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ORIGINAL ARTICLE

Identification of 13 Kinds Programmed Cell Death in Male Hepatocellular Carcinoma Based on WGCNA and Machine Learning Algorithm

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Abstract

Liver cancer is considered the sixth most common malignancy worldwide and is the second leading cause of cancer-related fatalities. The incidence rate in males is approximately three to four times greater than that in females. Additionally, male patients often experience higher rates of recurrence and poorer prognoses following a liver cancer diagnosis in comparison to women. Various forms of programmed cell death (PCD) have become significant disease phenotypes, with potential applications as targets for diagnosis and drug research aimed at male liver cancer patients. Transcriptome profiling related to liver cancer was sourced from the TCGA database. A set of 13 PCD-related genes (including pyroptosis, parthanatos, oxeiptosis, netotic cell death, necroptosis, lysosome-dependent cell death, ferroptosis, entotic cell death, disulfidptosis, cuproptosis, autophagy-dependent cell death, apoptosis, and alkaliptosis) was gathered from multiple public databases and literature reviews. Utilizing the Weighted Gene Co-expression Network Analysis (WGCNA) algorithm, transcriptomic datasets from the TCGA were examined to identify essential death genes within the core PCDs. Through the combination of three machine learning methodologies, we pinpointed three central PCD-related hub genes, specifically CDKN2A, CLTRN, and HGF, and conducted analyses on immune infiltration and ssGSEA. This study highlights three hub genes, identified through the integration of three machine learning algorithms with WGCNA analysis, as promising novel targets for the diagnosis and treatment of male liver cancer.

Keywords: Male hepatocellular carcinoma; WGCNA; machine learning; programmed cell death

Introduction

Background

Liver cancer is the sixth most common malignancy worldwide and the second leading cause of cancer-related mortality [1]. It primarily presents in two clinical subtypes: hepatocellular carcinoma (HCC), which makes up about 90% of cases, and intrahepatic cholangiocarcinoma (iCCA), responsible for approximately 10% [2]. In nearly all regions, men have an incidence rate that is three to four times higher than that of women [3]. Furthermore, male patients often experience higher recurrence rates and poorer outcomes after a liver cancer diagnosis compared to females [4, 5]. The exact reasons behind these sex-based disparities remain largely unclear.

Programmed cell death (PCD) represents a meticulously regulated biological mechanism crucial for preserving tissue homeostasis and disposing of damaged or superfluous cells. This process occurs in several phases, including recognition, initiation, execution, and activation of effector molecules [6]. he concept of PCD gained significant attention in 1972 when John Kerr, Andrew Wyllie, and Alastair Currie coined the

term "apoptosis" [7]. Over recent decades, numerous non-apoptotic forms of PCD have been identified, including pyroptosis, parthanatos, oxeiptosis, ferroptosis, apoptosis, alkaliptosis, entotic cell death, disulfidptosis, cuproptosis, necroptosis, netotic cell death, lysosome-dependent cell death, and autophagy-dependent cell death [6, 8]. Currently, many therapeutic strategies are focused on modulating PCD pathways to enhance drug efficacy and protect against cell damage.

Recent developments in gene chip technology have attracted significant interest, driving rapid advancements and the establishment of numerous biological data repositories [9, 10]. These innovations provide powerful tools and fresh perspectives for exploring various diseases. Concurrently, the integration of machine learning and bioinformatics in research has gained considerable traction in recent years [11-13]. Among these methods, weighted gene co-expression network analysis (WGCNA) has become a valuable approach for analyzing extensive gene expression datasets, identifying genes associated with liver cancer, and discovering potential biomarkers and therapeutic targets [14]. Ensuring the precise selection of differentially expressed genes for bioinformatics analysis is a critical global research focus. Comparative gene expression studies between case and control groups offer significant potential for enhancing disease understanding, diagnosis, and treatment strategies.

This research aims to explore how bioinformatics, machine learning, and WGCNA methodologies can enhance the study of male liver cancer. By examining gene expression profiles derived from male LICH and standard liver tissues, significant genes and pathways linked to the onset and advancement of male liver cancer were pinpointed. These candidate genes were then further refined using sophisticated machine learning methods. The results from this study are anticipated to improve male liver cancer diagnosis, treatment approaches, and patient outcomes. Given the complexity of molecular regulation in PCD, using bioinformatics tools to identify key regulatory molecules is crucial. This strategy could also aid in discovering novel therapeutic targets for male liver cancer, thereby enhancing treatment effectiveness.

Material and Methods

Data Processing and Download of the LICH Dataset

The TCGA-LICH dataset was obtained from The Cancer Genome Atlas (TCGA) database. Differential analysis of mRNA expression was conducted using the "Limma" software package [15]. To minimize the risk of false positives, adjusted P-values were evaluated within the GEO platform. The R package ggord facilitated visualization of thresholds for mRNA differential expression, while the pheatmap package was employed to generate heat maps representing expression patterns.

Enhancement of Functionality

To validate the potential roles of the identified targets, functional enrichment analysis was conducted. Gene Ontology (GO) is a commonly utilized approach for classifying gene functions into three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). Furthermore, KEGG pathway analysis offers valuable insights into gene functions and their connections to broader genomic contexts.

Co-Expression Networks are Built

The WGCNA technique facilitates the examination of gene expression patterns within gene sets [16]. Utilizing the WGCNA R package, gene networks were constructed and organized across multiple phases. Initially, clustering was executed to detect and eliminate any considerable outliers within the samples. Subsequently, automated algorithms were employed to create co-expression networks. A combination of hierarchical clustering and a dynamic tree-cutting approach was utilized to distinguish modules. To assess the correlations between modules and clinical characteristics, module membership (MM) and gene significance (GS) calculations were performed. Hub modules were defined as those exhibiting the highest Pearson correlation for MM, with a p-value of less than 0.05. A threshold of MM exceeding 0.8 and GS above 0.2 was established to select modules demonstrating strong connectivity and clinical significance. The genes within these modules were then chosen for additional analysis.

Identification of Hub Genes

The identified genes served as a basis for extracting feature genes pertinent to diagnosing male LICH. Support vector machines (SVM), which are a supervised machine learning approach for classification or regression tasks, depend on labeled training datasets. The SVM-RFE technique enhances feature subsets by eliminating less significant attributes, enabling the identification of the most predictive features [17]. In this study, LASSO regression was utilized as a fundamental methodology to optimize variable selection, employing the R package "glmnet." This approach leverages the minimum absolute shrinkage and selection operator (LASSO) technique, which is particularly effective in constructing linear models. The primary advantage of this method lies in its ability to retain only the most significant variables while simultaneously reducing model complexity. By incorporating 10-fold cross-validation, the model significantly improves both its predictive performance and its interpretability, ensuring that the selected variables contribute meaningfully to the analysis. Furthermore, the RandomForest algorithm was employed to assess and rank the importance of various genes. This algorithm identifies genes based on their relative importance value, with those exceeding a threshold of 0.25 classified as key contributors to the biological processes under study (18). The integration of data obtained through LASSO regression, SVM-RFE, and RandomForest allowed for a robust selection of critical feature genes. This cross-validation of findings not only reinforces the reliability of the selected genes but also guarantees a comprehensive evaluation of their significance.

Immune Infiltration Analysis by ssGSEA

Immune cell infiltration levels were investigated by comparing samples from male LICH tissues against those from normal male tissues. The Spearman rank correlation coefficient was calculated using the "corrplot" package in R to explore the relationships between immune cells and specific genes, providing further clarity on their interactions and potential implications in disease mechanisms.

Statistical Analysis

The statistical analyses and data visualization components of the study were conducted using GraphPad Prism version 5.0 or R version 4.1.1, ensuring thorough and accurate representation of the data. For the analysis of group differences, the Student's t-test was employed, which is a common statistical approach for comparing means between two groups. The diagnostic performance of the hub genes was particularly examined using ROC curve analysis, underscoring the effective assessment of their potential clinical relevance. Throughout all statistical analyses, a p-value threshold of less than 0.05 was established as the criterion for statistical significance, ensuring that the findings of this study would be both reliable and meaningful in advancing our understanding of the underlying biological processes involved.

Results

Identification of DEGs in Male LICH

In order to explore potential biomarkers for male liver intrahepatic cholangiocarcinoma (LICH), researchers conducted a retrospective analysis of data comprising 252 male LICH samples alongside 28 male control samples sourced from the TCGA-LIHC database. This comprehensive analysis led to the identification of a total of 129 differentially expressed genes (DEGs), all of which demonstrated significantly elevated expression levels, as illustrated in Figure 1A.

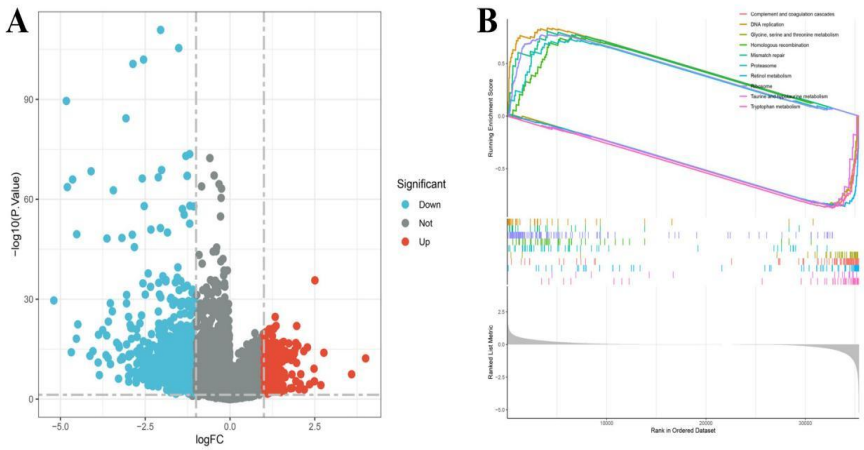

Figure 1. Analysis of DEGs in male LICH patients and healthy controls. (A) Volcano plot of DEGs. The red dots represent the up-regulated genes and the green dots represent the downregulated genes, while gray dots represent nonsignificant genes. (B) GSEA results in the male LICH group

Furthermore, a gene set enrichment analysis (GSEA) was performed to uncover the key biological pathways associated with these DEGs. The findings indicated that the most pertinent pathways were predominantly linked to several critical biological processes, including homologous recombination, glycine, serine, DNA replication, complement and coagulation cascades, and threonine metabolism, proteasome activity, and retinol metabolism, among others. These insights contribute to a deeper understanding of the molecular underpinnings of male LICH and could potentially guide future research aimed at identifying effective therapeutic targets or diagnostic biomarkers.

Weighted Gene Co-Expression Network Construction

The dataset was sourced from TCGA, comprising 28 normal male samples and 252 male LICH samples for clustering, as illustrated in Figure 2A. A soft threshold of 7 was applied to achieve high average connectivity, ensuring $R^2 > 0.9$, as shown in Figure 2B. Strongly correlated modules were merged using a clustering height limit of 0.25 (Figure 2C), leading to the identification of several modules for further analysis. These merged modules were subsequently visualized in a clustering tree (Figure 2D). A correlation analysis between the modules revealed no significant relationships (Figure 2E). Further transcriptional correlation analysis within the modules confirmed the robustness of the module classification, indicating no substantial inter-module connections (Figure 2F). To investigate the association between the modules and clinical characteristics, correlations between module eigengenes (ME) and clinical features were analyzed. Modules with significant correlations to LICH and normal samples ($|r| > 0.4$, $p < 0.05$) were selected for deeper investigation (Figure 2G). Clinically relevant modules, including grey, green, cyan, light cyan, midlight blue, dark turquoise, and dark grey, showed strong correlations with male LICH. All genes within these modules were further examined.

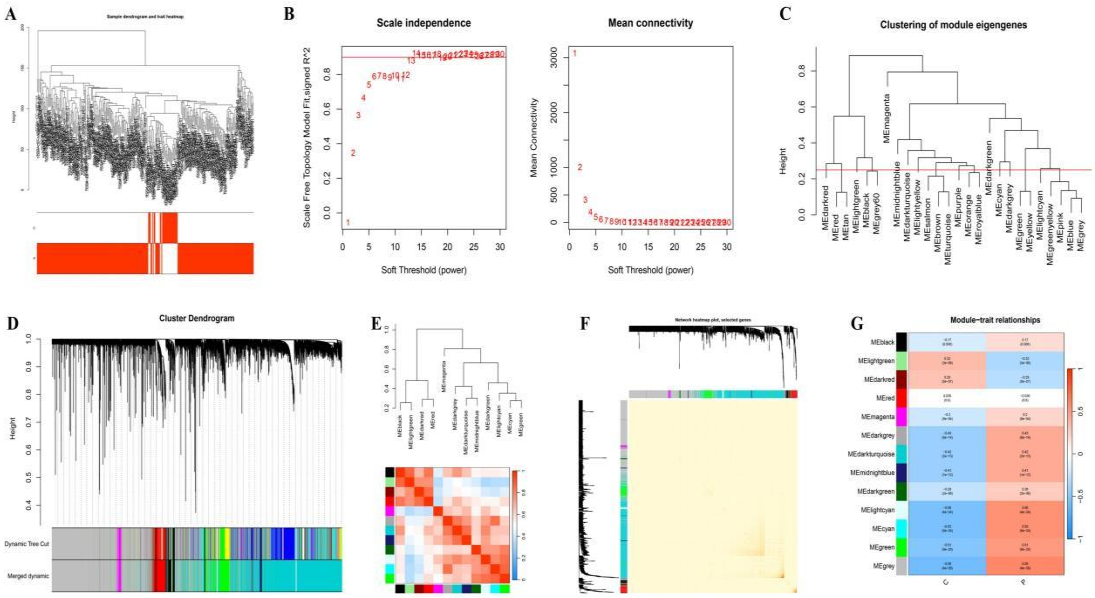

Figure 2. WGCNA co–expression network. (A) Sample clustering dendrogram of 280 samples of TCGA-LICH. (B) The scale-free fit index for various soft-thresholding powers (β) and the mean connectivity for various soft-thresholding powers. (C) Clustered dendrograms were cut at a height of 0.25 to detect and combine similar modules. (D) The original and combined modules under the clustering tree. (E) Collinear heat map of module feature genes. Red color indicates a high correlation and blue color indicates opposite results. (F) Clustering dendrogram of module feature genes. (G) Heat map of module–trait correlations. Red represents positive correlations and blue represent negative correlations.

DEGs and Functional Analysis of Critical Module Genes

By utilizing a Venn diagram to overlap the critical module genes, 13 types of PCD genes, and DEG genes, we identified a total of 21 genes (Figure 3A). Subsequently, functional analysis was conducted to explore the biological functions of these DEGs within the modules. The results of the GO enrichment analysis indicated that these DEGs were linked to various processes, including cell chemotaxis, regulation of apoptotic signaling pathways, negative regulation of cell-matrix adhesion, epithelial cell proliferation, cytoplasmic vesicle lumen, secretory granule lumen, and protein serine/threonine kinase inhibitor activity, among others (Figure 3B). KEGG pathway analysis identified connections with pathways including the p53 signaling pathway, hepatocellular carcinoma, melanoma, fatty acid biosynthesis, tyrosine metabolism, ferroptosis, fatty acid degradation, cellular senescence, and the cell cycle (Figure 3C). Additionally, the DO enrichment analysis revealed associations with mutiple cancers (Figure 3D). As depicted in Figure 3E, these DEGs are involved in the regulation of the p53 signaling pathway.

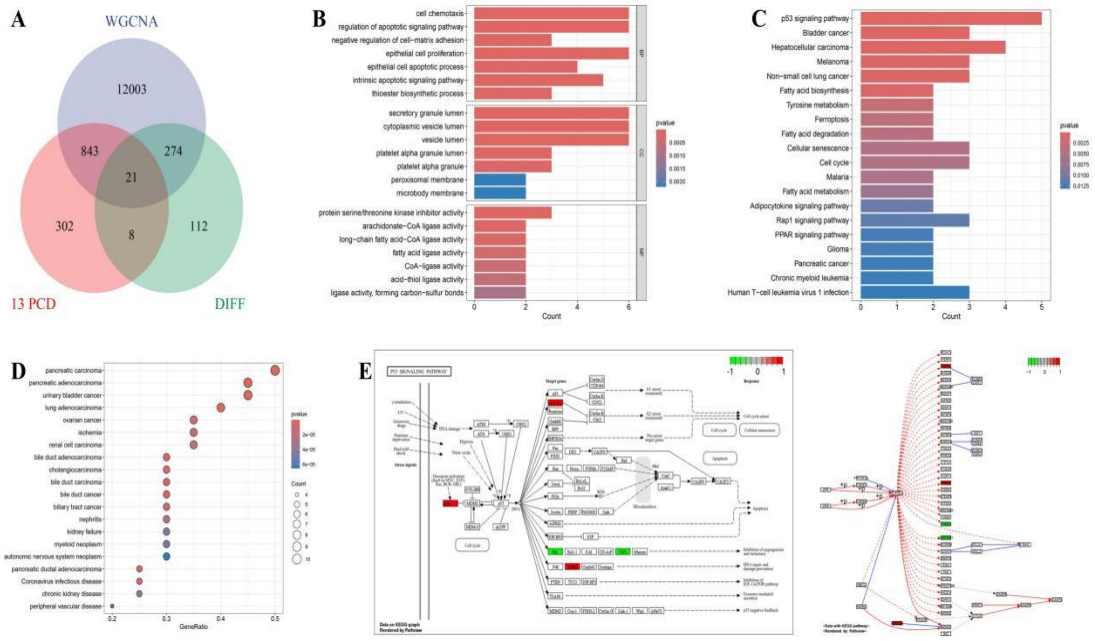

Figure 3. Functional analysis of key module genes merged with DEGs. (A) Venn diagram of key module genes versus DEGs. (B) GO analysis. (C) KEGG analysis. (D) DO analysis. (E) p53 signaling pathways. DEGs = differentially expressed genes, GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

Selection of Feature Genes

Three algorithms in machine learning were employed to pinpoint essential feature genes. The SVM-RFE approach indicated that the most accurate model included a total of 8 genes (Figures 4A, B) (Table 1). Through LASSO regression analysis, 7 predicted genes were chosen based on univariate variables that showed statistical significance (Figures 4C, D) (Table 1). Furthermore, RandomForest, in conjunction with feature selection, assessed the correlation between the error rate and the quantity of classification trees (Figures 4E, F) (Table 1), identifying 7 genes of relative importance. To ascertain the common genes, a Venn diagram was utilized, which highlighted three genes that were shared across all three methodologies (Figure 4G).

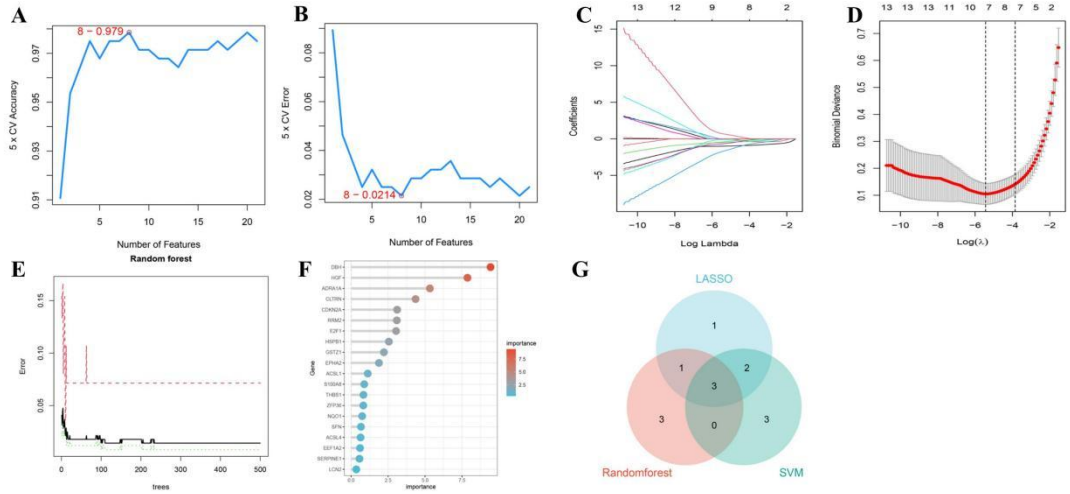

Figure 4. Screening hub genes by machine learning. (A, B) SVM-RFE algorithm. (C, D) LASSO regression algorithm. (E, F) RF algorithm. (D) Venn diagrams for three algorithms. LASSO, Least Absolute Shrinkage and Selection Operator; SVM-RFE, Support Vector Machine-Recursive Feature Elimination; RF, Random Forest.

Table 1. Hub genes screened by SVM-RFE, LASSO, and RF algorithms.

SVM-RFE algorithm	LASSO algorithm	RF algorithm
HGF	DBH	DBH
CDKN2A	CLTRN	HGF
CLTRN	HGF	ADRA1A
ACSL1	EPHA2	CLTRN
GSTZ1	GSTZ1	CDKN2A
EPHA2	CDKN2A	RRM2
PDK4	S100A8	E2F1
THBS1		

Immunological Infiltration in the LICH Group and Healthy Controls Using ssGSEA Analysis of Immune Correlation

Immune infiltration patterns in male LICH patients were further examined and compared to male healthy controls using ssGSEA. The analysis revealed that pathways such as MYC targets, WNT beta-catenin signaling, the G2M checkpoint, E2F targets, mTORC1 signaling, and mitotic spindle activity were notably upregulated in the male LICH group relative to the control group (Figure 5A). Specifically, CDKN2A was found to be linked with MYC targets, mitotic spindle, mitotic signaling, E2F targets, and the G2M checkpoint pathways, demonstrating a significant positive correlation when assessed using the "corrplot" package to explore the relationships among signature genes (Figure 5B). CLTRN was associated with adipogenesis, androgen response, bile acid metabolism, fatty acid metabolism, and showing a marked positive correlation with signature genes as well (Figure 5B). HGF, on the other hand, was connected to a range of pathways, including TGFβ signaling, reactive oxygen species, Notch signaling, myogenesis, apoptosis, mesenchymal transition, hypoxia, IL2 STAT3 signaling, IL6 JAK-STAT3 signaling, inflammatory response, angiogenesis, and allograft rejection, all of which exhibited significant positive correlations with signature genes (Figure 5B).

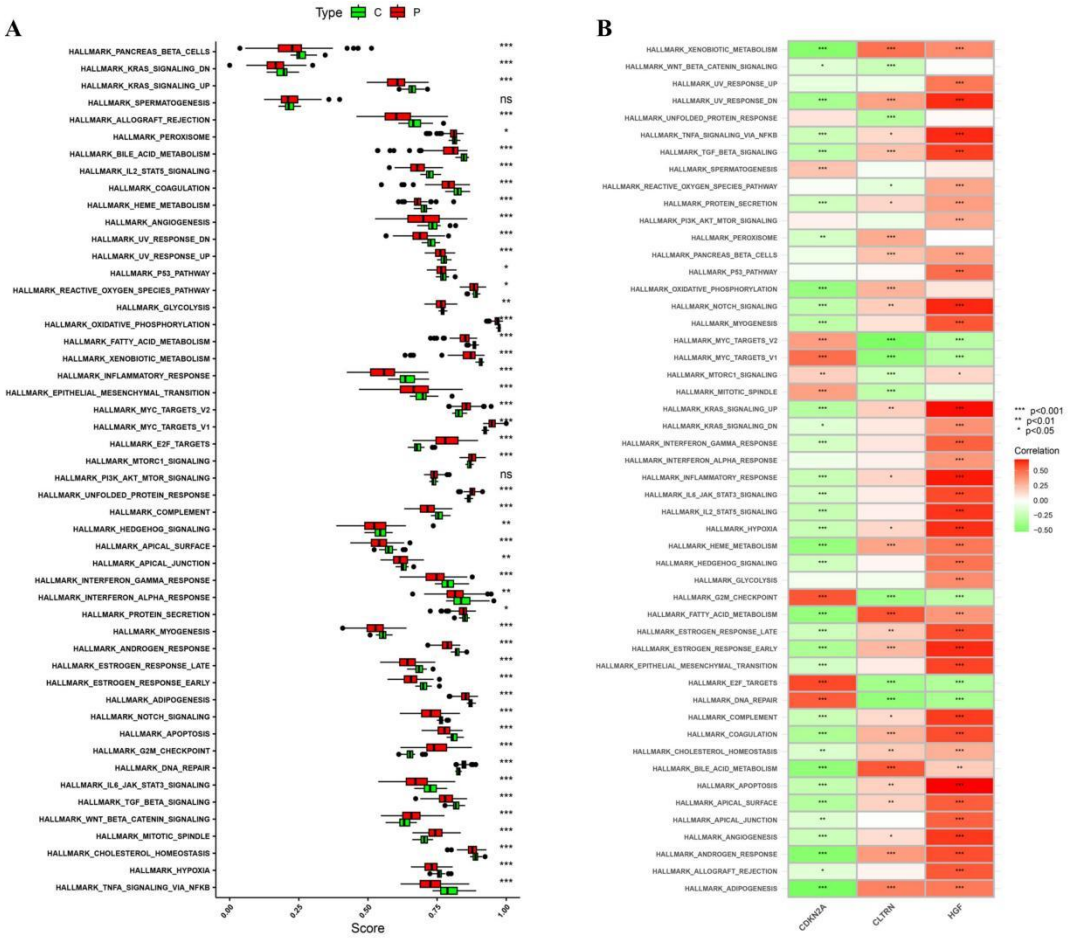

Figure 5. (A) ssGSEA analysis indicates differences in signaling pathways between the control and male LICH group. (B) ssGSEA shows the correlation between signaling pathway and diagnostic biomarkers (CDKN2A, CLTRN, and HGF, respectively).

These results suggest that CDKN2A, CLTRN, and HGF are potentially critical in regulating immune processes during the progression of male LICH.

Discussion

Hepatocellular carcinoma (HCC) stands as the most common type of primary liver cancer, characterized by elevated rates of both metastasis and recurrence [19]. Regrettably, the majority of HCC cases are identified at advanced stages, significantly diminishing the probability of successful treatment and resulting in a poor prognosis [2]). Recent studies propose that a systematic examination of particular gene sets may prove beneficial for predicting cancer outcomes [20, 21]. Nevertheless, despite these encouraging advancements, there is still an insufficiency of dependable diagnostic and therapeutic targets for male HCC based on 13 PCD genes. This gap is particularly evident in the context of the 13 PCD (programmed cell death) genes, underscoring the need for ongoing research to harness these genetic insights in the fight against this formidable cancer.

In our research, we employed Weighted Gene Co-expression Network Analysis (WGCNA) to identify gene modules that are closely associated with male LICH. WGCNA is an advanced regulatory network algorithm that constructs gene co-expression modules based on a scale-free topology, a concept established in previous studies [16, 22]. This methodology not only facilitates the identification of co-expression modules that correlate with clinical features but also aids in pinpointing potential biomarkers for diseases. The effectiveness of WGCNA has been validated through numerous studies, establishing it as a robust tool for the analysis of gene expression data. Importantly, it has demonstrated superior performance compared to other methods, whether weighted or unweighted, in the construction and analysis of gene network structures [23]. Furthermore, machine learning techniques provide an effective approach for

handling complex and voluminous genomic datasets, which often present significant challenges for traditional statistical algorithms [24, 25]. The application of these advanced methods has gained considerable traction in the identification of novel biomarkers, which are crucial for detecting diseases and forecasting treatment responses and disease outcomes [25]. As such, integrating machine learning with genomic analysis enhances our ability to uncover new insights in biomedical research and improve clinical decision-making.

In this research, we employed an extensive bioinformatics analysis to pinpoint differentially expressed genes related to programmed cell death (PCD) that are co-expressed between male LICH samples and normal tissue within the TCGA-LICH dataset. The results of GO analysis revealed that these 21 differentially expressed genes were primarily involved in processes such as cell chemotaxis, regulation of apoptotic signaling pathways, negative regulation of cell-matrix adhesion, epithelial cell proliferation, and the secretory granule lumen. KEGG pathway analysis, on the other hand, highlighted key pathways such as the p53 signaling pathway, hepatocellular carcinoma, melanoma, fatty acid biosynthesis, tyrosine metabolism, ferroptosis, fatty acid degradation, and cellular senescence. To narrow down the list, we applied several machine learning techniques and ultimately identified three significant genes: CDKN2A, CLTRN, and HGF.

The CDKN2A gene codes for the P16 protein, which plays a critical role in various cellular processes, including enhancing tumor cell proliferation, suppressing apoptosis in tumor cells, stimulating angiogenesis in the tumor stroma, and decreasing the sensitivity of cancer cells to chemotherapy [26]. Recent research indicates that alterations in the CDKN2A gene are linked to unfavorable outcomes in several types of cancer, including pancreatic ductal adenocarcinoma, bladder cancer, and pancreatic cancer. [27-30]. CLTRN (collectrin gene) has been linked to the tumor and demonstrated a clear response to radiation. CLTRN has been identified as a key gene associated with radiosensitivity in the context of radiation therapy. Research demonstrates that following irradiation in vitro, CLTRN plays a role in inhibiting the proliferation, migration, and invasion of HCC cells, pointing to its significance in the biological behavior of liver cancer cells [31]. Hepatocyte growth factor (HGF), which is primarily produced and secreted by specialized non-parenchymal cells commonly referred to as hepatocyte stellate cells, also plays a significant role in liver biology. Initially identified as a mitogen specifically for hepatocytes, HGF has been recognized for its broader function as a cytokine that exhibits a wide array of pleiotropic effects. This expanded understanding signifies that HGF is not merely a growth factor for liver cells but also participates in complex signaling pathways that can influence various cellular activities and responses, particularly in the context of liver health and disease [32].

Several limitations of the current study should be recognized. First, the study is based on a relatively small sample size, and data is exclusively sourced from the TCGA dataset. Second, the ssGSEA algorithm, which estimates immune cell infiltration based on gene expression, necessitates additional experimental validation.

Conclusion

We successfully screened three programmed cell death related hub genes (CDKN2A, CLTRN, and HGF) based on multi-omics, not only helping in male hepatocellular carcinoma diagnosis but also facilitating a deeper understanding of the disease's treatment modalities and etiology.

Acknowledgments

All authors have no acknowledgements to disclose.

Competing Interests

The authors declare that they have no conflict of interest.

Ethics Statement

Not applicable

Author Contributions

WH constructed this study. WH and XR performed figures plotted and writing. WH and XR were responsible for the critical reading of the manuscript. All authors contributed to the article and approved the submitted version.

Consent for Publication

All authors agreed to publish the article.

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