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Original Research

Multi-Dimensional Data Integration Reveals the Molecular Mechanism of Metabolic Reprogramming Associated With Low Asparaginase Expression in Hepatocellular Carcinoma

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Abstract

Background: To investigate the impact of low expression of asparaginase (ASPG) in hepatocellular carcinoma (HCC) on tumor metabolic reprogramming, tumor microenvironment interactions, and drug sensitivity, and to assess the potential of ASPG as a tumor suppressor gene through multi-dimensional functional mechanisms. **Methods**: Thus, ASPG expression in HCC was systemically evaluated by integrating global multi-center mRNA datasets, including RNA-seq and microarray data from 3967 HCCs and 2645 non-HCC samples, single-cell RNA sequencing (scRNA-seq) data from 10 HCCs and 8 adjacent normal tissues, spatial transcriptomics (STs), and internal immunohistochemistry data from 301 HCCs and matched adjacent liver tissues. A CRISPR-mediated gene knockout model was employed to examine the effects of ASPG deletion on HCC cell proliferation. ASPG-associated regulatory pathways were analyzed using Gene Set Enrichment Analysis (GSEA), GeneMANIA, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Changes in ligand–receptor interactions between low-expression ASPG (ASPG-low) and the tumor microenvironment were examined using CellChat, based on scRNA-seq data. Single-cell metabolism (scmetabolism) and single-cell flux estimation analysis (scFEA) were applied to infer metabolic activity and metabolite conversion pathways under ASPG-low conditions. Finally, correlations between ASPG expression and the IC50 values of anticancer drugs were assessed using OncoPredict. **Results**: Multi-dimensional analyses consistently showed that both ASPG mRNA and protein levels were significantly downregulated in HCC. CRISPR-mediated ASPG knockout was associated with significantly increased cell proliferation. Mechanistically, HCC cells with low ASPG expression were enriched in pathways related to alanine, aspartate, and glutamate metabolism, as well as amino acid biosynthesis, gluconeogenesis, and lipid metabolism. Cell–cell communication analysis revealed strong interactions between ASPG-low malignant hepatocytes and myeloid cells, with significant activation of the MIF–(CD74+CXCR4) and MIF–(CD74+CD44) signaling axes. Metabolic analysis demonstrated that the ASPG-low state was associated with alanine, aspartate, and glutamate metabolism, as well as the citric acid (TCA) cycle, thereby regulating the conversion of the aspartate–asparagine and glutamate–2OG metabolites. ASPG-low HCC was associated with resistance to drugs such as cisplatin, oxaliplatin, and gemcitabine, but increased sensitivity to lapatinib and paclitaxel. **Conclusion**: Low ASPG expression in HCC may drive aspartate metabolism and reprogramming of the TCA cycle, thereby influencing sensitivity to drug treatment.

Keywords: hepatocellular carcinoma; asparaginase; single-cell analysis; spatial transcriptomics; immunohistochemistry; metabolic reprogramming

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer type globally and one of the leading causes of cancer-related mortality [1]. HCC arises from hepatocytes and accounts for 75%–85% of primary liver cancer cases [2]. Known risk factors for HCC include alcohol consumption, metabolic diseases, and infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) [3]. Treatment options for early–stage HCC are surgical resection, liver transplant, and percutaneous image–guided ablation [4], while chemotherapy and immunotherapy are the best treatment strategies for advanced and unresectable disease [5]. More than 70% of HCC patients are diagnosed at intermediate or advanced stages, and only about 30% are deemed eligible for curative treatment. Due to the frequent recurrence and metastasis of HCC, the five-year survival rate is only 18% [6–8]. To improve the prognosis and treatment of HCC patients, novel biomarkers with high speci-

ficity and sensitivity are required, together with an understanding of their molecular regulatory mechanisms.

Metabolic reprogramming is one of the hallmarks of tumor cells, supporting their rapid proliferation and survival advantage through alterations in energy metabolism, enhanced biosynthesis, and maintenance of redox balance [9]. Aspartic acid, a key non-essential amino acid, plays a critical role in cell proliferation. It serves not only as a precursor for pyrimidine and purine nucleotide synthesis, but also helps in maintaining mitochondrial metabolism and redox balance via the malate-aspartate shuttle [10]. Its amide derivative, asparagine, also plays a pivotal role in tumor metabolism. The enzyme asparaginase (ASPG) catalyzes the hydrolysis of asparagine and demonstrates antitumor activity, indicating a significant role in regulating asparagine levels and related metabolic pathways [11]. Recent studies have shed light on the potential role of ASPG in different cancer types. ASPG was incorporated into a 13-inflammation-associated gene prognostic model for colorectal cancer (CRC), where its expression was found to correlate with expression of the PD-1 and PD-L1 immune checkpoint molecules, indicating that high-risk patients may be more sensitive to immunotherapy [12]. In colon adenocarcinoma, ASPG was identified as a key gene in a prognostic model based on 10 amino acid metabolism-related genes, which was further validated to predict patient responses to PD-1/CTLA-4 immune therapy [13]. These findings suggest that ASPG not only participates in the regulation of amino acid metabolism, but may also influence the tumor immune microenvironment and treatment response. Although ASPG was identified by machine learning methods as a potential diagnostic biomarker in HCC [14], the role of ASPG in HCC has not been specifically addressed, with most studies mentioning ASPG only in the context of a candidate diagnostic biomarker.

Consequently, this study systematically investigated the metabolism-related functional mechanisms of ASPG in HCC through multi-level expression characteristics. The ASPG expression pattern in HCC was analyzed using data obtained by different experimental approaches, including bulk RNA data (generated from global multi-center microarrays and RNA-seq), single-cell RNA sequencing (scRNA-seq), spatial transcriptomics (ST), and experimentally validated protein expression determined by immunohistochemistry (IHC). The results show that ASPG expression can alter the composition of various cell populations within the tumor microenvironment (TME), as well as the strength of ligand-receptor interactions. We also examined the regulatory role of ASPG in various signaling pathways related to metabolism, and the association between ASPG expression and sensitivity to anti-tumor drugs. The overall aim of this study was to clarify the biological significance of ASPG in HCC and to elucidate its role in metabolic reprogramming, thus providing a theoretical basis for the development of ASPG as a novel molecular biomarker and therapeutic target. A summary of the main findings is shown in Fig. 1.

2. Methods

2.1 Collection, Processing, and Analysis of Bulk RNA, scRNA-seq, ST, and IHC Data

2.1.1 Collection, Screening, and Integration of Global High-Throughput HCC-Related Datasets

The expression of ASPG mRNA in HCC tissues was assessed by collecting bulk RNA data from several high-throughput datasets, including The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/), and Genotype-Tissue Expression (GTEx, https://gtexportal.org/) databases. The inclusion and exclusion criteria were as follows: (1) datasets containing both HCC and control groups (normal liver tissue or adjacent non-cancerous tissue), with exclusion of metastatic cancer samples; (2) datasets with a sample size of $\geq$3, excluding duplicate samples; (3) human samples only, excluding animal samples. Subsequently, data from the same platform were merged, and outlier data points were removed. Batch and inter-group corrections were performed using the ComBat functionality of sva and limma-voom [15]. The specific process for filtering ASPG mRNA data is outlined in **Supplementary Fig. 1**.

2.1.2 Collection of scRNA-seq Data for HCC

The expression, distribution, and functional profile of ASPG within the HCC microenvironment were characterized using scRNA-seq data from GSE149614, consisting of 10 HCC samples and 8 adjacent samples. Cells were filtered based on the criteria of 200 $<$ nFeature_RNA $<$ 8000, and mt percentage $<$10%. Using Uniform Manifold Approximation and Projection (UMAP), cells were clustered and reduced at a resolution of 0.4. Based on information provided by GEO, cells were assigned a cell-type annotation as endothelial cells, fibroblasts, B cells, T/NK cells, myeloid cells, or hepatocytes. The infercnv method was used to identify malignant hepatocytes by considering endothelial cells and fibroblasts as reference cells [16,17]. The final analysis examined the distribution of ASPG expression across the various cell types.

2.1.3 Scoring of IHC Staining in HCC Tissues

A total of 301 HCC tissue samples and corresponding adjacent non-tumor liver tissues were collected from patients at the First Affiliated Hospital of Guangxi Medical University. The samples were fixed in formalin, embedded in paraffin, and deparaffinized with ethylenediaminetetraacetic acid (EDTA) buffer. Endogenous peroxidase activity was then blocked, and the tissues subsequently incubated at room temperature for 90 minutes with

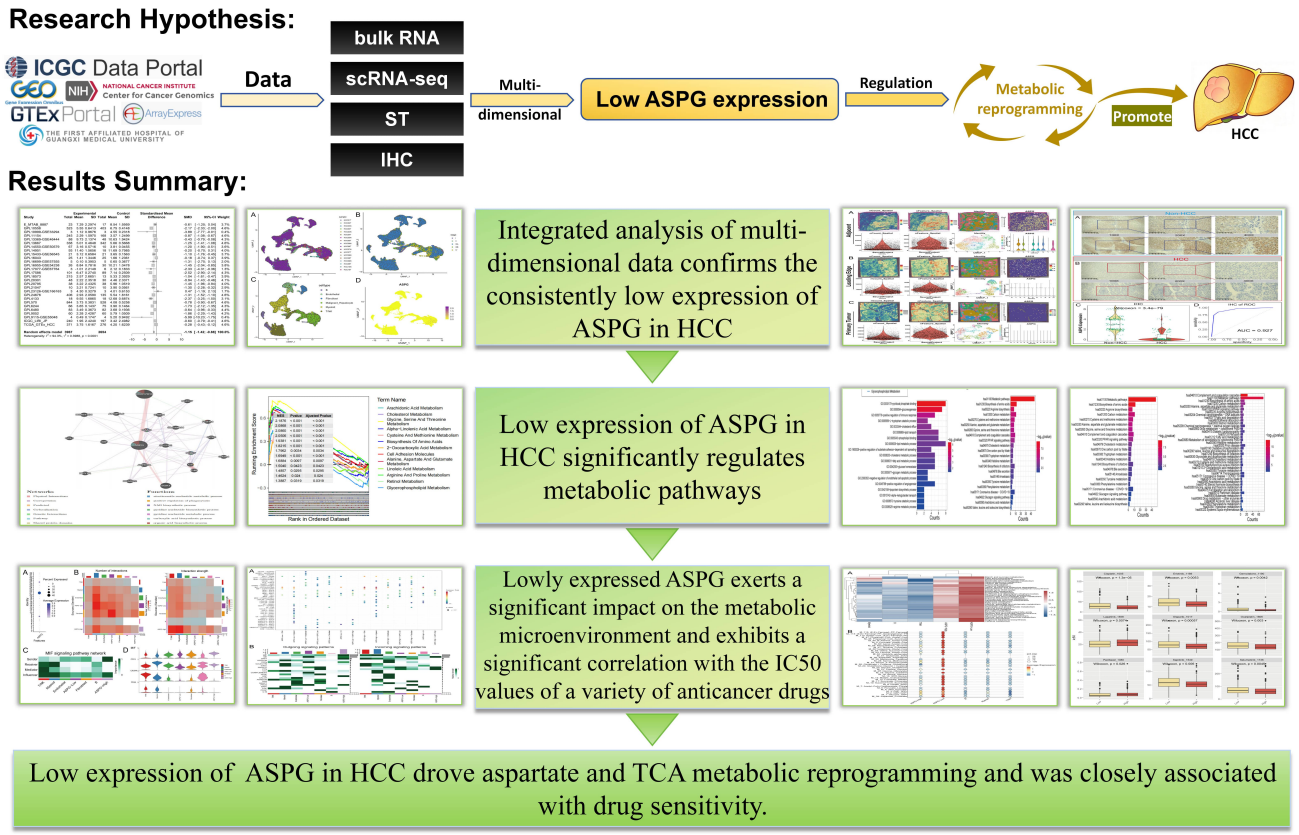

Fig. 1. Overview of key findings in the present study (Microsoft Corporation, Redmond, WA, USA). ST, spatial transcriptomics; IHC, immunohistochemistry; ASPG, asparaginase; HCC, hepatocellular carcinoma; TCA, the citric acid cycle.

an anti-ASPG polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. PA5-64429; dilution 1:500). This was followed by incubation with HRP-labeled Poly-HRP anti-Mouse/Rabbit IgG antibody mixture (Shanghai Changdao Antibody Diagnostic Reagent Co., Ltd., Shanghai, China; Cat. No. D-3004-15) for 30 minutes and DAB staining at room temperature. During the process.phosphate-buffered saline was used to wash the slides in order to eliminate any unbound primary and secondaryantibodies. After the staining procedure, the tissue microarrays underwent dehydration and were then covered with neutral resin before storage at room temperature. IHC scoring was independently assessed by two senior pathologists based on staining intensity, using the following scoring criteria: 0 (no staining), 1 (light yellow), 2 (light brown), and 3 (dark brown). The percentage of positive cells was scored as follows: 0 ($\leq$5% positive cells), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%), and 4 ($\geq$76%). The final IHC score was determined as the product of the intensity score and the percentage of positive cells (range: 0–12). Approval for this study was granted by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University (Approval Number: 2025-E0967). The research was carried out in full compliance with the principles outlined in the Declaration of Helsinki.

2.1.4 Acquisition and Processing of ST Data

ST data concerning adjacent, leading edge, and primary tumor tissues (HCC2N, HCC2L, and HCC2T) were sourced from a patient sample through the following website: http://lifeome.net/supp/livercancer-st/data.htm [18]. Data normalization, log transformation, centering, and scaling were performed using the SCTransform method and the Seurat package in R, with the parameter dim set to 1:15. Dimensionality reduction and clustering were carried out using the Seurat functions RunPCA, FindNeighbors, FindClusters, and RunUMAP. Spatial data was visualized using the SpatialDimPlot function.

2.1.5 ASPG mRNA Expression in Cell Lines and the Impact of Knockout on Proliferation

The expression level of ASPG mRNA in different HCC cell lines was evaluated based on data from the Cancer Cell Line Encyclopedia (CCLE). Additionally, knockout data for ASPG based on CRISPR were downloaded from the DepMap portal. Chronos scoring was utilized to evaluate the effects of gene knockout on cell proliferation. The higher the Chronos score, the stronger the proliferative effect of ASPG knockout.

2.2 Investigation of the Potential Molecular Mechanisms of ASPG in HCC

2.2.1 Preliminary Mechanistic Study of ASPG in HCC

Patients from the TCGA-LIHC dataset were divided into ASPG-high and ASPG-low expression groups based on the median value. Genes with differential expression between these two groups were then identified, and GSEA enrichment analysis was subsequently performed. A preliminary investigation was conducted on the molecular network regulatory mechanisms of ASPG by integrating GeneMANIA, shedding light on the potential role of ASPG as a modulator in HCC.

2.2.2 Investigating the Potential Regulatory Mechanisms of ASPG in HCC Based on Bulk RNA and scRNA-seq

In the bulk RNA dataset, Pearson correlation analysis was conducted to identify genes that showed a positive correlation with ASPG expression. Genes were selected based on the following criteria: correlation coefficient R $\geq$0.40, $p < 0.05$, and positive correlation in at least seven datasets. Standardized mean difference (SMD) of gene expression in HCC tissues were computed to identify low-expressed differential genes. Genes with SMD $<$0 and $p < 0.05$ were included in the analysis. Overlapping genes between the positively correlated genes and the low-expression differential genes were then subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses to investigate the regulatory mechanisms of ASPG within bulk RNA datasets.

To further analyze the potential mechanisms of ASPG in HCC at the scRNA-seq level, we screened the correlated genes related to ASPG in malignant hepatocytes ($R \geq 0.09$, $p < 0.05$) and subsequently performed GO and KEGG enrichment analyses [19].

2.2.3 Analysis of Cell Interactions Between ASPG-Low Expression Malignant Hepatocytes and Other Cell Types

In order to explore the role of ASPG in cell-to-cell communication within the HCC microenvironment, we re-clustered malignant hepatocytes according to their ASPG expression level (ASPG-high and ASPG-low). Using the "CellChat" package, we analyzed the interactions between ASPG-low malignant hepatocytes and other cell types, focusing on changes in ligand-receptor interactions between cells. This analysis provided insights into how low expression of ASPG influences intercellular communication and interactions in the HCC microenvironment [20].

2.3 Impact of Low ASPG Expression on Metabolism in the Tumor Microenvironment

In order to investigate the cellular metabolic flux and metabolite abundance, the scMetabolism tool was used to score individual cells from scRNA-seq data. This provides a means to further investigate the metabolic activity of different cell types [21]. Flux data analysis was performed using Single-Cell Flux Estimation Analysis (scFEA v1.1.2). This new probabilistic model incorporates a balance of fluxes and a graph neural network-based optimization solver. The method uses neural networks to extract complex information flow from the transcriptome to the metabolome. It also uses scRNA-seq data to infer cellular metabolic flux profiles and metabolite quantities [22–25].

2.4 Investigating the Role of Low ASPG Expression in HCC Drug Dosage

In order to evaluate the therapeutic potential of ASPG in HCC, we analyzed transcriptomic data from the TCGA LIHC and utilized the oncoPredict algorithm to systematically assess the correlation between ASPG expression level and the IC50 value of various anticancer drugs [26].

2.5 Statistical Analysis

Except where otherwise specified, all statistical analyses and computational algorithms were performed using R software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria). Differences in ASPG expression at both the mRNA and protein levels between HCC and non-tumor tissues were assessed using the Wilcoxon rank-sum test. To calculate the expression of bulk RNA ASPG, a fixed-effects model (I^2 $<$50%) was chosen due to the detected heterogeneity. A random-effects model was used to compute the SMD when deemed necessary. The pROC package was employed to construct receiver operating characteristic (ROC) curves. STATA 16.0 (StataCorp LLC, College Station, TX, USA) was utilized to develop the summary ROC (sROC) curve for assessing the level of ASPG expression through the area under the curve (AUC). A larger AUC value reflected more prominent expression differences [25]. In addition to the *Begg's* and *Egger's* tests ($p > 0.05$ indicating no publication bias), statistical significance was considered at $p < 0.05$.

3. Results

3.1 Identification of Consistent Low ASPG Expression in HCC Through Integrated Multi-Dimensional Data Analysis

3.1.1 Overall Low Expression of ASPG mRNA in HCC Tissues

In this study, 85 datasets were collected and merged into 28 platform datasets. The analysis of ASPG mRNA expression across these platform datasets found that 19 datasets showed significantly lower ASPG mRNA expression in HCC samples compared to non-HCC samples (AUC $>$0.7, $p < 0.05$, **Supplementary Figs. 2,3**). A comprehensive analysis integrating 28 independent platforms revealed that, across a total of 3967 HCC samples and 2645 non-cancerous control samples, ASPG exhibited consistently low mRNA expression levels in HCC (SMD = –1.15, 95% CI: –1.42 $\sim$ –0.88, Fig. 2A). The sROC curve indicated that low expression of ASPG mRNA in HCC samples had high

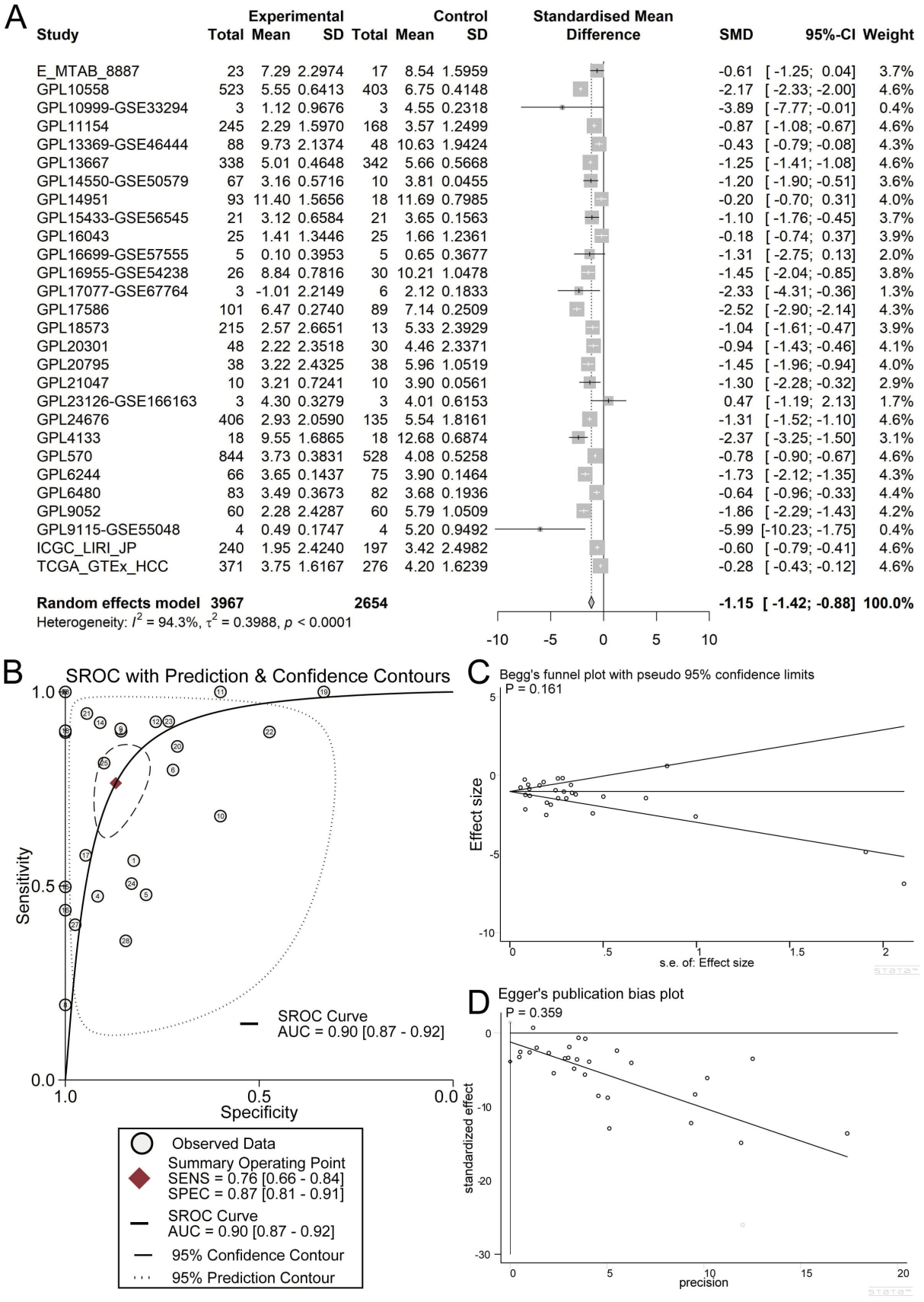

Fig. 2. Overall expression levels of ASPG mRNA in global HCC samples. (A) Forest plot of ASPG mRNA expression. (B) sROC curve. (C) *Begg's* test. (D) *Egger's* test.

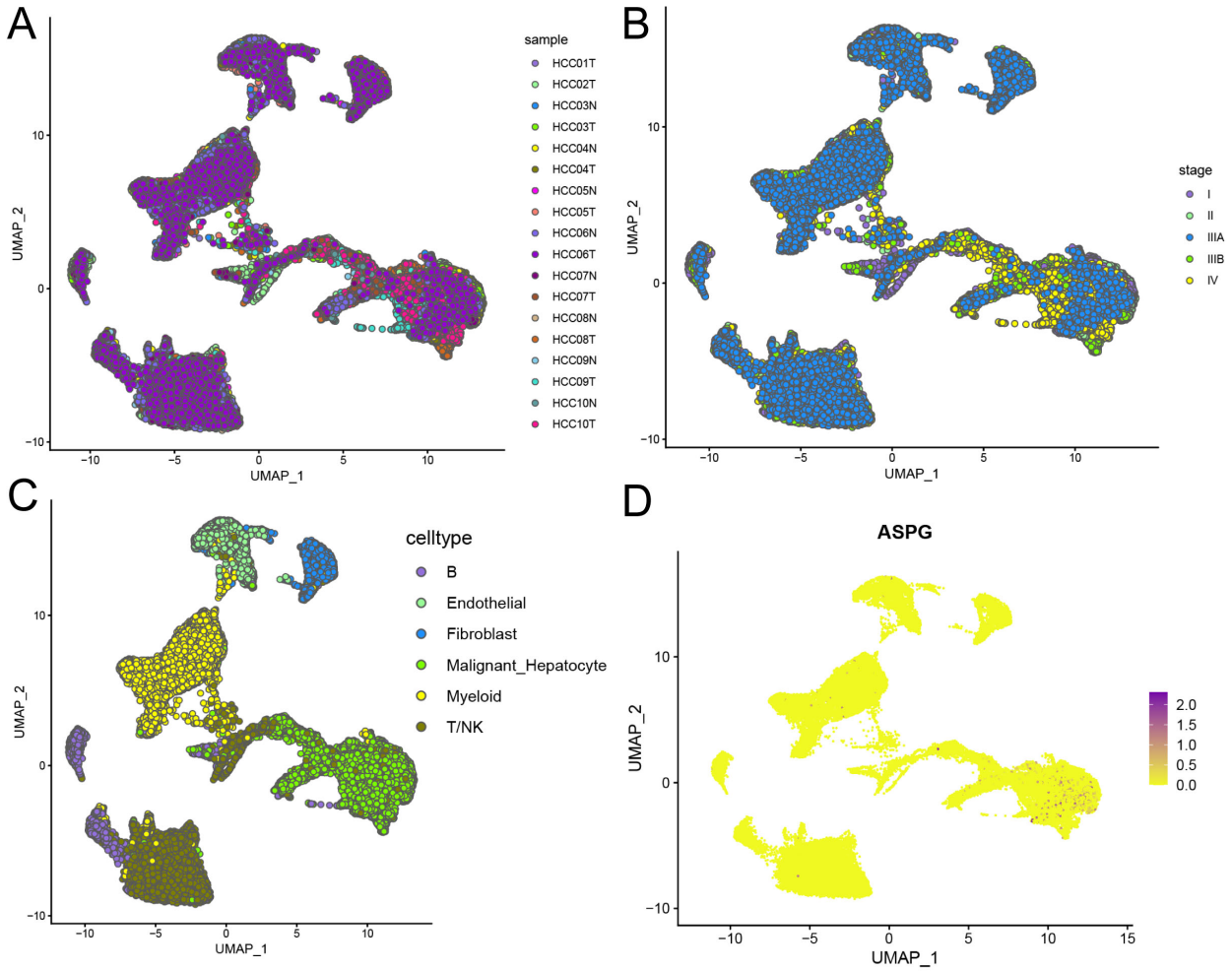

Fig. 3. Distribution of ASPG expression at the scRNA-seq level. (A) Clustering of HCC and non-HCC samples. (B) Distribution of samples across different clinical stages. (C) Distribution of annotated cell types. (D) Expression distribution of ASPG.

discriminatory power (AUC = 0.9, Fig. 2B). Both *Begg's* and *Egger's* tests revealed no significant publication bias ($p > 0.05$, Fig. 2C,D).

3.1.2 scRNA-seq Analysis of ASPG mRNA Downregulation in Malignant Hepatocytes

This study used scRNA-seq data from 8 non-HCC and 10 HCC samples (Fig. 3A). The HCC samples included clinical stages I, II, IIIA, IIIB, and IV (Fig. 3B). Following the processes of clustering and annotation, the samples were categorized into six cell types: B cells, endothelial cells, fibroblasts, malignant hepatocytes, myeloid cells, and T/NK cells (Fig. 3C). Notably, significant downregulation of ASPG was observed in malignant hepatocytes (Fig. 3D).

3.1.3 Analysis of ASPG Low Expression Profile Based on ST

Further exploration of the HCC expression characteristics using ST data revealed that ASPG was significantly downregulated in both the leading edge and primary tumor regions compared with adjacent tissues (Fig. 4A–C).

3.1.4 IHC Validation of ASPG Downregulation in HCC Tissues

IHC staining and scoring analysis of ASPG was performed on 301 HCC tissues and the corresponding adjacent non-HCC tissues. The staining in HCC was significantly lighter than that observed in non-HCC tissues, suggesting a lower expression level. The expression of ASPG in non-HCC and HCC tissues showed strong discriminatory power between the two tissue types ($p < 0.05$, AUC = 0.927; Fig. 5A–D).

3.1.5 The Effect of ASPG Expression on HCC Cell Growth and Proliferation

The expression of ASPG was evaluated in 8 different HCC cell lines, with the highest expression found in the Huh1 cell line (**Supplementary Fig. 4A**). Analysis of CRISPR knockout data from 21 HCC cell lines showed that ASPG knockout enhanced cell growth and proliferation in 17 of the cell lines (Chronos Score >0). Notably, the highest Chronos Score was observed in SKHEP1 (**Supplementary Fig. 4B**).

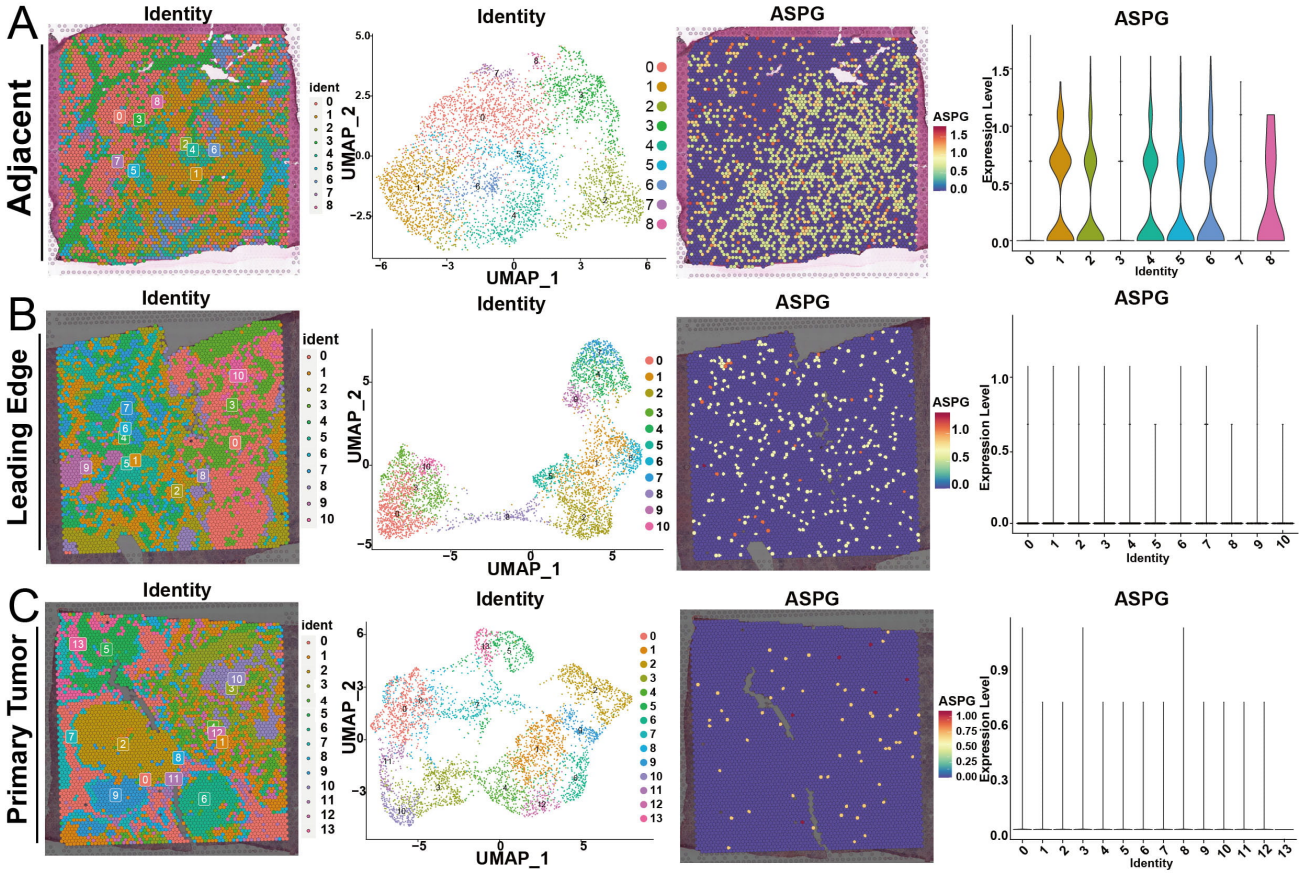

Fig. 4. Expression distribution of ASPG in ST samples (HCC-2 patient ST data). (A) ASPG expression distribution in Adjacent ST samples. (B) ASPG expression distribution in Leading Edge ST samples. (C) ASPG expression distribution in Primary Tumor ST samples.

3.2 Exploration of ASPG Pathways and Mechanisms in HCC

3.2.1 Regulatory Mechanisms Associated With Low ASPG mRNA Expression Based on Bulk RNA Data

This study employed GSEA enrichment analysis, GeneMANIA network analysis, along with GO and KEGG pathway analyses to investigate the regulatory pathways associated with low ASPG expression. Key pathways identified by GSEA include Glycine, Serine, and Threonine Metabolism, Alanine, Aspartate, and Glutamate Metabolism, and Biosynthesis of Amino Acids (Fig. 6A). GeneMANIA analysis suggested that functionally associated genes were primarily concentrated in biological processes such as nicotinamide nucleotide metabolic process, NAD biosynthetic process, and pyridine nucleotide metabolic process (Fig. 6B).

GO enrichment analysis showed significant associations with pyridoxal phosphate binding, gluconeogenesis, and lipid metabolic process (Fig. 6C). Furthermore, KEGG pathway analysis indicated that ASPG regulated several core metabolic hubs, including Metabolic Pathways, Biosynthesis of Amino Acids, Carbon Metabolism, and Arginine Biosynthesis (Fig. 6D).

Therefore, aberrant ASPG expression primarily regulates core pathways of metabolic reprogramming, including gluconeogenesis, amino acid metabolism, lipid metabolism, and carbon metabolism.

3.2.2 Molecular Mechanisms Regulating ASPG Expression in Malignant Hepatocytes Based on scRNA-seq Analysis

With regard to the specific regulatory role of ASPG in malignant hepatocytes, GO analysis revealed that the process of gluconeogenesis remained significantly enriched in these cells. This was accompanied by the regulation of energy metabolism-related processes, such as long-chain fatty acid metabolic process, aerobic respiration, and 2-oxoglutarate metabolic process (**Supplementary Fig. 5A**). KEGG pathway analysis further identified that ASPG regulated metabolic pathways, biosynthesis of amino acids, cholesterol metabolism, and one-carbon pool by folate, among others (**Supplementary Fig. 5B**).

3.2.3 Impact of Low ASPG Expression on the Interaction Network of Malignant Hepatocytes

Secondary clustering of the malignant hepatocytes resulted in 33 clusters. Based on the quantitative eval-

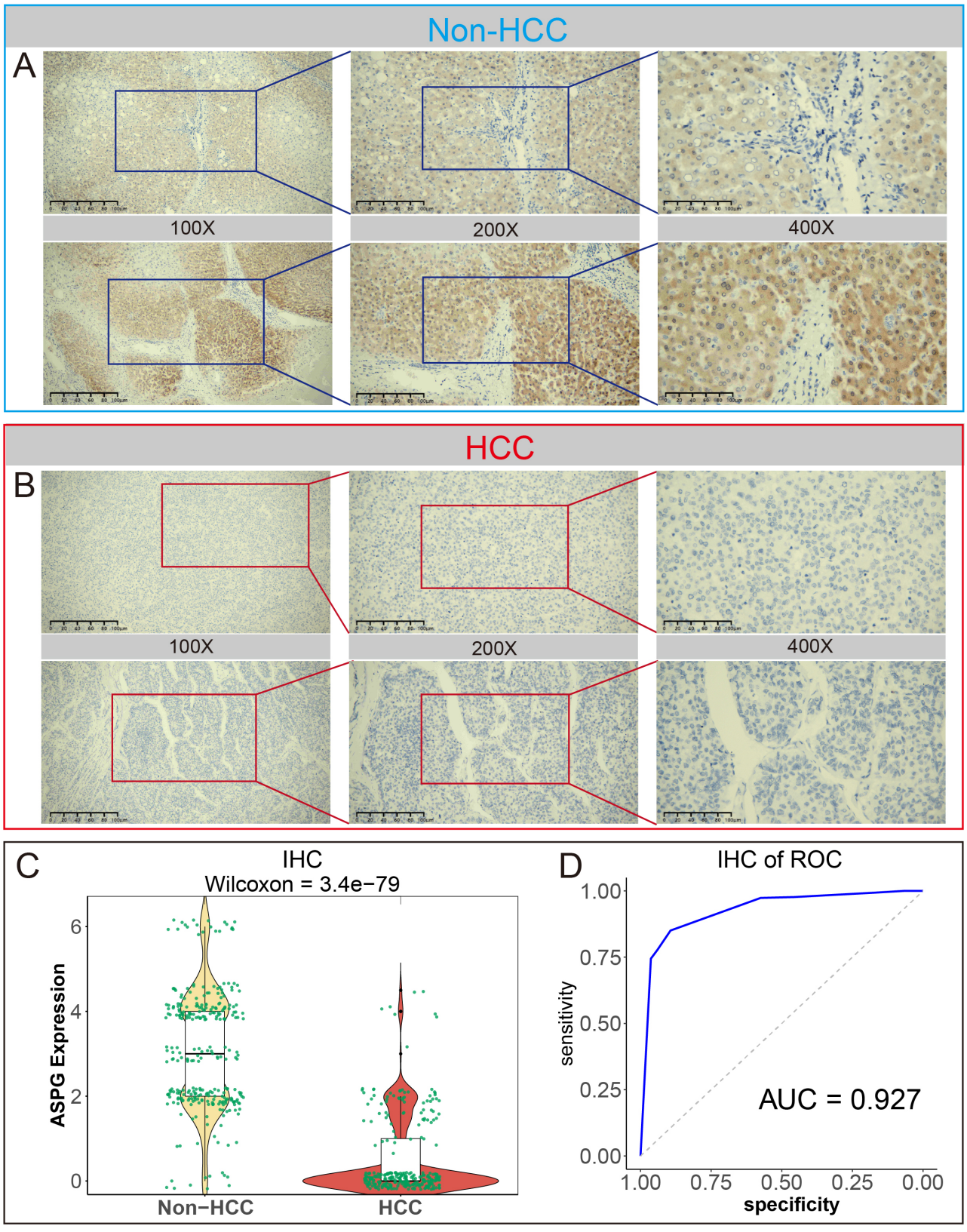

Fig. 5. IHC scoring analysis of ASPG protein expression. (A) IHC observation of ASPG expression in Non-HCC tissues (scale bar = 50 μm, 100 μm, 200 μm). (B) IHC observation of ASPG expression in HCC tissues (scale bar = 50 μm, 100 μm, 200 μm). (C) ASPG protein expression scoring in Non-HCC vs. HCC tissues by Wilcoxon test. (D) ROC analysis of ASPG protein expression scoring in Non-HCC vs. HCC tissues.

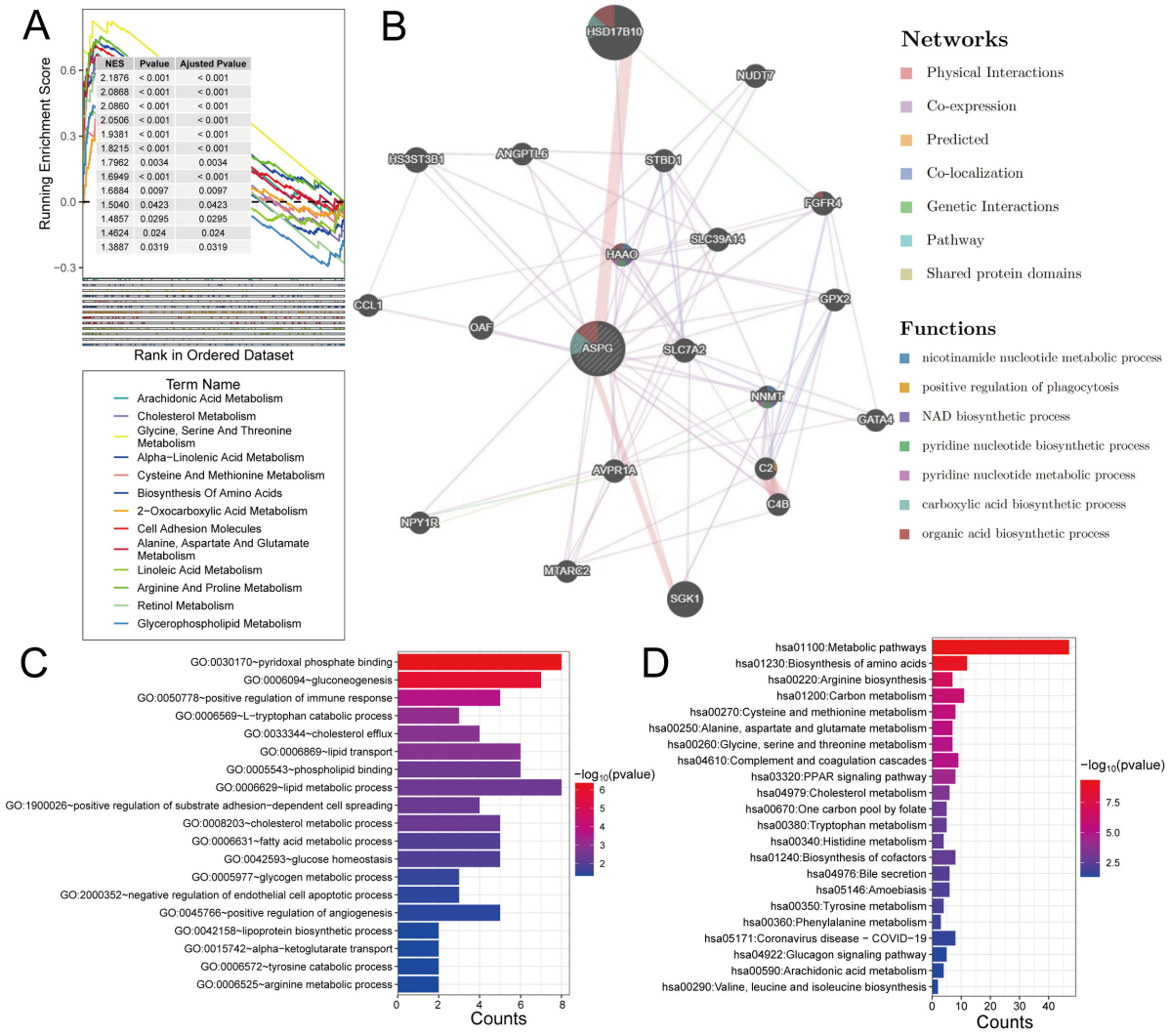

Fig. 6. Potential regulatory mechanisms of ASPG in HCC. (A) GSEA. (B) GeneMANIA analysis results. (C) GO enrichment. (D) KEGG pathway analysis.

uation of ASPG expression levels, clusters with significantly high ASPG expression (clusters 8, 21, and 27) were classified as ASPG-high, while the remaining clusters were designated as ASPG-low (Fig. 7A). Analysis of cell–cell communication revealed that, in terms of the number of interactions, ASPG-Low cells (as ligand-sending cells) exhibited a greater number of interactions with myeloid cells. In contrast, the interaction strength was stronger when ASPG-High cells (rather than ASPG-Low cells) acted as ligand senders to myeloid cells (Fig. 7B). Analysis of ligand–receptor interactions further revealed the MIF–(CD74+CXCR4) and MIF–(CD74+CD44) pathways exhibited significantly stronger interaction signals compared with other signaling pathways, with ASPG-low acting predominantly as the ligand (Fig. 8A). Comprehensive analysis of total signal input and output confirmed the pronounced activity of ASPG-low in the MIF signaling pathway (Fig. 8B). Expression levels of the MIF gene were significantly higher in the ASPG-low group (Fig. 7C,D).

3.3 Key Process Analysis of Metabolic Pathways Regulated by Low ASPG Expression

Metabolic scoring and flux analysis were performed on B cells, myeloid cells, T/NK cells, as well as on ASPG-high and ASPG-low populations, based on their interaction strengths with various cell types in the MIF pathway. Results from scMetabolism revealed that ASPG-low was significantly active in metabolic pathways, including Alanine, Aspartate, and Glutamate Metabolism, Arginine and Proline Metabolism, Pyruvate Metabolism, and the Citrate Cycle (TCA cycle) (Fig. 9A). Further analysis using scFEA suggested that ASPG-low may influence the conversion of metabolites such as Aspartate_Asparagine and Glutamate_2OG (Fig. 9B).

3.4 Low Expression of ASPG and Drug Sensitivity Analysis

According to drug sensitivity analysis, the IC50 values for cisplatin, erlotinib, gemcitabine, olaparib, oxaliplatin, sapitinib, and selumetinib were significantly lower in the

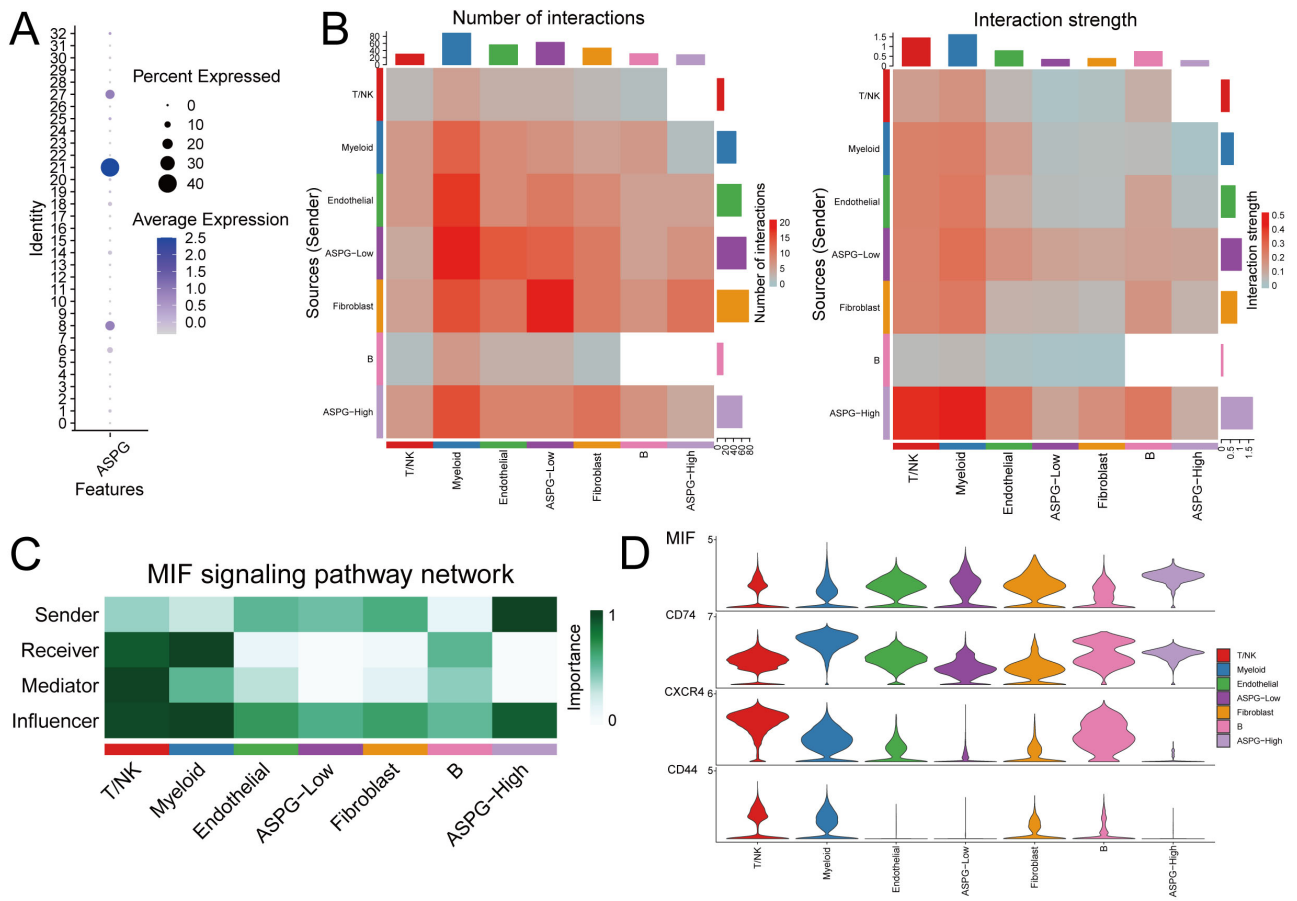

Fig. 7. Interactions between ASPG-Low and various cell types. (A) Secondary clustering of malignant hepatocytes. (B) Interaction quantity and intensity between different cell subgroups. (C) Role of cell subgroups in the MIF pathway. (D) Gene expression levels in the MIF pathway.

ASPG-high expression group compared with the ASPG-low expression group. In contrast, the ASPG-low expression group showed lower IC50 values for lapatinib and paclitaxel (Fig. 10).

4. Discussion

HCC is one of the most aggressive cancer types [27], and its malignant progression is strongly dependent on metabolic reprogramming [28]. As a key gene involved in metabolic regulation, the specific role and mechanism of ASPG in HCC remain poorly understood. The current study integrated bulk RNA, scRNA-seq, ST, and IHC multi-dimensional datasets to demonstrate the aberrant downregulation of ASPG mRNA and protein in HCC, along with associated impacts on the TME. CRISPR data revealed that ASPG knockout accelerated the growth of cells, while further mechanistic studies suggested that ASPG may potentially regulate biological behaviors related to metabolic reprogramming. Low expression of ASPG specifically affects the MIF pathway and enhances interaction with B cells, T/NK cells, and myeloid cells. Cancerous liver cells with low ASPG expression may be linked to altered amino acid metabolism, TCA cycle, and lipid metabolism. These processes are strongly involved in the metabolic conversion of malate to oxaloacetate, and of aspartate to asparagine. We also investigated whether low ASPG expression may be involved with drug resistance to cisplatin and gemcitabine, and sensitivity to lapatinib and paclitaxel. By applying a multi-platform analysis, we conducted a novel integrative study of ASPG in HCC. ASPG was found to have roles as a crucial metabolic regulator and as a driver of tumor progression. In addition, this work revealed the mechanism through which low ASPG expression induces metabolic reprogramming and oncogenesis, thus providing a novel theoretical basis for metabolic alteration in HCC.

The aberrant expression of ASPG may promote tumor progression. In addition to serving as a diagnostic molecular marker for treatment-related assessments in CRC, dysregulated expression of ASPG was associated with inhibitory effects in leukemia, possibly by directly exerting cytotoxic effects [11,12]. Furthermore, studies using machine learning have confirmed the significant diagnostic value of ASPG expression in HCC, thus hinting at a universal marker across different cancer types [14]. However, a complete picture of the role of ASPG in HCC is still lacking. The current research on HCC was per-

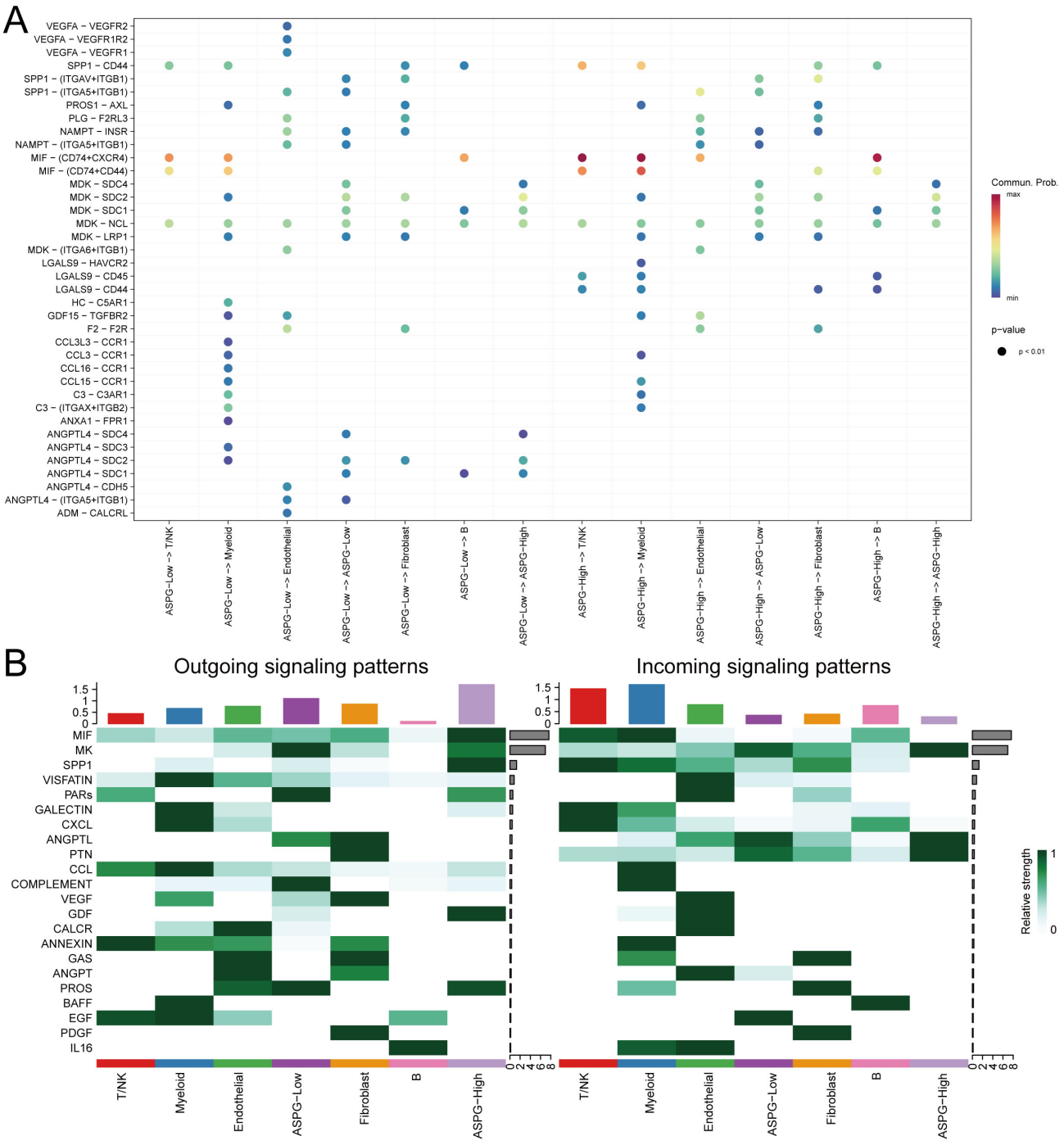

Fig. 8. Interactions in cell communication in ASPG-Low. (A) The overall interaction network between ASPG-Low and ASPG-High. (B) The overall signal strength of inputs and outputs.

formed using bulk RNA, scRNA-seq, spatial ST, and IHC to study the reduced expression of ASPG at the mRNA and protein levels, as well as the single-cell level. According to CRISPR data, ASPG knockout significantly promoted HCC cell proliferation, indicating that reduced ASPG expression leads to loss of its tumor-suppressive function and acquisition of a malignant phenotype. Notably, ST analysis demonstrated that ASPG expression is not only markedly reduced in the primary tumor mass, but also undergoes further downregulation at the tumor–stroma interface compared to adjacent non-tumor tissue. Accumulating evidence from multi-cancer spatial omics studies has shown that the invasive front is a critical functional boundary where tumor cells dynamically interact with their microenvironment. This is characterized by increased proliferative activity, enhanced invasive capacity, and profound metabolic reprogramming [29,30]. The spatially restricted low expression of ASPG at this interface strongly suggests involve-

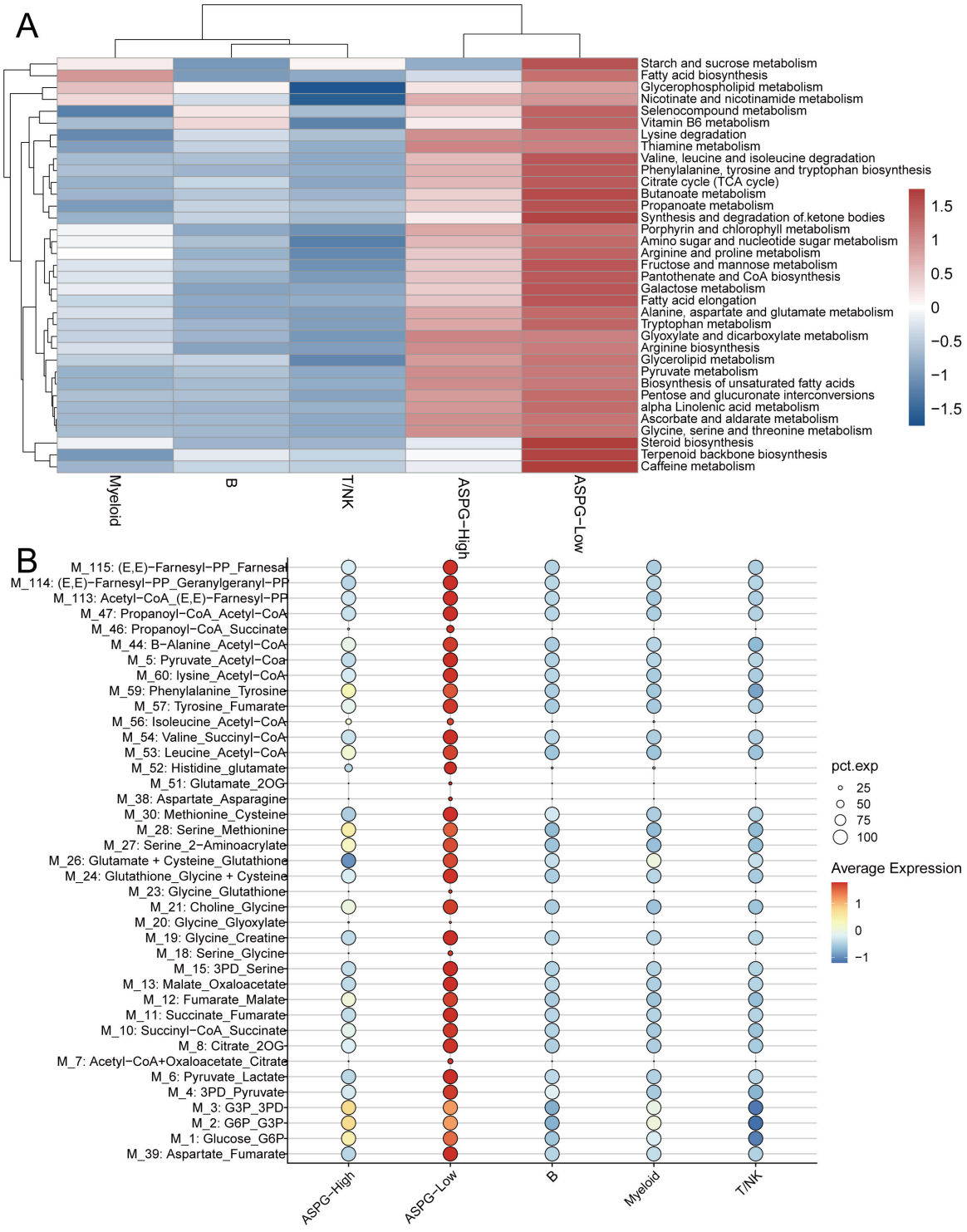

Fig. 9. Metabolic pathway analysis of ASPG-Low. (A) Metabolic pathway scores of ASPG-Low from scMetabolism. (B) Scatter plot of metabolite conversions of ASPG-Low from scFEA.

ment with the unique histopathological features and pro-invasive biological behaviors of the invasive niche. Collectively, our findings position ASPG as a significant tumor suppressor in HCC. Its inactivation—likely driven by underlying metabolic alterations—represents a key molecular event in hepatocarcinogenesis.

Metabolic reprogramming is one of the core features supporting the malignant progression of tumors. Such changes can promote tumor progression through dysregulation of the pathways involved in glucose metabolism, lipid metabolism, amino acid metabolism, pyrimidine metabolism, and oxidative metabolism [31]. As a metabolic

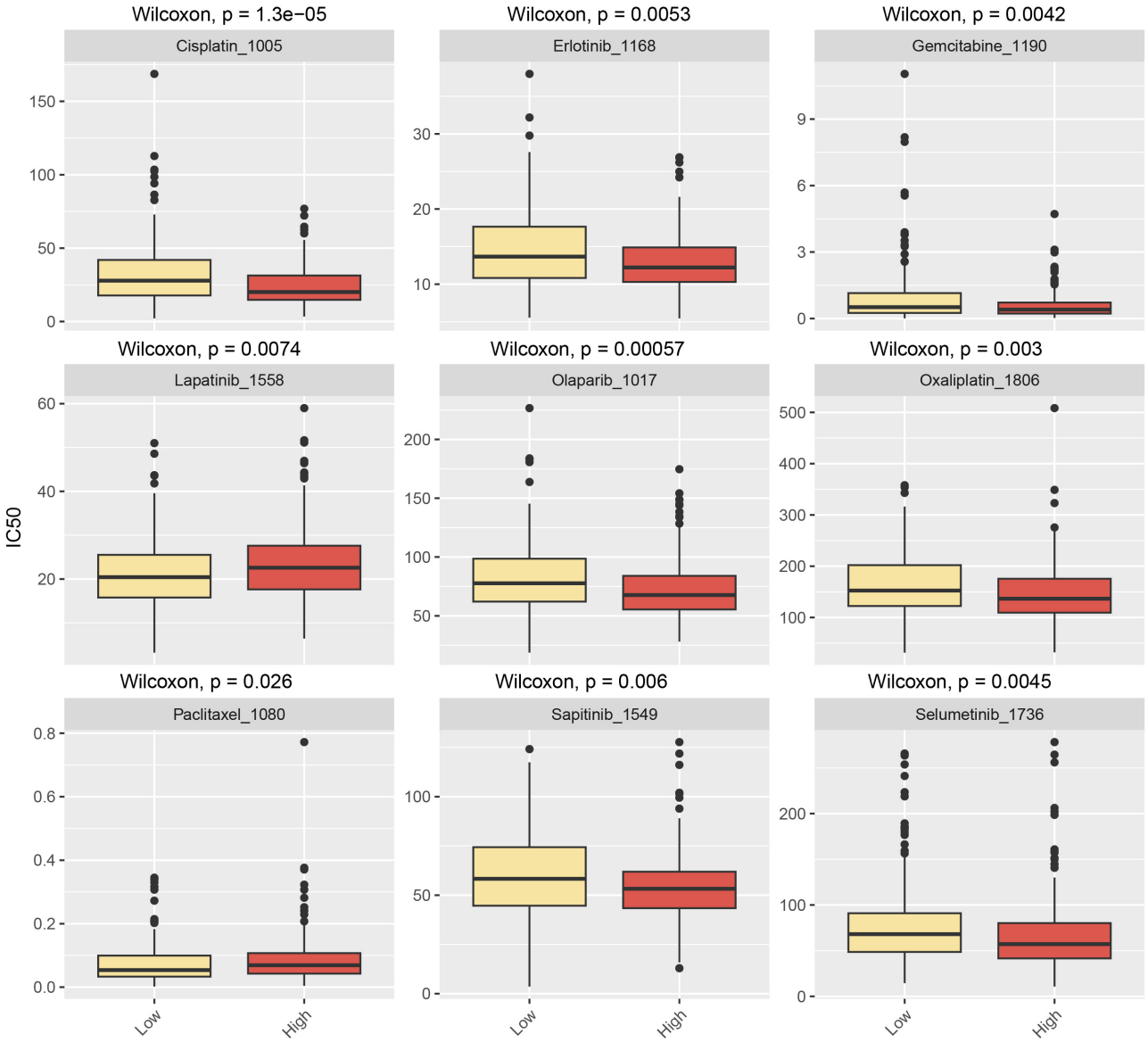

Fig. 10. Drug sensitivity analysis of ASPG low expression.

regulatory factor, ASPG catalyzes the release of asparagine residues via specific reactions. The amino group of asparagine is also released by asparagine transaminase (AsnAT) for amino acid biosynthesis, thereby potentially regulating asparagine metabolism [32]. As a metabolic gene, ASPG is also implicated in the development of cancer, and has been identified as a key gene related to amino acid metabolism in CRC [33,34]. Furthermore, ASPG was shown to coordinate a unique CAR-T cell phenotype skewed toward central memory T cells and asparagine metabolic reprogramming, thereby enhancing anti-tumor immunity [35]. Mechanistic analyses in the present study revealed that low ASPG expression affected broad metabolic pathways, including the regulation of amino acid biosynthesis, and specifically the metabolism of alanine, aspartate, and glutamate. Interestingly, scFEA results also indicated that low ASPG expression promoted the conversion of aspartate to asparagine. This suggests that ASPG deficiency leads to the abnormal accumulation of asparagine in tumor cells, contributing to core tumor metabolic reprogramming and promoting protein translation and tumor proliferation.

As a key regulatory factor in energy metabolism, ASPG has been confirmed as a potential hub for glucose metabolism. Studies have shown that liver ASPG levels are negatively correlated with human insulin sensitivity. In a mouse model of metabolic dysfunction-related fatty liver disease, knockout of the *Aspg* gene was shown to remodel the liver factor secretion profile, systematically increasing insulin sensitivity and maintaining glucose homeostasis in the body [36]. The present study further explored the metabolic regulatory function of ASPG from the perspective of metabolic flux, linking its role from the macroscopic glycogen metabolic process to specific intracellular path-

ways. Our findings revealed that the effects of low ASPG expression in HCC were not merely linear changes, but instead involved a process of coordinated metabolic reprogramming. These effects regulated the glycogen metabolic process, potentially via the TCA cycle, thereby altering the storage-release balance of glucose in the liver, while concurrently enhancing the conversion of glutamate to 2-oxoglutarate (2OG). As a key intermediate of the TCA cycle, the increased level of 2OG directly promotes mitochondrial energy metabolism [37–39]. Our study confirmed the macro-level role of ASPG in glycogen metabolism, and more importantly also delineated a precise axis from "low ASPG expression" to "enhanced glutamate-2OG metabolic flux", which drove "TCA cycle activation and optimized energy metabolism". Our discovery of this axis shifts the understanding of ASPG function from associative to mechanistic, offering potential new strategies for HCC treatment.

In the context of cell–cell interactions, hepatocytes with low ASPG expression were found to promote signaling through the MIF (CD74/CXCR4/CD44) axis. This intensifies their crosstalk with myeloid cells, activating mTORC1 and subsequently increasing the expression of MIF [40]. Furthermore, our scFEA analysis revealed an increase in the glutamate metabolic flux in ASPG-low hepatocytes. Glutamate can be converted to α-ketoglutarate (α-KG) [41], which not only regulates epigenetic modifications [42] but also stabilizes HIF-1α, thereby enhancing MIF transcription [43]. From an immunological perspective, MIF signaling promotes tumor proliferation via the WNT/β-catenin/c-MYC pathway [44] and simultaneously drives immune evasion by inducing M2 macrophage polarization, suppressing $CD8^+$ T cell infiltration, expanding regulatory T cells (Tregs) and tumor-associated neutrophils (TANs), and mediating glucocorticoid resistance [40,45,46]. Collectively, these findings suggest that low ASPG expression may foster a pro-tumorigenic microenvironment through an integrated metabolic–immune mechanism, thus providing a theoretical basis for the role of enhanced MIF signaling in linking metabolic reprogramming to immunosuppression.

The notion of low ASPG expression as a potential therapeutic target for HCC aligns with the emerging paradigm of "metabolic intervention" in cancer treatment. Indeed, a variety of drugs currently included in the spectrum of oncological therapies are known to exert their effects through direct or indirect modulation of tumor cell energy metabolism. Several studies have reported on the intrinsic relationship between metabolic reprogramming and chemotherapy responses. In cholangiocarcinoma, higher α-D-glucose levels were observed in non-responders to gemcitabine and cisplatin, while metabolites involved in the TCA cycle were significantly lower, suggesting that metabolic states determine chemosensitivity [47]. In patient-derived xenograft models of pancreatic ductal adenocarcinoma, proteins regulated by gemcitabine combined with nab-paclitaxel were shown to inhibit oxidative phosphorylation and the TCA cycle [48]. Finally, a study in HCC patients showed that limiting glucose uptake and targeting the HSF1/AMPKα2 signaling axis could potentially prevent chemoresistance to oxaliplatin [49]. This framework is consistent with the role of ASPG in remodeling the TCA cycle. As ASPG hydrolyzes asparagine to aspartate—an anaplerotic precursor of oxaloacetate—the loss of asparagine may impair TCA cycle flux and trigger compensatory, glutamine-driven accumulation of α-KG. This mechanism has been implicated in the resistance to gemcitabine/cisplatin by biliary and pancreatic cancers [50–52]. Moreover, enhanced oxidative phosphorylation in ASPG-low cells may further promote chemoresistance [43,51]. Further validation of the effectiveness of targeting specific amino acid metabolic pathways in cancer therapy was reported in acute lymphoblastic leukemia, where exogenous ASPG was used to deplete asparagine and thereby inhibit tumor progression [53–55]. Within the broader context of metabolic intervention, our findings provide the additional insight that HCC patients with low ASPG expression exhibit resistance to oxaliplatin and gemcitabine, but sensitivity to paclitaxel. This distinct drug response profile strongly suggests that low ASPG expression defines a molecular subtype of HCC with specific metabolic vulnerabilities. We hypothesize that by reprogramming tumor energy metabolism, the loss of ASPG impairs the efficacy of drugs that rely on specific metabolic processes, while potentially increasing sensitivity to agents that target the microtubule system. Consequently, ASPG represents not only a potential standalone therapeutic target, but also a promising biomarker for guiding precision chemotherapy in HCC.

5. Limitations

This study has several limitations that should be acknowledged. Although significant associations were identified between ASPG expression and metabolic phenotypes in the current analysis, the precise molecular mechanisms underlying these relationships remain to be fully elucidated. As the present research is based on observational and correlational analyses, no causal inferences can be drawn, which may restrict the interpretability of the observed associations. These findings therefore require further verification through well-designed functional experiments, including *in vitro* cellular models and *in vivo* animal studies, to clarify potential causal links. In addition, this study did not explore the subcellular localization of the ASPG protein, the biological functions of its specific transcript isoforms, or its potential post-translational modifications and interaction networks. These omissions may limit a comprehensive understanding of ASPG's regulatory roles in hepatocellular carcinoma. Furthermore, the study did not establish feasible approaches to specifically activate ASPG in HCC subtypes with low ASPG expression, which constrains immediate translational implications for precision therapy of HCC.

6. Conclusion

This is the first comprehensive study on the phenotype of HCC with low ASPG expression. Our findings can inform future research into the rewiring of aspartate and TCA flux as a therapeutic approach. Moreover, our study revealed a strong correlation between ASPG expression and drug sensitivity in HCC.

Availability of Data and Materials

The datasets supporting the conclusions of this article can be found at multiple public databases. The single - cell dataset is available from GEO (https://www.ncbi.nlm.nih.gov/gds/). The ST dataset can be accessed from the following database (http://lifeome.net/supp/livercancer-st/data.htm; HCC2N, HCC2L, and HCC2T). The bulk RNA datasets are accessible from the following sources: TCGA (https://portal.gdc.cancer.gov/); GEO (https://www.ncbi.nlm.nih.gov/gds/); GTEx (https://www.gtexportal.org/home/); ICGC_LIRI_JP (https://platform.icgc-argo.org/); ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress). The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YWD and YLD were responsible for the execution of the experiments, systematic analysis of the data, and drafting the initial manuscript. YXT, RLS, KSN, WQ, ZDC and LX provided key assistance in optimizing the experimental design and were responsible for the rigorous implementation of data collection and statistical analysis. YWD and YLD focused on deepening the data analysis and made detailed revisions and improvements to the manuscript. ZBF and DDX as the core planners of the research, not only proposed the overall research concept but also guided the preparation and revision of the paper throughout, providing comprehensive oversight of the entire research process. Finally, all authors reviewed the manuscript and unanimously approved the final version. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was carried out in accordance with the guidelines of the Declaration of Helsinki. Ethical approval was obtained from the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University (Approval Number: 2025-E0967). Written informed consent was obtained from all participants included in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL49215.

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