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Identification of Critical Genes Associated with Intrahepatic Cholangiocarcinoma: Weighted Gene Co-expression Network Analysis

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Abstract: Intrahepatic cholangiocarcinoma (ICC) is a liver cancer originating in the bile duct and is characterized by its complex nature and poor prognosis. While immunotherapy holds promise for modulating the tumour microenvironment and impeding cancer growth, its role in ICC remains largely unexplored. This study aims to identify critical immune-related genes that may influence the progression of ICC. We employed an *in silico* approach to analyze gene expression data from human ICC samples, sourced from the Gene Expression Omnibus database. We performed differential gene expression analysis in conjunction with weighted gene co-expression network analysis (WGCNA) to investigate the relationships between differentially expressed genes (DEGs) and 22 immune cell types, which were estimated using the CIBERSORT algorithm. Hub gene identification and pathway enrichment analysis were performed to pinpoint potential regulatory genes and their associated molecular pathways. Nine hub genes, namely *AGXT*, *MBL2*, *SERPINC1*, *RHOA*, *TTR*, *SLC2A2*, *PLG*, *C8A*, and *SOX9,* were found to be positively associated with CD4 naïve T cells, with pathways linked to cancer progression and poor prognosis. This study leverages an *in silico* systems network biology approach to uncover critical genes associated with ICC. Such findings may aid in the development of multimodal and multiagent immunotherapeutic approaches for disease treatment.

Keywords: intrahepatic cholangiocarcinoma, differential gene expression analysis, tumor microenvironment, weighted gene co-expression network analysis, system biology and bioinformatics

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is a cancer that arises in the bile ducts within the liver. It is the second most common type of primary liver cancer, representing 10–20% of liver cancer cases and 8–20% of biliary tract cancers [1]. ICC is a highly aggressive malignancy with a poor prognosis due to its silent progression, lack of specific symptoms, and resistance to chemotherapy. Over the recent decades, there has been a rise in the incidences of ICC, with potential underreporting resulting from inadequate diagnosis and misclassification [2, 3].

The five-year survival rate for ICC is approximately 9%, improving to 25% when detected early but dropping to as low as 2% in cases of metastasis. Most patients are diagnosed at an advanced stage, and even after radical surgical resection, tumor recurrence, and post-operative deaths remain common [4, 5].

ICC is characterized by a fibrous desmoplastic stroma predominantly composed of cancer-associated fibroblasts. This stroma creates a highly reactive tumor microenvironment, which is enriched with immunosuppressive and pro-tumorigenic immune cells. Among these cells, CD8+ and CD4+ T cell subsets play a significant role in the tumor's immune landscape, contributing to its aggressive nature and immune evasion strategies. Given the complex nature of ICC, understanding its molecular underpinnings is crucial for the identification of novel therapeutic targets that play a pivotal role in treating this disease [6, 7].

Complex interactions within the tumor microenvironment have a critical impact on the course of cancer development, progression, and regulation. The tumor microenvironment consists of various components, including stromal cells like cancer-associated fibroblasts, and an array of immune cells from innate and adaptive immune system. These include tumor-associated macrophages, neutrophils, natural killer cells, T and B lymphocytes, and endothelial cell [8]. This highly heterogeneous milieu plays a significant role in shaping the molecular and cellular characteristics of the tumor immune microenvironment, ultimately affecting how the disease progresses by shifting the balance between immune suppression and cytotoxicity near the tumor site [9].

The term "immunosurveillance" refers to the ability of the host immune system to detect and eliminate tumor cells as "non-self" [10]. While effective immune responses have the potential to either eradicate malignant cells or disrupt their phenotypes and functions, cancer cells often develop mechanisms to evade immune surveillance. In contrast to conventional treatments like chemotherapy and radiation therapy, immunotherapy harnesses the body's own immune system to identify and destroy cancer cells [11, 12].

Various types of immunotherapies, including immune checkpoint inhibitors, adoptive cell transfer, and cancer vaccines, offer diverse strategies to enhance the immune response against malignancies [13]. Clinical trials and studies provide evidence of the effectiveness of immunotherapy across different cancer types,

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leading to significant improvements in patient outcomes [14]. For instance, CAR-T cell therapy, a type of adoptive cell transfer, has shown notable success in treating specific blood cancers, including acute lymphoblastic leukemia and non-Hodgkin lymphoma [15].

Systems bioinformatics is an interdisciplinary field that merges systems biology with classical bioinformatics to explore dynamic molecular interactions. This approach helps in understanding the mechanisms behind various biological functions and the pathophysiology of diseases [16]. Various computational methods enable comprehensive analysis of large datasets, accelerating the identification of potential therapeutic targets. Cancer studies that focus on identifying differentially expressed genes (DEGs) play a significant role in understanding the molecular differences driving disease onset and progression. Weighted gene co-expression network analysis (WGCNA) is another cutting-edge bioinformatics method that identifies gene modules associated with clinical features [17]. Additionally, hub gene identification and pathway analysis through bioinformatics techniques has become important in cancer research. These methods allow for the identification of key regulatory genes and complex molecular pathways that drive tumorigenesis, providing valuable insights into the mechanisms driving cancer progression [18].

Although recent studies have investigated the potential of immunotherapy in altering the tumor microenvironment to slow cancer progression, its application in ICC remains largely underexplored [19]. Thus, investigating the molecular mechanisms driving the onset and progression of ICC and understanding their relationship with the immune microenvironment presents an untapped opportunity for identifying novel therapeutic targets, particularly for advancing immunotherapy approaches. In this study, we aim to fill this gap by identifying key immune-related genes associated with ICC progression through an integrative bioinformatics approach. Using gene expression data from the gene expression omnibus(GEO) database, we performed (i) differential gene expression analysis, (ii) immune cell profiling via the CIBERSORT algorithm, and (iii) WGCNA. Additionally, hub gene identification and pathway enrichment analysis were employed to uncover critical molecular pathways and regulatory genes driving ICC progression. The insights gained from this study may provide a foundation for future research aimed at developing targeted immunotherapeutic strategies.

2. Research Methodology

2.1. Data retrieval

Gene expression profiles under the series identifier GSE32225 were obtained from the GEO database [20]. This dataset consisted of gene expression profiles from formalin-fixed, paraffin-embedded ICC tissues, collected from 149 patients with ICC and 6 normal liver samples.

2.2. Screening for differentially expressed genes

The retrieved data were processed and analyzed in RStudio, where DEGs were identified using the limma package.

2.2.1. Pre-processing of data

The gene expression data from GSE32225 underwent systematic pre-processing to ensure its quality and usability for analysis. A log2 transformation was applied to stabilize variance and enhance interpretability for statistical analyses and visualization. Normalization was performed with the aim to adjust the technical variations and correct the bias in the microarray data ensuring correct downstream analysis. Quantile normalization was performed for this purpose which makes distributions identical in statistical properties.

2.2.2. Identifying differentially expressed genes

Differential expression analysis was conducted using the limma package. A contrast was created between normal and ICC samples to calculate empirical Bayes' differential expression statistics. Genes were classified as differentially expressed if they satisfied an adjusted p-value < 0.05 and a log fold-change (logFC) threshold of ± 1.5 [21].

2.3. Identification of immune cell types using CIBERSORT

CIBERSORT is a tool designed to estimate the proportions of different cell types within a complex gene expression data. In the present study, the mixture file contained the expression data of 155 samples with 22,037 genes from series identifier GSE32225 while the LM22 signature file available in CIBERSORT containing 22 distinct immune cell types was used as the reference file [22].

2.4. Identifying key modules using weighted gene co-expression network analysis

The WGCNA R package was utilized to identify clusters of highly correlated genes (modules) based on their expression profiles. For this analysis, genes with a coefficient of variation (CV) > 0.1 were selected to ensure sufficient variability. Immune cell infiltration data, derived from CIBERSORT, was incorporated as the clinical trait file to establish biologically relevant associations [17, 22]. An optimal soft-thresholding power was determined through systematic testing of multiple values. This ensured the adjacency matrix effectively captured network connectivity, with values normalized to a continuous range between 0 and 1. The resulting network was structured to approximate a power-law distribution, reflecting the hierarchical organization typical of real-world biological systems.

2.5. Building gene interaction networks using STRING database

DEGs identified using the Limma package, along with genes from the turquoise module associated with CD4 naïve T cells (highlighted as the key module in WGCNA), were analyzed using the STRING database to construct protein-protein interaction (PPI) networks [23]. The resulting PPI network consisted of nodes representing genes and edges signifying functional or physical interactions between them. This approach facilitated the identification of critical gene interactions and regulatory hubs within the biological pathways of interest.

2.6. Identification and validation of hub genes from complex interactome using CytoHubba and MCODE plugin of Cytoscape

Cytoscape software was used to visualize and analyze PPI networks obtained from the STRING database. The CytoHubba and MCODE plugins were used to identify densely connected regions or clusters in a network, which may be representative of functional modules or complexes. The validation of the hub genes obtained from CytoHubba was done using the MCODE plugin of

Cytoscape. Four MCODE sub-networks were obtained for the merged network [24].

2.7. Functional enrichment analysis of the identified genes using Enrichr

Enrichr, a web-based tool, provides several types of analyses, including gene set enrichment analysis, pathway enrichment analysis, and transcription factor binding site analysis. KEGG pathway and GO terms such as molecular function, cellular component, and biological process associated with the identified hub genes were obtained using Enrichr [25].

An overview of our methodology is provided in the graphical abstract (Figure 1), which outlines the key steps of the analysis.

3. Results

3.1. Identification of DEGs

The dataset retrieved from the series identifier GSE32225 consisted of 155 samples, including 149 ICC and 6 normal samples. Differential gene expression analysis with a cutoff of <0.05 adjusted p-value and ± 1.5 logFC resulted in the identification of 386 DEGs from a total of 22,037 genes (Figure 2). Among the identified DEGs, 287 genes were up-regulated while 99 genes were down-regulated (Supplementary Table).

3.2. Calculating immune cell types using CIBERSORT

In the mixture file, individual samples were represented as columns, with each row corresponding to a gene. The LM22 signature matrix from CIBERSORT was utilized to estimate the proportion of 22 immune cell types in each ICC sample. The output displayed the relative fractions of immune cell types across all samples, where columns represented the immune cell types from the signature file, and rows indicated the abundance of each cell type for a given sample. The results were expressed as relative proportions, normalized to a total of 1 across all immune cell subtypes. To evaluate the reliability of these estimations, *p*-values, Pearson's correlation coefficients, and root mean square error values were generated for each sample. The results were saved in CSV format and subsequently used as input for the clinical trait file in downstream analyses (Supplementary Table).

3.3. Identifying key modules associated with ICC using weighted gene co-expression network analysis

To examine the correlation between immune cells and ICC samples, we used WGCNA on a gene expression matrix of 1,983 genes retained after applying a CV filter [26]. The immune cell infiltration file obtained from CIBERSORT facilitated the assessment of how immune infiltrates correlated with the ICC samples.

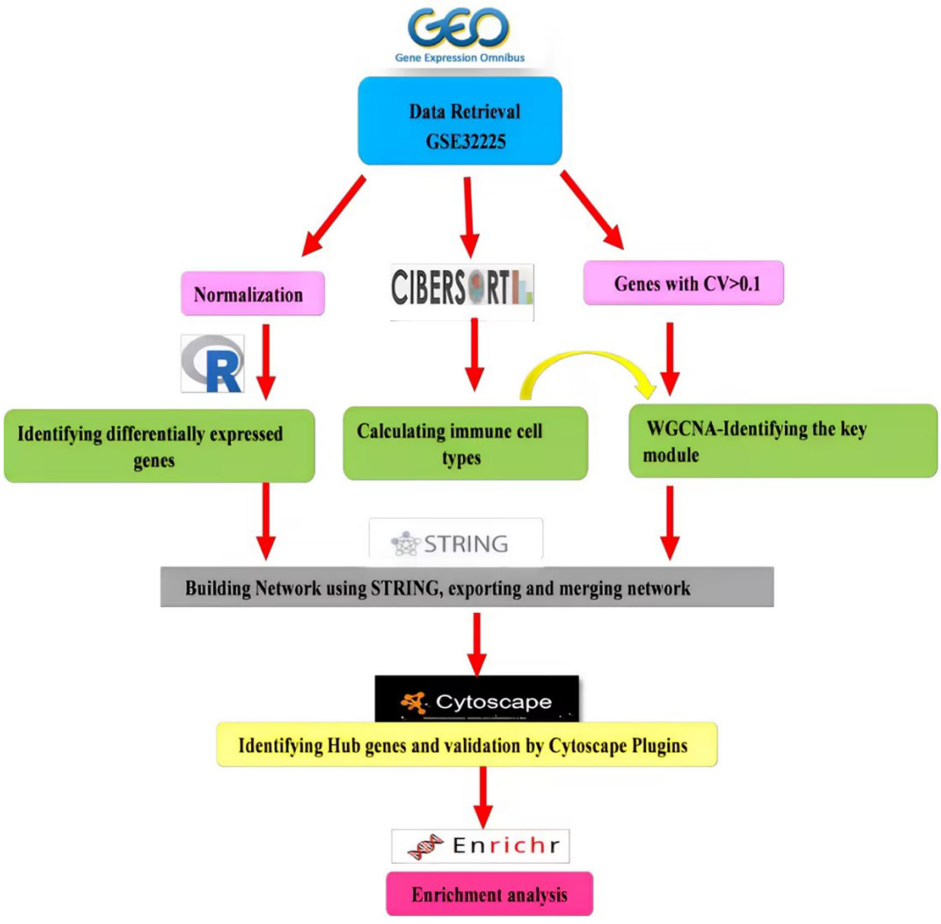

Figure 1. Graphical abstract of the research methodology

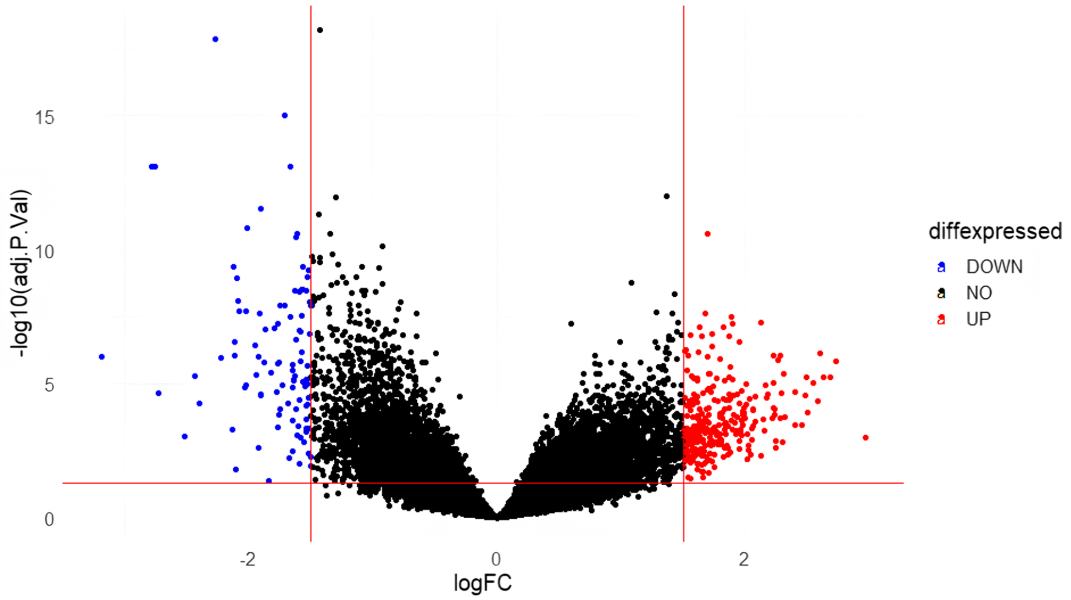

Figure 2. Volcano plot for 386 differentially expressed genes [287 up-regulated genes (shown in red) and 99 down-regulated genes (shown in blue)] obtained from GSE32225.

The soft threshold power (β) of 5 corresponded to the lowest value at which the scale-free topology fit index curve leveled off, achieving a high R^2 value (Figure 3). The adjacency matrix was converted into a topological overlap matrix, and modules were identified through hierarchical clustering of the gene dendrogram.

Figure 4 presents a clustering dendrogram, where each leaf represents one of the 1,983 filtered genes. Highly co-expressed genes are grouped into dense, interconnected branches. The dynamic tree cut method was employed to define clusters, with the algorithm partitioning the dendrogram into branches to identify modules. Each module represents a set of highly co-expressed genes, visually distinguished by different colors. Four module colors—gray, blue, turquoise, and brown—were identified. Notably, the gray module comprises genes that could not be assigned to any specific cluster.

A module-trait relationship graph (Figure 5) illustrates the correlation between gene modules and associated immune cell types. The correlation strength is visually represented by a gradient from green (low correlation) to red (high correlation). Among the modules, the turquoise module showed the highest positive correlation (0.49) with CD4 naïve T cells. This module, comprising 344 genes, was identified as the key module, with CD4 naïve T cells as the immune cell type of interest. The

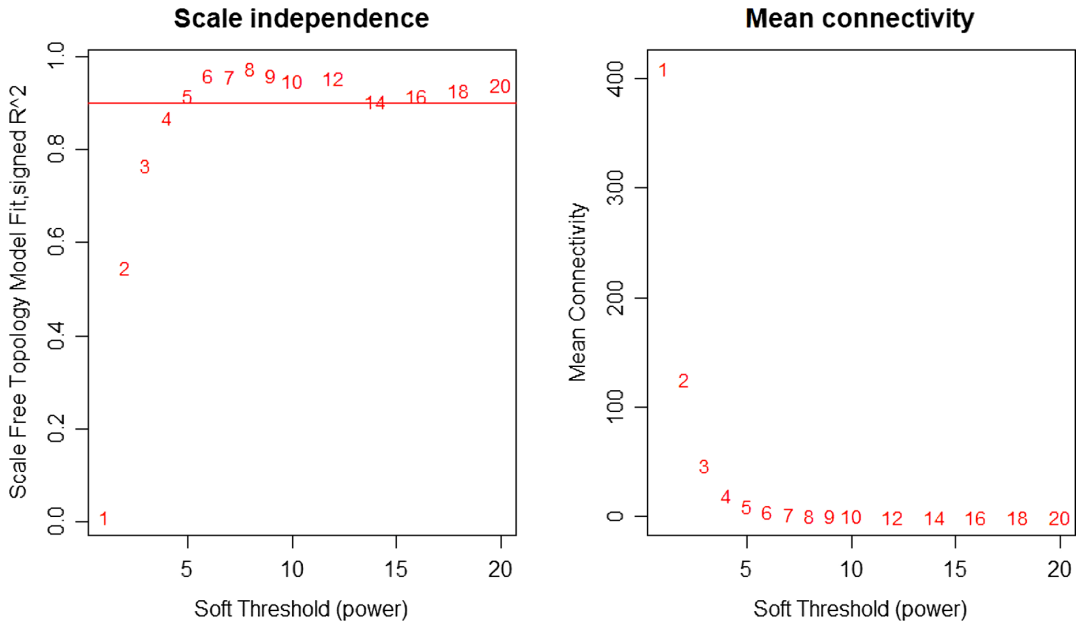

Figure 3. Scale-free fit index and mean gene connectivity analysis. A soft threshold power (β) of 5 was selected, as this is the lowest value where the scale-free topology fit index curve stabilizes. The R^2 value of 0.90 (indicated by the red horizontal line) served as the cutoff. The mean connectivity of genes decreases with increasing β.

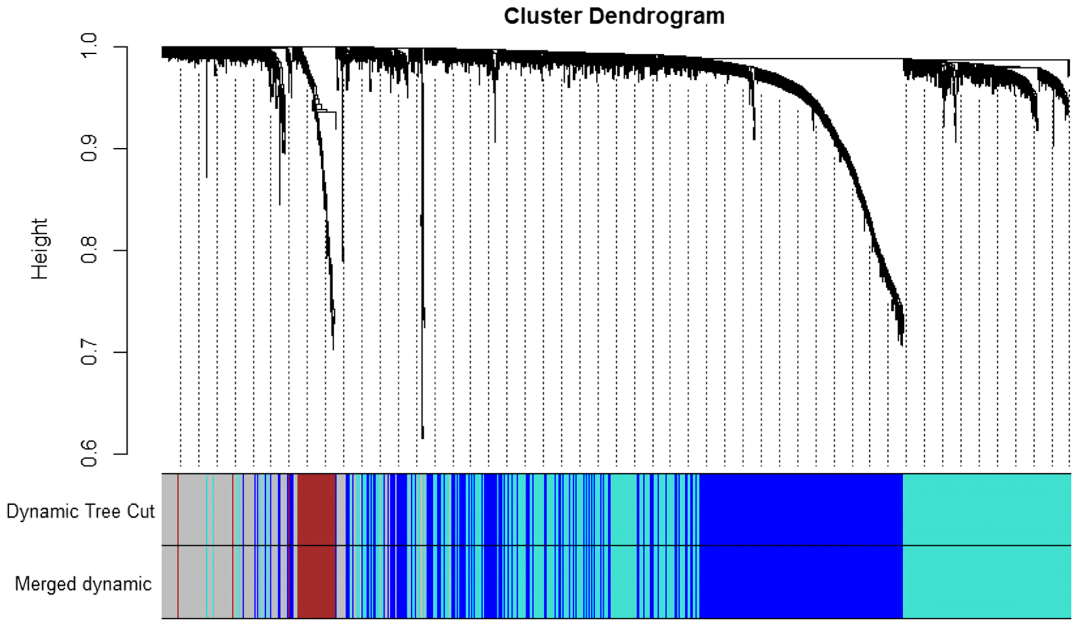

Figure 4. Cluster dendrogram displaying genes as short vertical lines. The branch group genes with similar expression profiles. Dynamic tree clustering identified four distinct clusters, color-coded as gray, brown, blue, and turquoise.

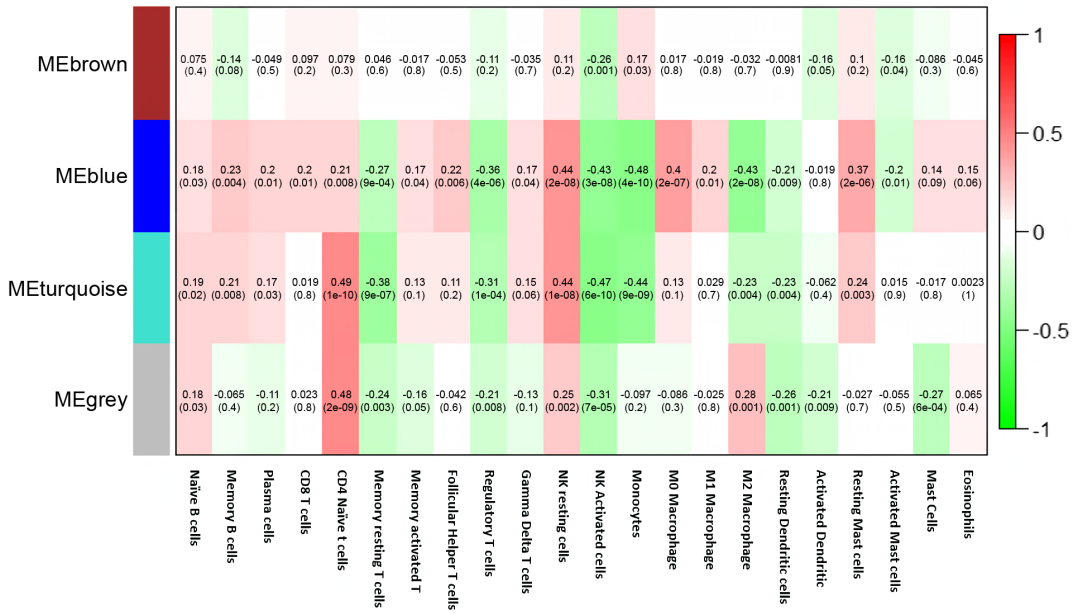

Figure 5. Module trait relationship between the identified modules and the immune cells. The heatmap is color-coded from green to red and the significance increases as we move from green to red. The highest correlation was represented by CD4 naïve T cells and genes within the turquoise module.

strong positive correlation between module membership and gene significance highlights its potential association with ICC (Supplementary Table).

3.4. Building protein-protein interaction (PPI) network using STRING

To comprehensively understand the molecular interactions of the 386 DEGs identified by the Limma package and the 344 genes from the turquoise module of CD4 naïve T cells (identified as the key module from WGCNA), STRING database was used. For the 386 DEGs, the PPI network consisted of 359 nodes representing genes and 573 edges indicating connections between genes. Notably, 27 genes lacked homologs for Homo sapiens in the STRING database, reducing the number of nodes to 359.

Similarly, the PPI network for the 344 genes identified through WGCNA was subjected to STRING analysis. This analysis resulted in a network comprising 323 nodes and 233 edges, with 21 genes unidentifiable by the STRING database for Homo sapiens, leading to 323 nodes.

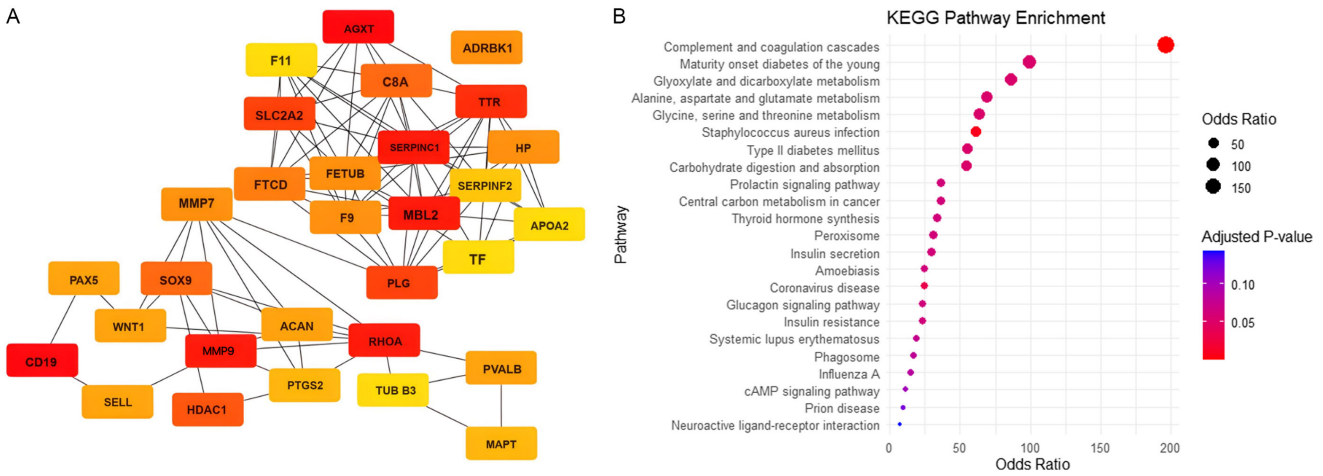

Figure 6. (A) Hub genes detected by the CytoHubba plugin. The significance decreases as the color moves from yellow to red. (B) KEGG pathway enrichment analysis for the 9 identified hub genes. Odds ratio quantifies the strength of association between your input gene list and the genes annotated to a specific pathway. Adjusted *p*-value indicates the statistical significance of the identified term.

3.5. Identification of hub genes and validation by Cytoscape plugins

Both PPI networks obtained from the STRING database were imported into Cytoscape and combined using the network merge tool. This resulted in a unified network with 736 nodes and 1,514 edges. The merged network was analyzed using the CytoHubba plugin, which calculated node scores using eleven different scoring methods. The top 30 nodes ranked by the degree method were identified, with their significance visualized using a color gradient from red to yellow. Highly significant genes were highlighted in red, followed by orange and yellow (Figure 6(A)). Among these, the top 12 ranked genes were identified as particularly significant and further validated using the MCODE plugin in Cytoscape.

To refine our analysis, we compared the genes obtained from the top four clusters of MCODE and the top-ranked genes from CytoHubba. Genes common to both sets were identified to ensure a robust selection, and from these, the top-ranked genes (up to rank 12) were finalized for further analysis (Table 1). Consequently, 9 genes namely AGXT, MBL2, SERPINC1, RHOA, TTR, SLC2A2, PLG, C8A, and SOX9 were shortlisted for enrichment analysis.

3.6. Enrichment analysis using Enrichr

To better understand the biological relevance of the nine selected hub genes, enrichment analysis using Enrichr was performed. The analysis revealed that the complement and coagulation cascade pathway was significantly enriched among the identified hub genes in the KEGG pathway analysis (Figure 6(B)). In terms of Gene Ontology analysis, the most significantly enriched cellular component was the membrane attack complex, the most enriched molecular function was fructose transmembrane transporter activity, and the most enriched biological process was cellular response to heparin. (Supplementary Table). These results suggest that the selected genes are involved in cancer progression and migration.

4. Discussion

In this study, we identified nine immune-related hub genes—AGXT, MBL2, SERPINC1, RHOA, TTR, SLC2A2, PLG, C8A, and SOX9—as potential regulators in the progression of ICC. These genes were found to be significantly associated with immune cell infiltration, particularly CD4 naïve T cells, and their interactions were linked to pathways promoting cancer progression and immune evasion. Our findings provide new insights into the potential immunomodulatory role of these genes and their involvement in tumor progression, which is largely underexplored in ICC [27–30].

C8A encodes the alpha chain of a complex known as complement component 8, which plays a crucial role in the complement system of the immune response by forming a membrane attack complex [27]. High levels of C8A have been identified as a negative indicator of immune response and have been associated with hepatocellular cancer progression [28, 29]. A recent study further emphasized the significance of C8A in the immune landscape of ICC, where immune-related pathways were broadly down-regulated in small duct-ICC, however, complement-related signatures, including C8A, were an exception, showing upregulation, indicating its potential role in the distinct immune profile of small duct-ICC. This upregulation, alongside reduced tumor-infiltrating lymphocyte signatures, highlights a complex interplay between immune evasion and complement activation in the progression of this cancer subtype [30].

RHOA (Ras Homology Family Member A) encodes a small GTPase belonging to the Rho family, functioning as a molecular switch in key signal transduction pathways that regulates cell migration and invasion. Overexpression of RHOA has been strongly associated with tumor cell metastasis, proliferation, and apoptosis suppression, particularly in cholangiocarcinoma and other cancers [31]. It is also recognized as a crucial biomarker for poor prognosis in hepatocellular carcinoma, highlighting its broader relevance in liver-related malignancies [32]. These observations align with our findings, which identify RHOA as a hub gene in ICC, underscoring its significant role in tumor progression.

Transthyretin (TTR) is primarily produced in the liver and plays an essential role in the growth, differentiation, and function of hepatic cells. Variations in TTR levels are strongly associated with severe liver diseases, making it a potential diagnostic biomarker for cholangiocarcinoma [33]. In addition to its transport functions, TTR has been recognized for its anti-inflammatory properties, particularly its ability to inhibit the production of IL-1, a key pro-inflammatory cytokine. This anti-inflammatory effect

Table 1. Cross-comparison between genes obtained CytoHubba and MCODE sub-networks

Rank	Genes_CytoHubba	MCODE_Subcluster_1	MCODE_Subcluster_2	MCODE_Subcluster_3	MCODE_Subcluster_4
1	CD19	FTCD	PLG	MAPT	ACAN
1	AGXT	F9	SERPINC1	PVALB	RHOA
3	MBL2	SLC2A2	TF	TUBB3	PTGS2
3	SERPINC1	C8A	HP		SOX9
3	RHOA	F11	TTR		
3	TTR	AGXT	APOA2		
7	MMP9	MBL2			
8	SLC2A2	FETUB			
8	PLG				
10	HDAC1				
11	C8A				
11	SOX9				
14	FTCD				
14	F9				
14	MMP7				
14	FETUB				
19	HP				
19	ADRBK1				
19	PAX5				
19	SELL				
19	WNT1				
19	PVALB				
24	ACAN				
24	MAPT				
24	PTGS2				
26	SERPINF2				
26	TUBB3				
27	F11				
27	TF				
27	APOA2				

positions TTR as a potential suppressor gene in liver cancers, highlighting its dual role in both tumor biology and immune modulation. These findings suggest that TTR could influence both the progression of ICC and the regulation of inflammation within the hepatic tumor microenvironment [34].

SLC2A2 encodes the GLUT2 transporter, essential for glucose uptake in the liver, and its overexpression is linked to cancer cell metabolism and poor prognosis in carcinomas. High levels of SLC2A2 are associated with poor patient outcomes in hepatocellular carcinoma, highlighting its potential as a prognostic marker [35]. Similarly, PLG, which is activated to plasmin, plays a key role in cancer metastasis by aiding tissue invasion and remodeling. Increased PLG activity is associated with tumor cell invasiveness, making it a potential target for cancer progression, particularly in metastasis and angiogenesis [36].

SOX9 is a transcription factor crucial for embryonic development and the formation of intrahepatic bile ducts. In adults, SOX9 is highly expressed in biliary epithelial cells, and its overexpression in ICC is associated with biliary infiltration and poor prognosis [37]. SOX9 promotes cell migration and invasion through epithelial-to-mesenchymal transition. Additionally, it has been linked to chemotherapy resistance in ICC by regulating Chk1 activation and multidrug resistance genes [38].

AGXT encodes alanine-glyoxylate and serine-pyruvate aminotransferase, an enzyme involved in the conversion of L-alanine to pyruvate and glyoxylate to glycine within liver peroxisomes. Elevated levels of serine and glycine contribute to oncogenic progression, and targeting their availability is being explored as a cancer therapy [39, 40]. AGXT has been linked to glyoxylate detoxification and the metabolism of platinum-based chemotherapies. Depletion of AGXT impairs cell self-renewal and accelerates hepatocellular carcinoma progression, leading to poor prognosis [5].

MBL2 encodes mannose-binding lectin 2, a protein secreted by the liver during the acute-phase response. It plays a crucial role in the innate immune system and immune surveillance against malignancies. MBL2 is associated with the complement pathway and immune regulation, with studies linking its expression to cancer susceptibility, progression, and prognosis. In particular, its gene expression patterns have been identified as potential diagnostic factors for ICC [41].

SERPINC1 encodes antithrombin, a serine protease inhibitor produced by hepatocytes, which plays a role in coagulation. By inhibiting cancer cell proliferation and migration, SERPINC1 can suppress tumor growth. Its overexpression has been associated with apoptosis induction and macrophage polarization in hepatocellular carcinoma. Notably, SERPINC1 has been identified as a potential biomarker for ICC, suggesting its relevance in cancer progression and prognosis [39].

The KEGG pathway identified in the present study is reported to promote oncogenesis as it can assist the escape of tumor cells from immunosurveillance promoting angiogenesis, sustain cellular proliferation, make cells insensitive to apoptosis, and participate in tumor cell invasion and migration [42]. The molecular function reflects the characteristic of cancer cells for an enhanced uptake and consumption of sugar. Due to high nutritional requirements,

the sugar transporters are deregulated in cancer cells so that they can incorporate higher level of sugar which supports their growth and proliferation [43]. The identified cellular component and biological processes identified are reported to promote cell differentiation, proliferation, and migration along with the reduction of apoptosis.

In conclusion, this study highlights the critical role of nine immune-related hub genes in the progression of ICC, emphasizing their connections to immune cell infiltration, immune evasion, and tumor-promoting pathways. These findings provide novel insights into the molecular landscape of ICC and its immune microenvironment. The identified genes play multifaceted roles in tumour biology, from influencing immune responses to driving cancer progression and metastasis. Our findings align with and extend previous research, offering a more comprehensive understanding of ICC biology underscoring the potential of these genes as targets for future translational research to combat ICC more effectively.

5. Conclusion

Our study aimed at advancing the present understanding of the complex molecular landscape of ICC and its interaction with the immune microenvironment. We employed a systems-level bioinformatics analysis which identified nine key genes, namely AGXT, MBL2, SERPINC1, RHOA, TTR, SLC2A2, PLG, C8A, and SOX9. These genes can play potential roles in ICC progression and may serve as promising candidates for further investigation as diagnostic or therapeutic targets in ICC. Furthermore, our characterization of immune cell infiltration highlights the substantial presence of naïve CD4 T cells within the ICC microenvironment. This finding suggests that naïve CD4 T cells play a pivotal role in modulating the immune response to ICC.

Understanding the intricate interactions between these immune cells and the identified genes may provide avenues for novel immunotherapeutic strategies. These findings provide a foundation for future research aimed at developing targeted therapies and improving patient outcomes in ICC. Further experimental validation of the identified genes and their interactions with immune cells will be crucial to translate these findings into clinical applications.

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Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Author Contribution Statement

Tammanna R. Sahrawat: Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Aayushi Notra:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration.

Supplementary Information

The supplementary table is available at https://doi.org/10.47852/bonviewMEDIN52024009.

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