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Multi-omic analysis of glycolytic signatures: Exploring the predictive significance of heterogeneity and stemness in immunotherapy response and outcomes in hepatocellular carcinoma

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Conceptualization, FZ; writing original draft preparation, SZ; visualization, SZ and YP; data resources, YP and LT; supervision, validation, and funding acquisition, FZ; review and editing, FZ. All authors reviewed and approved the final manuscript.

Keywords

HCC, heterogeneity, Stemness, Glycolysis, Immunotherapy

Abstract

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Background: Hepatocellular carcinoma (HCC) is a global health challenge with complex pathophysiology, characterized by high mortality rates and poor early detection due to significant tumor heterogeneity. Stemness significantly contributes to the heterogeneity of HCC tumors, and glycolysis is crucial for maintaining stemness. However, the predictive significance of glycolysis-related metabolic genes (GMGs) in HCC remains unknown. Therefore, this study aimed to identify critical GMGs and establish a reliable model for HCC prognosis.

Methods: GMGs associated with prognosis were identified by evaluating genes with notable expression changes between HCC and normal tissues retrieved from the MsigDB database. Prognostic gene characteristics were established using univariate and multivariate Cox regression studies for prognosis prediction and risk stratification. The "CIBERSORT" and "pRRophetic" R packages were respectively used to evaluate the immunological environment and predict treatment response in HCC subtypes. The HCC stemness score was obtained using the OCLR technique. The precision of drug sensitivity prediction was evaluated using CCK-8 experiments performed on HCC cells. The miagration and invasion ability of HCC cell lines with different riskscores were assessed using Transwell and wound healing assays.

Results: The risk model based on 10 gene characteristics showed high prediction accuracy as indicated by the receiver operating characteristic (ROC) curves. Moreover, the two GMG-related subgroups showed considerable variation in the risk of hepatocellular carcinoma (HCC) with respect to tumor stemness, immune landscape, and prognostic stratification. The in vitro validation of the model's ability to predict medication response further demonstrated its reliability.

Conclusions: Our study highlights the importance of stemness variability and inter-individual variation in determining the HCC risk landscape. The risk model we developed provides HCC patients with a novel method for precision medicine that enables clinical doctors to customize treatment plans based on unique patient characteristics. Our findings have significant implications for tailored immunotherapy and chemotherapy methods, and may pave the way for more personalized and effective treatment strategies for HCC.

Contribution to the field

Tumor heterogeneity in hepatocellular carcinoma (HCC) is primarily attributed to stemness, which is, in turn, critically regulated by glycolysis. However, the clinical significance of glycolysis-related metabolic genes (GMGs) in HCC prognosis is still poorly understood. Thus, the objective of this investigation is to identify crucial GMGs and develop a robust prognostic model for HCC. By employing receiver operating characteristic (ROC) curves, we developed a 10-GMG-based risk model that exhibits high predictive accuracy, which was subsequently validated. Furthermore, two distinct GMG-related subtypes were identified, exhibiting significant differences in tumor stemness, immune landscape, and prognostic stratification, indicating a considerable degree of heterogeneity in HCC risk. Notably, these findings also suggest that patterns in immunotherapy and chemotherapy responses may be associated with HCC heterogeneity and stemness diversity among patients. In vitro validation confirmed the predictive value of our model for drug response. In summary, this study provides clinicians with a potential strategy for precision medicine targeting HCC heterogeneity by utilizing the 10-GMGs model.

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Keywords: HCC, heterogeneity, stemness, glycolysis, immunotherapy

Abstract

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Methods: GMGs associated with prognosis were identified by evaluating genes with notable expression changes between HCC and normal tissues retrieved from the MsigDB database. Prognostic gene characteristics were established using univariate and multivariate Cox regression studies for prognosis prediction and risk stratification. The "CIBERSORT" and "pRRophetic" R packages were respectively used to evaluate the immunological environment and predict treatment response in HCC subtypes. The HCC stemness score was obtained using the OCLR technique. The precision of drug sensitivity prediction was evaluated using CCK-8 experiments performed on HCC cells. The miagration and invasion ability of HCC cell lines with different riskscores were assessed using Transwell and wound healing assays.

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Conclusions: Our study highlights the importance of stemness variability and inter-individual variation in determining the HCC risk landscape. The risk model we developed provides HCC patients with a novel method for precision medicine that enables clinical doctors to customize treatment plans based on unique patient characteristics. Our findings have significant implications for tailored immunotherapy and chemotherapy methods, and may pave the way for more personalized and effective treatment strategies for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and is responsible for a significant proportion of cancer-related deaths worldwide [1]. Established risk factors for HCC include liver cirrhosis and metabolic syndrome, which have a negative impact on the prognosis and reduce overall survival rates [2-5]. While recent advances in conventional treatments such as chemotherapy, surgery, and radiation therapy have shown promise, HCC recurrence and metastasis remain significant challenges [6]. Current prognostic models for liver cancer, which rely on clinical indicators like grade and TNM stage, may have limited accuracy [7-10]. Therefore, there is a need for new biomarkers that can accurately predict survival and help identify specific therapy targets for HCC. Molecularly targeted treatments represent a promising avenue for the future treatment of hepatocellular carcinoma.

Malignant neoplasms are distinguished by their unquenchable demand for energy to fuel their growth. Consequently, cancer cells utilize a complex network of interrelated metabolic pathways [11-13]. With their impressive metabolic flexibility, cancer cells can rewire crucial metabolic pathways like glycolysis to satisfy their heightened energy requirements [14]. One instance of such metabolic reprogramming is the Warburg effect, which Warburg originally proposed in 1956. This phenomenon

involves heightened glucose uptake, lactate accumulation, and increased ATP synthesis in cancer cells [15, 16]. Aerobic glycolysis, which is a key characteristic of the Warburg effect, not only facilitates the proliferation of cancer cells, but also promotes invasion and metastasis by creating an acidic microenvironment [17]. Several types of cancer, including breast, pancreatic, and gastric cancers, exhibit this phenomenon [18-20]. While the role of aerobic glycolysis in the initiation, progression, and pharmacological management of hepatocellular carcinoma (HCC) has been widely studied [21-24], the prognostic relevance of genes involved in glycolysis remains poorly understood.

In this investigation, we analyzed clinical and sequencing data from the TCGA-LIHC database to explore the potential correlations between gene expression markers (GMGs) and the survival outcomes of hepatocellular carcinoma (HCC) patients, as well as the genetic changes associated with these outcomes. Through LASSO analysis, we identified eleven GMGs that demonstrated robust associations with HCC. Subsequently, utilizing the cumulative weights of these GMGs, we performed patient stratification to classify individuals into high- and low-risk groups, revealing contrasting immunological landscapes and stemness features between the two groups. Notably, our in vitro assays employing GMGs as predictors of chemotherapy sensitivity yielded a high degree of predictive accuracy. Taken together, our results present a novel predictive framework for HCC that may facilitate the creation of customized treatment plans tailored to individual patients' unique risk profiles.

2. Materials and Methods

2.1 Acquisition of Data from the TCGA Portal

The Cancer Genome Atlas (TCGA) is a comprehensive database of genomic cancer information for 33 cancer types, integrating gene expression patterns and clinical data [25]. We accessed the TCGA database (https://portal.gdc.cancer.gov/) to obtain HCC data relevant to our study.

2.2 Retrieval of GMGs

The Molecular Signatures Database (MsigDB) provides access to 200 glycolysis-related genes [26].

2.3 Identification of a Prognostic GMGs Signature

Our univariate Cox regression analysis identified 45 genes significantly associated with HCC patient survival. LASSO regression analysis was then employed to select the most relevant genes, revealing 10 core genes that formed the basis of a risk signature. We determined patient risk scores based on their unique gene expression profiles [27].

2.4 Evaluation of Infiltrating Immune Cells

We utilized the ssGSEA and CIBERSORT R packages [28, 29] to evaluate immune cell infiltration. Using the CIBERSORT algorithm, we generated immune cell type scores for each tissue sample and assigned a score to each sample based on inferred immune cell type scores.

2.5 Prediction and Validation of Chemotherapy Response

We utilized the "pRRophetic" R package to calculate IC50 values of drugs to predict and confirm treatment efficacy in HCC cells [30]. Sensitivity of the drugs to HCC cells was then determined using the CCK-8 test.

2.6 Analysis of Functional Enrichment

Functional enrichment analysis was conducted using the GSVA method and the "c2.cp.kegg.v7.4.symbols.gmt" database [31, 32].

2.7 Transwell Assays for Invasion and Migration

To investigate cellular invasion, we employed Transwell chambers (Corning, Lowell, MA, USA) that were precoated with Matrigel. HepG2 and Huh7 cells were seeded in the top chamber without FBS, while the bottom chamber was supplemented with DMEM containing 15% FBS. After a 48-hour incubation period, cells that remained in the top chamber were gently removed using a cotton swab. The cells that had invaded the bottom chamber were stained with 1% crystal violet and fixed with 4% paraformaldehyde (PFA) for 30 min. The cells were then counted under a light microscope. To assess cellular migration, we used the same Transwell chambers but without Matrigel coating. Each experiment was performed in triplicate.

2.8 Wound-Healing Migration Assay

To assess the migratory capacity of HepG2 and Huh7 cells, we conducted a wound-healing migration assay. Cells were seeded in 6-well plates and allowed to reach 100% confluence within 24 hours. A sterile 10-l micropipette tip was used to create a wound in the cell monolayer, and the detached cells were gently removed. The wound closure was monitored and evaluated at 24 hours using an inverted microscope (OLYMPUS, Japan) in five randomly selected fields. The wound closure was calculated as (W0h - W24h)/W0h x 100%, where W is the width of the scratch. Each experiment was conducted in triplicate.

2.9 Statistical Analysis

Statistical analysis was performed using the R 4.1.3 program. The significance level was set at P-values < 0.05 and False Discovery Rates (FDR) (q) < 0.05. The results of the Student's t-test were presented as mean and standard deviation (SD) for the two groups. We used P < 0.05*, P < 0.01**, and P < 0.001*** as the levels of statistical significance.

3. Results

3.1 Gene Signature Construction

WWe aimed to construct a gene signature related to glycolysis metabolism (GMGs) by utilizing the Glycolysis_Hallmark gene collection, consisting of 200 genes, obtained from the MsigDB website. In this study, we utilized 55,316 gene expression profiles from the TCGA database, which included 370 tumor samples and 50 samples of nearby normal tissue, as well as information on hepatocellular carcinoma (HCC). Using the "limma" R package and applying a logFC filter of 1 and adj.P. Val. Filter of 0.05, we identified 59 GMGs that were differentially expressed in HCC tumor and nearby normal samples (**Figure 1A**). Additionally, using the "survival" and "survminer" R packages, we identified 45 GMGs that were significantly associated with patient survival at P < 0.05 and km score < 0.05 (**Figure 1B**), enabling us to investigate the potential impact of GMGs on HCC patient survival. Notably, with the exception of four GMGs, all others acted as unfavorable prognostic indicators. A lasso analysis was performed using the 45 GMGs to develop an HCC predictive model (**Figure 1C**), which was validated by a time-dependent ROC curve showing high accuracy at one, three, five, and seven years (AUC=0.803, 0.72, 0.683, and 0.611, respectively) (**Figure 1F**).

Subsequently, based on the median Riskscore, we stratified the 370 HCC patients into two distinct subgroups, high-risk and low-risk, and found that the high-risk group had significantly shorter overall survival time compared to the low-risk group (**Figure 1E**), with median survival times of 2.6 and 6.7 years, respectively. Finally, we generated a heatmap to illustrate the expression patterns of the top ten GMGs across different Riskscore groups (**Figure 1D**), providing additional support for the prognostic significance of our GMG signature in the evaluation of HCC.

3.2 Analysis of HCC Subtypes

To investigate the mRNA expression patterns of the 10 GMGs, we performed a comparative analysis of their expression levels in normal and tumor groups (**Figure 2A**). Notably, we observed that the expression of these genes was significantly elevated in tumor tissues when compared to adjacent non-tumor tissues ($P < 0.001$), with TALDO1 exhibiting the highest level of expression. The mRNA expression patterns of the 10 GMGs in high-risk and low-risk categories (**Figure 2B**) were consistent with those depicted in Figure 1A. To evaluate the prognostic value of each GMG, we generated Kaplan-Meier curves (**Supplementary Figure S1**) and found that all 10 GMGs were significantly associated with unfavorable clinical outcomes ($P < 0.05$). Further studies are required to elucidate the molecular mechanisms underlying the dysregulated expression of these genes in HCC and explore the potential for developing novel therapeutic interventions.

3.3 Enrichment Analysis

Dysregulation of various signaling pathways is known to contribute to altered tumor microenvironments and tumorigenesis. In this study, we compared gene expression levels between high-risk and low-risk groups (**Figure 3A**) to identify differentially expressed genes. Our analysis revealed a significant enrichment of pathways related to cytoplasmic processes in high-risk HCC patients (**Figure 3B**). Furthermore, gene ontology (GO) enrichment analysis in the high-risk group demonstrated a marked activation of the biological process of cytoplasmic translation (**Figure 3C**). This pathway was significantly overrepresented in the differentially expressed genes between high-risk and low-risk groups (**Figure 3D, E**), highlighting its potential as a therapeutic target for HCC treatment.

3.4 Immune Infiltration Patterns in HCC Patients with Different Risk Profiles

In this study, we utilized a set of 10 GMGs to explore immune infiltration patterns in heterogeneous risk profiles of patients with hepatocellular carcinoma. The Lasso method was applied to conduct dimensionality reduction and clustering analysis, and our results, depicted in **Figure 4A**, successfully distinguished HCC patients across different risk categories. To further evaluate the immune cell landscape, we assessed immune cell abundance across different risk scores (**Figure 4B**). Our findings revealed a higher infiltration of regulatory T cells (Tregs) and macrophage M0 in high-risk patients, compared to that of macrophage M1 (**Figure 4C, D**)..

One of the GMGs investigated in this study was G6PD, a crucial metabolic enzyme involved in glycolysis. Our results, presented in **Figure 5**, indicated a positive correlation between G6PD and STC2 expression levels and the infiltration of M2 macrophages. Conversely, the expression levels of CENPA and HMMR were negatively correlated with the presence of CD4 memory resting T cells, while B3GAT3 and SAP30 expression levels were negatively correlated with naive B cells. Our results were consistent with the riskscore distribution, as shown in **Figure 5A**. Additionally, significant associations were found between STC2 and HMMR with multiple immune cell types, as

revealed by our correlation analysis between the ten GMGs and various immune cell types (**Figure 6A, B**).

3.5 Relationship between GMG Expression and Immunotherapy Response

In our previous investigation, we identified unique immunological microenvironments in patients with high-risk and low-risk hepatocellular carcinoma (HCC). Specifically, the high-risk group exhibited elevated levels of Macrophage M0 and Tregs, which have immunosuppressive properties. Effective activation of CD4 memory T cells is essential for a positive response to immune therapy, as these cells display varying degrees of sensitivity to immunotherapeutic modalities. Notably, we observed a substantial increase in the expression of ten GMGs in HCC patients who exhibited positive responses to Anti-PD-L1 and Anti-PD-1 immunotherapies. This discovery suggests that these genes may serve as potential biomarkers for predicting the effectiveness of immune checkpoint blockade (ICB) treatment in HCC patients (**Figure 7A, B**).

The glycolytic pathway, with G6PD as a key enzyme, plays a critical role in the metabolism of cancerous cells. We detected a marked increase in G6PD protein expression in the tissues of patients with hepatocellular carcinoma (HCC) (**Figure 8A**). To assess the effectiveness of immune checkpoint blockade (ICB) in HCC patients at high and low risk, we utilized the TIDE algorithm, an innovative approach that integrates G6PD expression levels and HBV infection factors (**Figure 8B**). Our analysis revealed a positive correlation between elevated G6PD expression levels and immune response score, independent of HBV status. We further classified HCC patients into high and low G6PD expression groups and observed a significant upregulation of immune checkpoint markers, including PDCD1 and CD274, in the high G6PD expression group (**Figure 8C, D**). Additionally, our Cibersort analysis revealed substantial differences in immune cell infiltration levels among normal, low-risk, and high-risk HCC patient tissue samples (**Figure 8E, F**). Taken together, our results suggest that G6PD expression levels may serve as a promising biomarker for predicting response to ICB in HCC patients..

3.6 Stemness Scores in Hepatocellular Carcinoma Patients

Hepatocellular carcinoma (HCC) exhibits significant heterogeneity, which affects both tumor progression and treatment response. To investigate the stemness phenotype as a contributing factor to this heterogeneity, we assessed stemness scores in HCC patients stratified by risk level. Our analysis revealed significantly higher stemness ratings in HCC patients than in healthy liver tissue (**Figure 9A**). Moreover, high-risk HCC patients displayed significantly elevated stemness scores compared to low-risk individuals (**Figure 9B**). Notably, even among low-risk patients, we observed a significant positive correlation between risk ratings and stemness index ($R = 0.31$; **Figure 9C**). Our results underscore the pivotal role of stemness in HCC pathogenesis and suggest that targeting stemness may represent a promising therapeutic approach for HCC. Nevertheless, a better understanding of the underlying mechanisms linking stemness to HCC risk stratification is needed and warrants further investigation.

3.7 Anticipation of drug responsiveness and authentication

In this study, we aimed to evaluate the efficacy of personalized therapy for managing hepatocellular carcinoma (HCC) in patients with diverse risk profiles by assessing variations in chemotherapeutic drug sensitivity. Specifically, we measured the IC50 concentrations of nine chemotherapeutic agents in HCC subgroups categorized according to high and low risk scores, as illustrated in Figure 10. Our analysis revealed significant inter-subgroup heterogeneity in IC50 values, with Etoposide exhibiting

the most pronounced disparity. Additionally, we confirmed the enhanced susceptibility of HCC patients with high-risk scores to Etoposide, as demonstrated in **Supplementary Table S1**. To assess drug treatment efficacy, we selected Huh7 and HepG2 cells as representatives of the subgroups with high and low risk scores, respectively, and determined their IC50 values for Etoposide using the CCK-8 assay (**Figure 11B**). Notably, the IC50 of Etoposide in Huh7 cells was significantly lower than that in HepG2 cells, lending support to the potential therapeutic benefits of Etoposide chemotherapy for patients with high-risk scores, as identified by our analysis. These findings are consistent with our drug sensitivity prediction results (**Figure 11D**) and underscore the promise of personalized therapy in improving the efficacy of HCC treatment across varying risk scores.

3.8 Invasion and Migration Abilities between HCC cell lines under different risk

To explore potential disparities in migratory and invasive capacities across HCC cell lines stratified by risk scores, we conducted transwell assays at 24h and 48h time points. Our findings revealed that, at 24h after cell inoculation, a greater number of Huh7 cells with elevated risk scores traversed the transwell chambers and reached the bottom of the wells. This trend was even more evident at 48h post-inoculation (**Figure 11A, C**). Furthermore, the scratch assay corroborated the greater migratory ability of the Huh7 cell line in comparison to the HepG2 cell line, in line with their respective risk scores (**Figure 11E, F**).

4. Discussion

Accurate and timely diagnosis of hepatocellular carcinoma (HCC) is essential for optimizing patient outcomes. However, the high heterogeneity of HCC tissue poses a significant challenge to the accuracy of current clinical classification systems [33]. Moreover, the complex molecular mechanisms involved in HCC create obstacles for identifying effective therapeutic targets [13, 34, 35]. The Warburg effect, a phenomenon in which tumor cells rely on aerobic glycolysis to evade apoptosis, plays a crucial role in maintaining cellular function, particularly in malignancy [36-38]. As the liver plays a vital role in energy metabolism, HCC tumorigenesis is inextricably linked to glycolysis. Previous studies have demonstrated that HCC growth, metastasis, and resistance to treatment are closely associated with glycolytic metabolism [39-43]. Therefore, the construction of accurate prognostic models utilizing machine learning techniques that incorporate glycolysis-related genes is essential for precise diagnosis, individualized therapy, and the prediction of clinical outcomes in HCC patients [44].

To identify potential prognostic markers for hepatocellular carcinoma (HCC), our study employed a screening strategy utilizing a panel of 200 metabolic genes linked to glycolysis. By conducting a differential gene expression analysis, we identified 10 glycolysis-related metabolic genes (GMGs) that were significantly correlated with HCC prognosis. Subsequently, we employed this subset to establish a predictive model for HCC prognosis (**Figure 1C**). Our model effectively predicted overall mortality rates among HCC patients over different time periods, including 1, 3, 5, and 7 years following diagnosis. These results suggest that our GMGs-based prediction model may have significant clinical applications in the decision-making process for HCC patients. Moreover, our findings indicate that GMGs may serve as a promising new class of prognostic biomarkers for HCC.

The tumor microenvironment (TME) plays a crucial role in the pathogenesis of cancer [45], and recent studies have demonstrated the promotion of malignancy within TME via exosome-mediated signaling [46, 47]. Reliable prognostic indicators in HCC include patterns of immune infiltration within the TME [48, 49] , with regulatory T-cells (Tregs) contributing to an immunosuppressive

milieu that supports cancer cell survival while impeding immune surveillance [50]. In addition, increasing evidence indicates that neutrophils serve as key immunosuppressive regulators in the TME of various malignancies, including HCC [51]. Our study reveals a significant association between elevated levels of neutrophil and Treg infiltration and increased GMG expression, as demonstrated in **Figure 4C**. Although M2 macrophages have been extensively studied for their role in promoting tumor development in HCC, recent investigations have highlighted the ability of M0 macrophages to inhibit T cell-mediated anti-tumor responses [52]. For instance, the miR-149-5p/MMP9 signaling pathway has been identified as a mechanism through which M2 macrophages facilitate HCC cell motility and invasion [53].

The metabolic shift towards glycolytic metabolism that leads to lactate accumulation and polarization of macrophages towards an M2-like phenotype is a defining characteristic of cancer development [54, 55]. This change may explain the differential enrichment of M0 and M2 macrophages observed in individuals with higher GMGs expression, as depicted in **Figure 4D**. Cancer therapy often involves harnessing the immune system to detect and eradicate cancer cells. Numerous immunotherapy approaches have been explored, including checkpoint inhibitors, adoptive cell transfer, and cancer vaccines [56-59]. M0 macrophages in the tumor microenvironment have been shown to inhibit T cell-mediated anti-tumor responses and to secrete tumor-promoting factors [52]. PD-1 and PD-L1 have close associations with macrophages [60]. Additionally, lactic acid can elevate PD-1 expression in Tregs within glycolytic tumor microenvironments [61]. Our study revealed higher PD-1 and PD-L1 mRNA expression levels in the high GMGs group than in the low GMGs group (**Figure 8C, D**), which could indicate the presence of Tregs and macrophages. This finding may account for the improved response to anti-PD-1 and anti-PD-L1 therapy observed in the high GMG group of HCC patients (**Figure 7**).

In order to gain a comprehensive understanding of the etiology of hepatocellular carcinoma (HCC), it is crucial to investigate not only the interactions between tumor cells and immune cells but also the dysregulation of signaling pathways within tumor cells, as these factors can profoundly impact the initiation and progression of HCC [35]. This study identified several pathways, including cytoplasmic translation, fibrinolysis, blood microparticles, and cytosolic ribosomes, that were significantly enriched in HCC patients with elevated gene module groups (GMGs) and may therefore affect their response to chemotherapy (**Figure 3C, E**). Based on the identification of 10 GMGs, we also identified nine potential therapeutic agents that could be effective for certain subtypes of HCC (**Figure 9**). To validate our predictions, we used etoposide as a test substance and a classification system based on the transcript levels of GMGs in HCC cell lines, differentiating between Huh7 cells with high GMG levels and HepG2 cells with low GMG levels. Our results revealed that Huh7 cells exhibited a lower IC50 after exposure to various doses of etoposide (**Figure 11B**). Moreover, Huh7 cells exposed to etoposide under the same therapeutic conditions and dosage exhibited greater cytotoxicity than HepG2 cells (**Figure 11C**). These findings not only demonstrate the effectiveness of our methodology but also lend strong support to the drug sensitivity predictions we made using GMGs.

In recent years, there has been a growing emphasis on investigating the potential association between glycolytic metabolism-related genes and tumor development. Emerging evidence has highlighted the pivotal role of glycolytic gene expression levels in shaping the tumor microenvironment, which in turn modulates the efficacy of chemotherapy and immunotherapy for HCC patients. Therefore, personalized treatment regimens tailored to the individual glycolytic profiles of patients are of paramount importance. Gene expression profiling has been demonstrated in numerous studies to be a valuable tool for accurate classification of tumor tissue [62-64].

Hepatocellular carcinoma (HCC) is a malignant tumor characterized by pronounced intratumoral heterogeneity, which is closely associated with tumor growth and therapeutic resistance, and has been linked to an increased risk of treatment failure and unfavorable prognosis. Cancer stem cells (CSCs) are a subset of cells within tumors that possess unique self-renewal and multipotency capabilities, and have been implicated in driving tumor heterogeneity, as well as contributing to treatment resistance and disease recurrence. Thus, the role of stemness in HCC heterogeneity was investigated by evaluating stemness levels in patients with varying risk scores. As illustrated in **Figure 9A**, the stemness score of HCC patients was significantly higher than that of normal liver tissue. Furthermore, high-risk HCC patients exhibited a markedly elevated stemness score compared to their low-risk counterparts, as indicated by the results presented in **Figure 9B**. Notably, our analysis revealed a positive correlation between risk score and stemness index in HCC patients, as shown in **Figure 9C**. These data highlight the potential of our 10-gene model as an accurate predictor of stemness index in HCC patients, and suggest its potential use in identifying new therapeutic targets for intervention in high-risk populations.

Our study aimed to examine the heterogeneity and stemness of hepatocellular carcinoma (HCC) patients and the corresponding changes in their microenvironment through patient stratification based on gene expression levels within the glycolysis pathway. Our findings demonstrated considerable differences in immune infiltration patterns and prognosis among HCC patients with distinct levels of glycolytic metabolic gene (GMG) expression. Remarkably, we discovered a strong association between the degree of dryness and the probability of survival in HCC patients. We developed a glycolysis-related model using ten genes, which displayed a high degree of accuracy in predicting patient outcomes. This model has significant implications as a prognostic tool for HCC. To reinforce the clinical relevance of our model, we conducted cell toxicity experiments to assess its capacity to predict chemotherapeutic sensitivity. Our results provide crucial information for physicians to make informed decisions regarding HCC treatment.

5. Conclusion

Tumor heterogeneity in hepatocellular carcinoma (HCC) is primarily attributed to stemness, which is, in turn, critically regulated by glycolysis. However, the clinical significance of glycolysis-related metabolic genes (GMGs) in HCC prognosis is still poorly understood. Thus, the objective of this investigation is to identify crucial GMGs and develop a robust prognostic model for HCC. By employing receiver operating characteristic (ROC) curves, we developed a 10-GMG-based risk model that exhibits high predictive accuracy, which was subsequently validated. Furthermore, two distinct GMG-related subtypes were identified, exhibiting significant differences in tumor stemness, immune landscape, and prognostic stratification, indicating a considerable degree of heterogeneity in HCC risk. Notably, these findings also suggest that patterns in immunotherapy and chemotherapy responses may be associated with HCC heterogeneity and stemness diversity among patients. In vitro validation confirmed the predictive value of our model for drug response. In summary, this study provides clinicians with a potential strategy for precision medicine targeting HCC heterogeneity by utilizing the 10-GMGs model.

6. Data Availability Statement

We utilized data from the TCGA database, available at http://cancergenome.nih.gov, in our study. To access the raw data used in our investigation, we utilized the Jianguoyun website, located at: https://www.jianguoyun.com/p/DWu7E70QovD_Chj87_YEIAA.

7. Author Contributions

Conceptualization, FZ; writing original draft preparation, SZ; visualization, SZ and YP; data resources, YP and LT; supervision, validation, and funding acquisition, FZ; review and editing, FZ. All authors reviewed and approved the final manuscript.

8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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