-regulated and 46 down-regulated, were recognized as DEGs with a threshold of adjusted *p*-value 0.05 and were found to be identical in two approaches (limma and GEO2R) (Fig. 2). After that, these 50 DEGs were chosen for further analysis. Positive regulation of collagen fibril

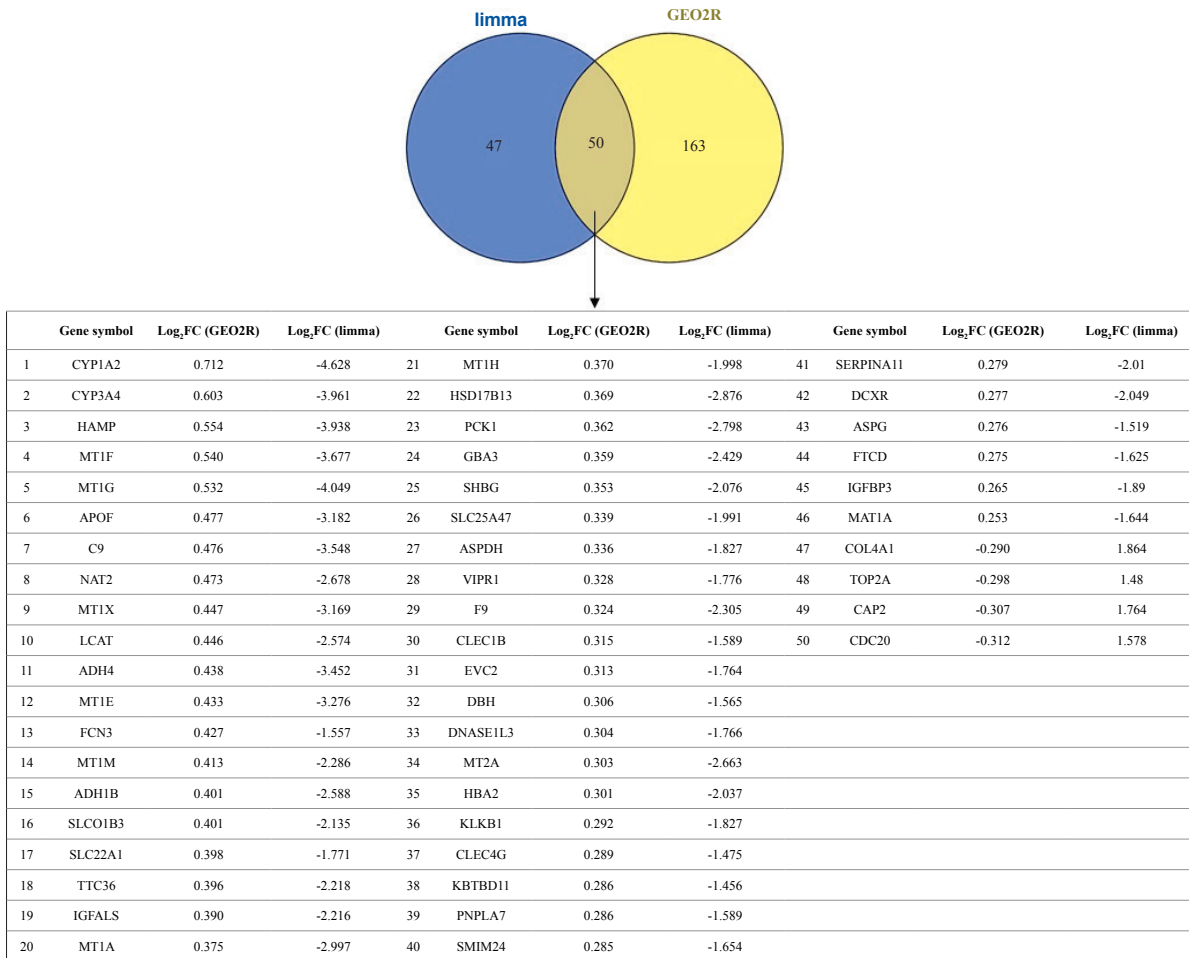


Figure 2. DEG analysis of GSE39791 using two different approach (limma package of R and GEO2R online software). The logFC of the gene expression is presented under the Venn diagram. A total of 50 genes were considered as DEG and were selected for hub-g.

organization, cellular response to amino acid stimulus, and the amino acid response was the most critical biological functions of the DEGs (**Fig. 3**).

4.2. Identification of WGCNA Modules

Quantile-normalization, probe ID conversion, and averaging were performed in the data preprocessing phase of the study. There were 4000 genes included in WGCNA as a result of the obvious broad range of expression values. By using sample clustering, we were able to identify 20 outliers among 144 samples. As a result, 124 samples were included in the analysis (**supplementary data 1**). Afterward, $\beta=10$ was chosen as soft-threshold power, and the weighted co-expression network of HCC patients and normal samples was reconstructed. Thus, the hierarchical clustering dendrogram found 10 modules, which are shown in different colored branches of the dendrogram. Each module's number of genes varied from 49 (green-yellow) to 706 (turquoise) (**Table 1**). In addition, 738 genes were not allocated to any modules (designated as grey).

4.3. Identification of WGCNA Module and Enrichment Analysis

For each module, eigengenes were generated to assess the connection of the modules with the presence of illness in samples and module-module correlation. As previously stated, the blue module was strongly connected with HCC illness ($r=0.92$, $p\text{-value}=3.00E-44$) (**Fig. 4A** and **Table 1**). The most important pathways connected to the blue module and DEGs were investigated using the ClueGO program. Extracellular exosome, extracellular space, positive regulation of cell proliferation and serine-type endopeptidase activity, were important biological functions of the blue module (**Fig. 4B**). We also used the KEGG pathways online website to examine the gene function of the blue module, as well as the desired hub genes with their co-expression genes in the signaling pathways. The enriched hub genes were mainly observed in chemical carcinogenesis, caffeine metabolism, cholesterol metabolism, drug metabolism, and glycerophospholipid metabolism (**Fig. 4B**).

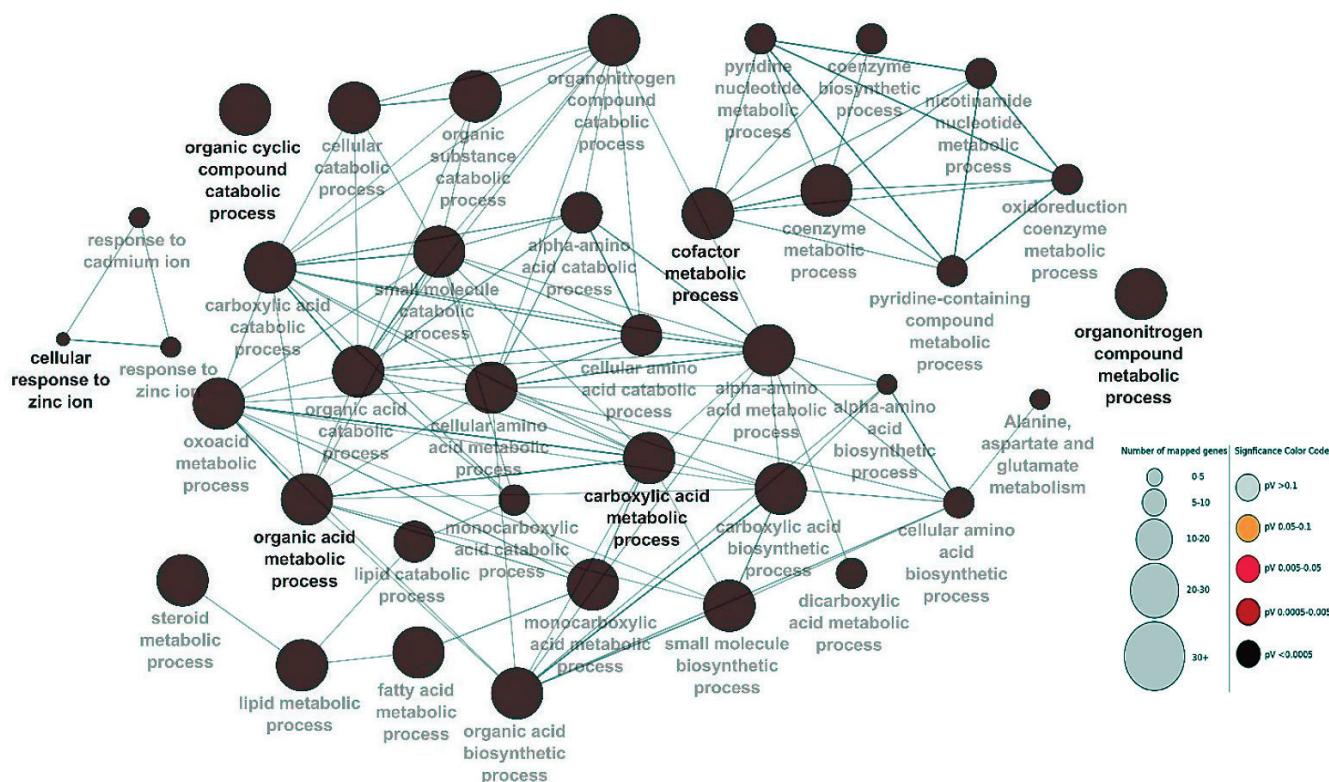


Figure 3. Processes and pathways identified within the DEGs. Gene ontology and pathway analysis were performed using significant genes across all datasets. Node size corresponds to the number of associated genes, and node color reflects the statistical.

Table 1. Module colors characteristics.

Module color	Correlation	p-value	#Genes	Module Preservation	
				GSE82177	GSE121248
Black	-0.84	1.00E-28	156	6.9	15
Blue	0.92	3.00E-44	665	11	23
Brown	0.54	4.00E-09	449	3.1	23
Green	-0.21	0.03	324	3.2	5.5
Greenyellow	0.5	5.00E-08	49	4.7	8.6
Grey	-0.15	0.1	738	1.6	14
Magenta	-0.73	1.00E-18	558	1.4	8.8
Pink	-0.4	3.00E-05	156	8.2	13
Red	-0.076	0.4	199	6.8	14
Turquoise	0.4	3.00E-05	706	1.1	2.4

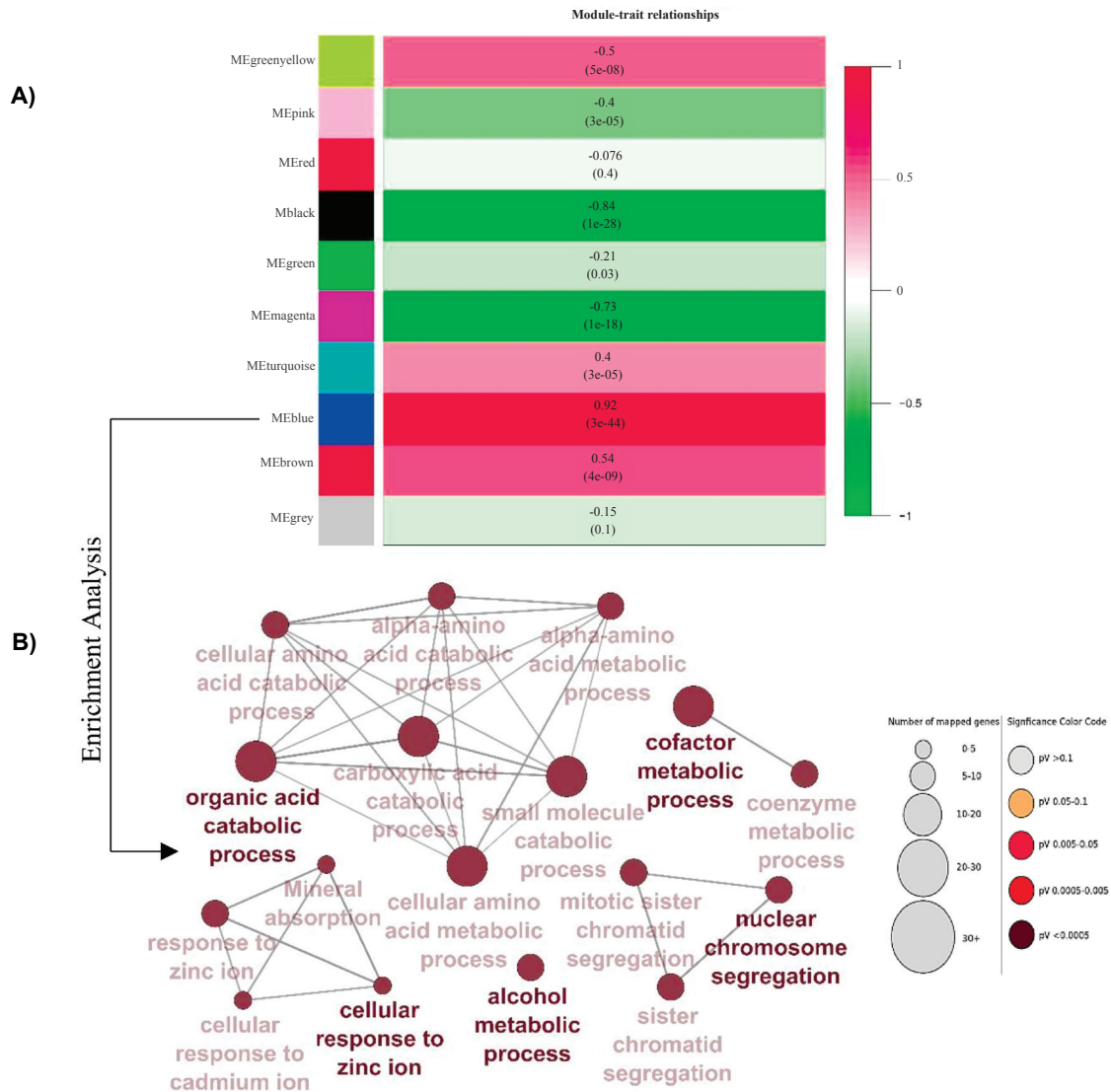


Figure 4. Module-trait relationship and enrichment analysis of blue module. **A)** Module-trait relationship of GSE39791. Each row corresponds to a module eigengene and the column corresponds to HCC status. Numbers in each cell represent the corresponding.

4.4. Module Preservation Analysis

The result of module preservation showed that Z_{summary} scores were equal to 11 and 23 for GSE82177 and GSE121248 datasets, respectively (**Table 1**). These results suggest that the blue module, which was significantly associated with HCC had high preservation in both datasets.

4.5. Hub-Genes Detection and Enrichment Analysis

The association between the blue module's properties (MM and GS) resulted in discovering hub-genes of interest that were strongly connected with HCC pathogenesis. The correlation between features (MM and GS) of the blue module led to detection hub-genes of interest that were highly associated with HCC pathogenesis. *APOF*, *NAT2*, *LCAT*, *TTC36*, *IGFALS*, *ASPDH*, and *VIPR1* were among the hub-genes investigated (**Fig. 5**). The blue module's co-expression network was reconstructed using GeneMANIA online database and visualized using Cytoscape software.

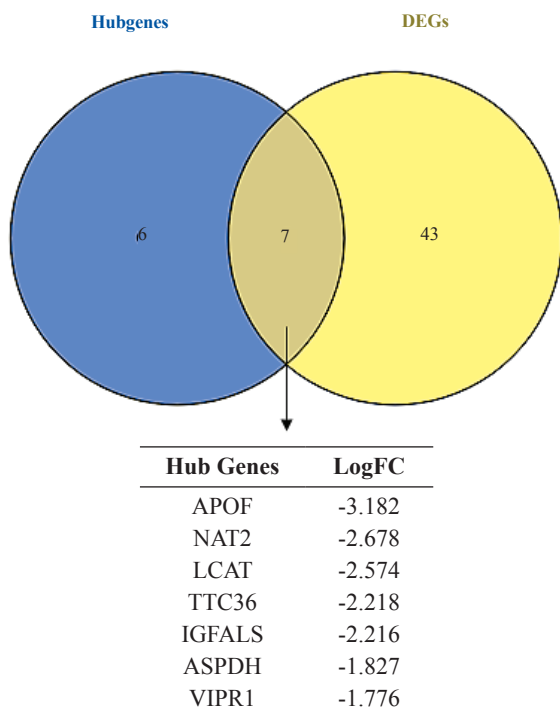


Figure 5. Similarity assessment between DEGs and hub genes of the blue module using a Venn diagram. A total of 7 hub genes which were similar in both list were chosen and then imported to GeneMANIA to construct a co-expression network.

4.6. Identification and Validation of Common-Hub Genes

A total of 7 hub genes with significant differential expression were found among normal and HCC samples in the blue module through validation by GEPIA online tool. Moreover, IHC results obtained from the HPA database showed variances in the expression levels of these hub genes (**supplementary data 2-3**). Survival analysis was performed to estimate the relationship between these 7 hub genes and the prognosis of the disease using the GEPIA database (**supplementary data 4**).

4.7. MicroRNAs as Upstream Regulators for Common Hub-Genes

Predicted hub-gene miRNAs were examined by the miRWalk database to discover any putative molecular processes. The experimentally validated miRNAs were shown in **Figure 6**. As a result, the *VIPR1* hub gene is regulated by *has-miR-1224-3p*, *has-miR-3940-3p*, and *has-miR-6749-3p* miRNAs (**Fig. 6**).

4.8. Drug-Target Network Construction

We searched for known HCC drug targets in the blue module to assess its drug development potential. In addition, we looked for drug targets that have not yet been approved for use in the treatment of HCC in the module we used. These targets in the module included: *LCAT* (Testosterone Propionate, Prednisolone, Streptozotocin, Testosterone), *NAT2* (Thalidomide, Sulfamethazine, Paclitaxel, Aspirin, Docetaxel, Isosorbide Dinitrate, Cyclophosphamide, Alcohol, Isoniazid, Interferon Alfa-2a, Pyrazinamide) and *VIPR1* (Azithromycin, Azelaic Acid, Secretin, Bepridil) (**Fig. 6**). The existence of targets in the module of interest revealed that these drugs may impact HCC and could be evaluated as suitable candidates for further study in this respect.

5. Discussion

Prognoses for HCC are still unknown despite recent advances in diagnostic and therapeutic techniques (16). The important public health aim nowadays is the development of biomarkers and targeted therapy approaches for the early detection and treatment of HCC, respectively, and finally, aid to reduce the mortality rate of the illness worldwide (17). To this

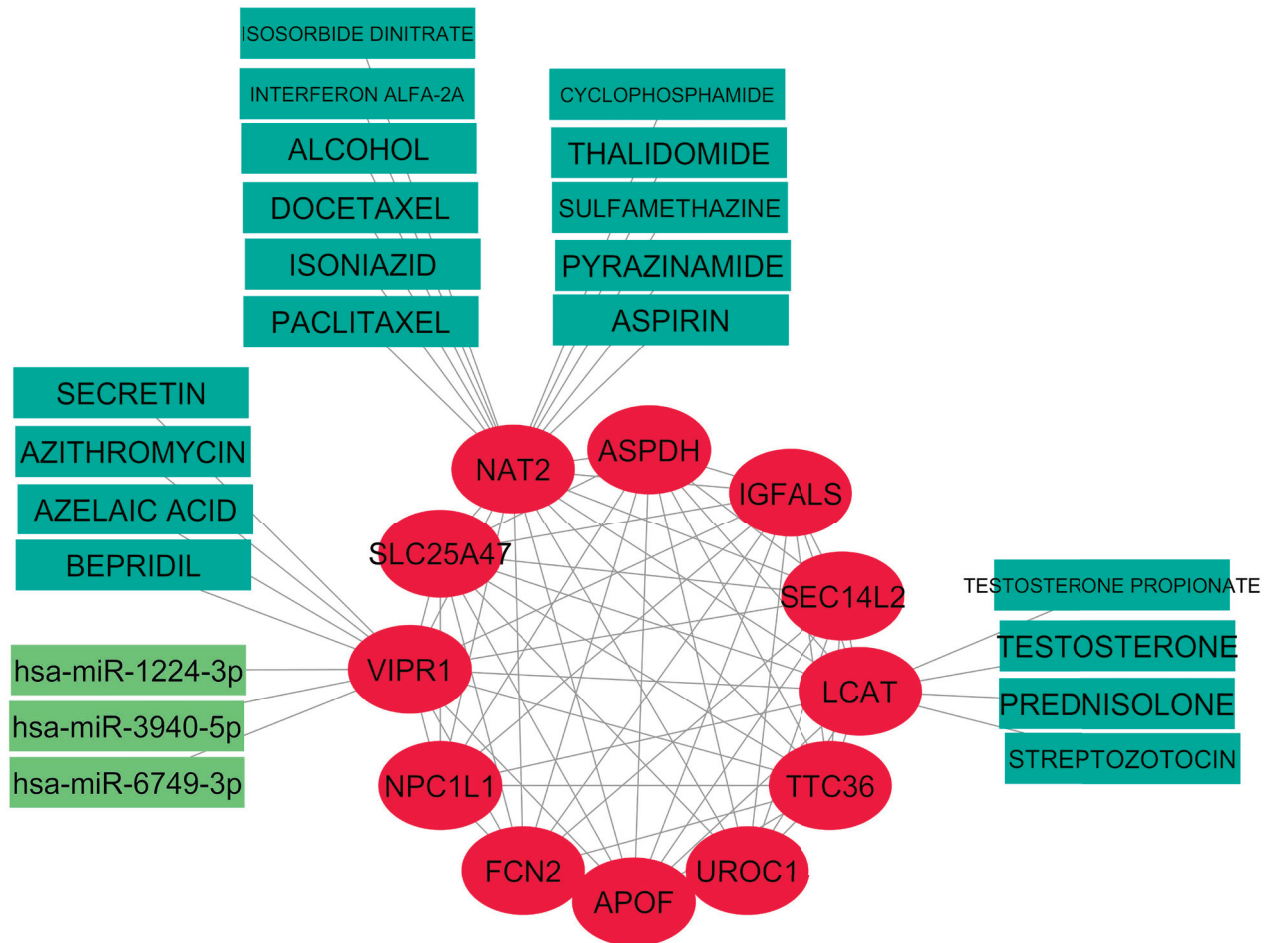


Figure 6. Co-expression network of selected hub-genes with related miRNAs and drugs. Experimentally validated miRNAs were downloaded from the miRWalk database for each gene. FDA approved drugs were acquired from DGIDB database for each gene.

end, we performed a WGCNA-based analysis to discover biomarkers related to HCC progression. The GSE39791 dataset was used for this study, which contained 72 HCC samples and 72 normal samples. An analysis of co-expression networks by WGCNA found a highly conserved blue module with 665 genes to be strongly linked to HCC. The top 13 experimentally validated hub genes of this module were *APOF*, *NAT2*, *LCAT*, *TTC36*, *IGFALS*, *ASPDH*, and *VIPR1* (**Fig. 5**). The result of hub genes enrichment using g: Profiler found that peroxisome organization and metabolic pathways were the two biological processes with the highest association to KEGG. Numerous studies have shown that peroxisomes play a significant role in the development of cancer. Prostate cancer (18, 19), colorectal carcinomas (20), liver cancer, ovarian cancer,

and bladder cancers (21), all express enzymes involved in peroxisomal lipid processing. Additionally, using *in vivo* mouse models, modulating the expression of the genes involved in peroxisome degradation (18) and/or chemically inhibiting peroxisomal lipid processing (19), can diminish tumor growth *in vivo* mice models across a wide range of cancers (18, 19).

The *NAT2* gene, for example, is highly expressed in the liver and has previously been associated with an increased risk of developing HCC. Both Gelatti *et al.* (22) and Yu *et al.* (23) identified a significant correlation between *NAT2* genetic variants and HCC susceptibility among chronic HBV carriers who were smokers. Recently, Jiang *et al.* were conducted the first systematic gene- and gene-set-based association study of HCC. Their analysis highlighted *NAT2* and several

other potential genes significantly related to HCC risk. Our results also confirmed the significant role of *NAT2* in HCC pathogenesis (**supplementary data 2-3-4**), it could be a potential target for HCC treatment. In our study, drug repositioning analysis (**Fig. 6**) suggested some FDA-approved drugs like Aspirin for the treatment of HCC through regulation of the *NAT2* gene. The effect of Aspirin on cancer risk decrease has been examined in various research (24-26). In the case of HCC treatment, the findings of recent long-term cohort study indicate that daily aspirin therapy may be connected to a decreased risk of HCC progression in patients with chronic hepatitis B (27).

Based on **Figures 5, supplementary 2-4**, another significantly correlated hub gene was *LCAT*. The *LCAT* gene is an extracellular cholesterol esterifying enzyme in which cholesterol esterification is required for cholesterol transportation. Free cholesterol in the macrophage efflux due to ATP binding cassette transporter A1 (*ABCA1*) transporter and apolipoprotein A1 (*APOA1*) is esterified into an acyl chain via *LCAT* in the cholesterol esterification pathway. The cholesterol acceptors *APOA1* drive cholesterol for bile secretion from the periphery to the liver (28). The *LCAT* gene defect may cause hypercholesterolemia, leading to accumulation of cholesterol in macrophages and other immune cells, promoting inflammatory responses, including Toll-like receptor (TLR) signaling, inflammatory activation, and bone marrow and spleen monocyte and neutrophil production (29). Also, the previous microarray-based research demonstrated that down-regulation of *LCAT* was related with HCC progression (30). Accordingly, the result of our study showed that the *LCAT* gene is not only a promising biomarker for prognosis but also could be a good target for HCC therapy. Regarding this, drug-target network interaction analysis showed 3 drugs that target the *LCAT* gene, including testosterone, streptozotocin, and prednisolone (**Fig. 6**).

Prednisolone as a glucocorticosteroid has many approved applications in the treatment of numerous kinds of cancer such as leukemia, lymphoma, and multiple myeloma as well as HCC (31). The next drug is testosterone which affects the gene expression of important HDL metabolism regulators such as Apo-I, hepatic lipase (HL), scavenger receptor B1 (SR-BI), and *ABCA1* due to defects in the *LCAT* gene function and cholesterol esterification. Hence, testosterone, despite

dropping HDL cholesterol, intensifies the transport of reverse cholesterol and may have good potential in HCC treatment (32).

MiRNAs regulate about 50 percent of mRNAs in mammalian cells (33). So, any changes in miRNAs expressions can lead to disruption of the cellular functions and cause disease. Numerous studies have shown that these non-coding RNAs play a crucial role in the development and development of tumors (34). miRNAs have been identified as either tumor suppressors or oncogenes and function as regulators in various oncogenic and tumor-suppressing pathways (35). In many malignancies, these RNAs are expressed differently in malignant samples compared to normal ones or at different stages (36). In the literature, it is clear that miRNAs have a strong association with cancer, which suggests that they could be used in the diagnosis and prognosis of cancer (37). Many researchers have also screened different miRNAs in the tissue, plasma, or sera of HCC patients (38). So, we constructed a bipartite miRNA-mRNA network which showed 3 experimentally validated miRNAs that regulated our co-expression network via the *VIPRI* hub gene.

6. Conclusion

In summary, the findings of the WGCNA investigation on three datasets of HCC showed a substantially associated module containing 7 hub-genes, which can consider potential genes in HCC pathogenesis. Also, according to the drug-target network, we found 19 potential drugs with FDA approval that can potentially use for HCC treatment through the regulation of three hub genes of the co-expression network. The three experimentally validated miRNAs also regulated our co-expression network via the *VIPRI* hub gene, according to our findings.

Conflict of interest

The authors declare that there is no conflict of interest.

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Data Availability Statement

The processed data, which were required to reproduce these findings, are available in supplementary data.

Author's Contribution

Mohsen Khorashadizadeh, as the corresponding author, contributed to the study design, guide all aspects of study implementation. Hengameh Sharifi contributed to data analysis and drafting of the manuscript. Hossein Safarpour contributed to the interpretation of the data as well as critically revised the manuscript for intellectual content. Maryam Moossavi critically revised the manuscript for intellectual content. All authors reviewed and approved the final version of the manuscript.

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