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Comprehensive Analysis Based on Machine Learning and Cell Differentiation Trajectory for Predicting Prognosis and Response of Immunotherapy in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is a highly maligant tumor of digestive system,patients often have a short survival time after diagnosis. Therefore, a novel prognostic model is urgently needed.

Methods: This study aims to examine the trajectory of HCC cell differentiation and its clinical implications. Additionally, we develop a prognostic signature for HCC differentiation, known as HDRG-ps, utilizing a comprehensive machine learning approach.

Results: HDRG-ps exhibits autonomous prognostic significance in terms of overall survival and consistently demonstrates robust and effective performance. Furthermore, HDRG-ps outperforms conventional clinicopathological features as well as 91 previously published signatures in terms of accuracy. Additionally, the high-risk group demonstrates sensitivity to Sorafenib, Sunitinib, Paclitaxel, and Crizotinib, whereas the low-risk group experiences greater benefits from Erlotinib. Notably, patients with high score display high expression of CTLA4 and PDCD1, which may allow them to benefit from immunocheckpoint inhibitors. Conclusions: HDRG-ps is a robust and reliable tool that could improve the prognosis for individual HCC patients.

Keywords: Hepatocellular Carcinoma; Machine learning; differentiation trajectory; scRNA-seq

Introduction

Globally, primary liver cancer ranks sixth in terms of morbidity among all cancer types, and it is the second most common tumor of the digestive tract. Furthermore, it is responsible for the fourth highest number of cancer-related deaths [1]. Among the various histological types of primary liver cancer, hepatocellular carcinoma (HCC) exhibits the highest incidence rate. It frequently emerges in individuals with a background of hepatitis B virus (HBV) infection, with many patients experiencing simultaneous HBV hepatitis and liver cirrhosis [2]. Despite advances in methods of diagnosis methods and treatment ways over the last decades, radical surgery operation is still the preferred therapy for patients with HCC. But even after successful surgery, patients with advanced HCC often have a low OS and PFS time in five years due to the elevated frequency of recurrence and metastasis [3-7].Currently, clinicopathological factors such as the TNM staging system and alpha- fetoprotein (AFP) are the few ways to predict the prognostication of HCC patients. But these factors are limited by low sensitivity and accuracy which constrain their clinical utility [8,9]. Hence, there is a compelling need for a novel clinical model that can more accurately predict the prognosis of patients with HCC to be established and used to outline personalized and individualized forms of treatment.

During tumorigenesis and progression, intratumoral heterogeneity frequently arises due to reprogramming at the genomic and epigenomic levels, as well as DNA replication errors. These alterations result in widespread abnormal cell division and differentiation [10]. Novel molecular phenotypes with high heterogeneity were also generated among these pathogenesis processes [11]. The identification of tumor marker genes and targeting therapy based on these genes provide a future that we can cure cancer completely; however, the existence of heterogeneity is a great challenge for cancer treatment. Fortunately, the development of scRNA-seq offers us an opportunity to depict genetic diversity at the cellular level [12]. Recently, a new bioinformatic algorithm named “Monocle 2” has been widely used in cancer cell classification and molecular separation. This process is referred to as cell differentiation trajectory analysis, and its close association with tumorigenesis and prognosis has been extensively demonstrated in various cancers [13-15].In our study, based on the HCC scRNA-Seq expression profile and TCGA-LIHC transcriptome, we filtrated the HCC cell differentiation-related genes (HDRGs) based on cell differentiation trajectory analysis and constructed a predictive signature through machine learning. Significantly, the model exhibited a dependable predictive capability and has the potential to impact the choice of immunotherapies for patients with HCC.

Materials and Methods

Obtaining and Preparing Single-Cell Data

The single-cell sequencing data were sourced from GEO, under the registration number GSE149614. In the R environment, the "Seurat" package was applied to pre-process the single-cell sequencing data. Cell annotation is consistent with that of Gangqiao Zhou's team [16]. Standard single-cell processing procedures were implemented in the R (version 4.1.2) environment to ensure quality control. Specifically, genes expressed in fewer than three cells were eliminated, and cells expressing fewer than 200 genes were excluded from the analysis. To conduct principal component analysis (PCA), we selected the top 2000 genes that exhibited high variability. Subsequently, we utilized the 30 most significant principal components (PCs) for cluster analysis. In order to eliminate batch effects among the samples, the "Harmony" method was applied. For downsizing the cells, the TSNA method was employed. Cell clustering was accomplished using the "FindClusters" function, employing a resolution of 0.5.

Pseudo Time Analysis

For the pseudo-time analysis, we utilized version 2.24.1 of the "monocle" package, first creating the object for the proposed pseudo time analysis using the newCellDataSet function, and then selecting the high variant genes using the detectGenes function. Finally, cell differentiation trajectories were inferred using the high variant genes.

To identify genes specific to each differentiation state, we utilized the "FindAllMarkers" function from the "seurat" package. Genes were considered differentially expressed if their |logFC| was greater than 0.585 and their adjusted P value was below 0.05.

Bulk Transcriptome Data Download and Processing

Download transcriptome information of 50 normal liver tissues and 374 HCC tissues from TCGA (https://portal.gdc.cancer.gov/). Also, download the clinical information for the appropriate sample. Transcriptomic data of 82 liver cancer samples with clear survival time records were sourced from the GSE54236 database in GEO.

Machine Learning Techniques were Employed to Construct Prognostic Signatures

We employed a diverse set of 10 machine learning algorithms for our analysis, including Lasso, Ridge, stepwise Cox, CoxBoost, random survival forest (RSF), generalized boosted regression (GBM), supervised principal components (SuperPC), partial least squares regression for Cox (plsRcox), elastic network (Enet), and survival support vector machine (survival-SVM). In our methodology, we employed one algorithm to filter the variables and another algorithm to construct the prognostic signature. If the resulting prognostic signature comprised less than 5 genes, it was deemed invalid. Throughout the process, we integrated a total of 99 combinations of machine learning algorithms. Subsequently, Harrell's concordance index (C-index) was calculated for each signature, and the optimal signature was determined based on the highest average C-index value.

Enrichment Analysis

We subjected the DEGs between the two groups to GO and KEGG analysis using the R package "clusterProfiler." To visualize the enrichment terms, we utilized the R packages "ggplot2" and "GOplot" in various plots.

Tumor Microenvironment Analysis and Immune Cell Infiltration Analysis

In order to assess the tumor mutation burden (TMB) within each risk group, we utilized the R package "maftools." To determine the levels of infiltrating immunocytes and activation levels of various immune-related pathways, we employed the R package "gsva" and utilized the ssGSEA method. Moreover, we compared the expression levels of multiple immunotherapy target genes across different risk groups using the TCGA-LIHC dataset.

Statistical Analysis

All statistical analyses were performed utilizing R software (version 4.1.1) and Strawberry Perl software. A significance level of $P<0.05$ was employed to ascertain statistical significance.

Results

Identification of HCC Differentiation-Related Genes Through scRNA-Seq Data

In our study, the single-cell sequencing data from 10 HCC samples were extracted from GSE149614. After a pre-process of quality control and normalization and cell annotation, 14781 cancer cells remained for subsequent analysis(Figure 1A-D). Figure 1E revealed that the sequencing depth and mitochondrial gene sequences were unrelated, whereas Figure 1F demonstrated a significant positive correlation between the sequencing depth and the number of detected genes. Out of the total 16,288 genes analyzed, 2000 exhibited significant variability.

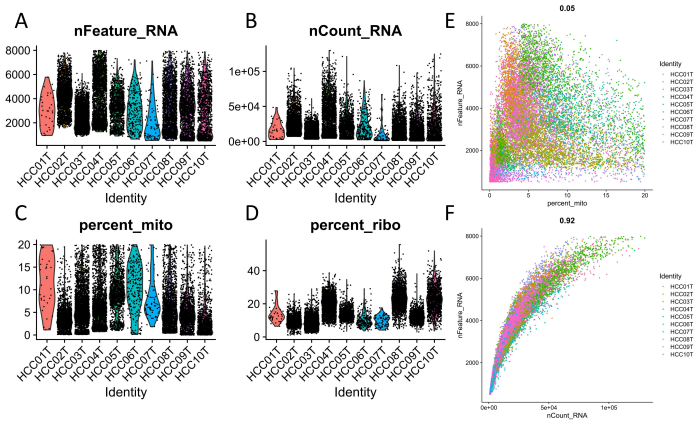


Figure 1

Applying the tSNE algorithm, 14781 HCC cells were categorized into 10 distinct clusters, despite their origin from different tumor tissues. This finding emphasized the high heterogeneity of HCC as a tumor (Figure 2A). Then five different subsets were identified from cancer cells by the Pseudotime and trajectory analysis(Figure2 C-D). We subsequently performed differential analysis to further explore HCC differentiation-related genes (HDRGs). As a result, there were 451 HDRGs in subset I, 302 HDRGs in subset II, 727 HDRGs in subset III, 508 HDRGs in subset IV and 586 HDRGs in subset V, a total of 1211 HDRGs were ultimately identified in HCC. SPP1 and GLUL were highly expressed in subset I, which were positively correlated with multiple metabolic pathways. Subset II had high expression levels of IGHG4,IGLC2,IGKC and IGHG1, all of which were involved in the synthesis of immunoglobulins. AGR2 and CCL26 were highly expressed in subset III, which were positive correlated with epithelial cell differentiation and endothelial cell proliferation. Because of the high expression levels of NTS and NQO1, HCC cells in subset IV had functional changes in blood circulation and metabolism. In subset V, the expression of APOH and APOA1 were extremely active, indicated a non-dynamic status of immune response in cancer cells.The results suggested that HCC cancer cells may first undergo metabolic changes, then gradually developed into a state of low immune response(Figure 2B).

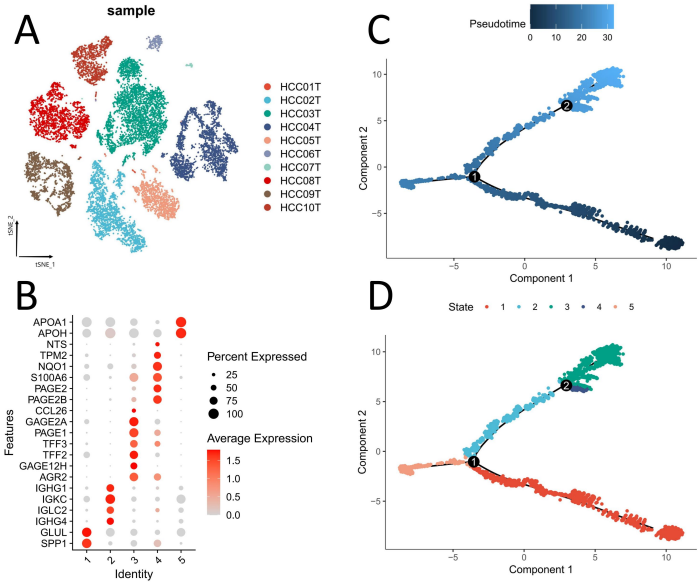


Figure 2

Establishment and Validation of the Novel HDRGs Predictive Signature

Based on the expression profiles of 1211 HDRGs, we performed univariate Cox analysis to identify prognostic HDRGs in TCGA-LIHC, GSE54236 and the meta-cohort. A total of 81 overlapping prognostic HDRGs were extracted for further machine learning-based integrative procedure,included 46 protect-genes and 35 risk-genes(Figure 3).

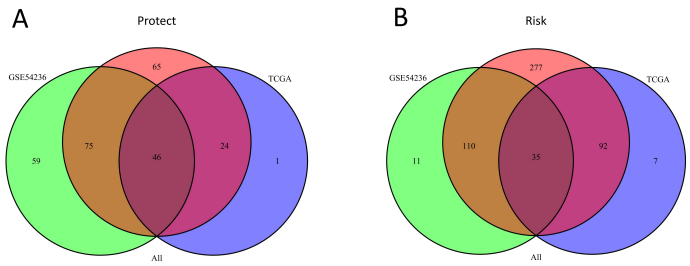


Figure 3

In the TCGA-LIHC dataset, we integrated a total of 99 combinations of machine learning algorithms and further calculated the C-index of each model across GSE54236 and the meta-cohort as validation datasets(Figure 4A). Remarkably, the most effective model was a combination of stepwise Cox (direction = both) and Efficient neural network (Enet, alpha=0.7), boasting the highest average C-index (0.734). Notably, this combination model exhibited the highest C-index across all validation datasets. A final set of 28 HDRGs were identified and involved in a risk scoring (RS) signature(Supplementary table1).

	coef	exp(coef)	se(coef)	z	Pr(>|z|)	
ADI1	-0.17547	0.83907	0.12227	-1.435	0.151271	
ALDH2	0.30664	1.35885	0.14362	2.135	0.032759	*
APOC3	0.41752	1.51819	0.18246	2.288	0.022123	*
CLIC1	-0.30375	0.73805	0.1306	-2.326	0.020034	*
CYP2C9	-0.29037	0.74798	0.11582	-2.507	0.012174	*
CYP3A4	0.24757	1.28091	0.10964	2.258	0.02395	*
CYP4A11	0.34314	1.40937	0.12851	2.67	0.007581	**
F12	0.29021	1.3367	0.15671	1.852	0.064049	.
HMGCS2	-0.21587	0.80584	0.14159	-1.525	0.127355	
KNG1	0.34379	1.41029	0.19346	1.777	0.075554	.
LEAP2	-0.14839	0.86209	0.10769	-1.378	0.168238	
LSM4	-0.27884	0.75666	0.12351	-2.258	0.023964	*
MAGEA4	0.21061	1.23443	0.07109	2.963	0.00305	**
PABPC1	0.19946	1.22074	0.1124	1.774	0.075984	.
PAH	-0.37278	0.68882	0.14637	-2.547	0.010869	*
PLG	0.24958	1.28349	0.17558	1.421	0.155179	
PON1	-0.24335	0.784	0.10806	-2.252	0.024318	*
PPIA	0.21028	1.23402	0.12772	1.646	0.099667	.
RAN	0.15299	1.16531	0.13574	1.127	0.259714	
S100A10	0.24719	1.28042	0.09211	2.684	0.007284	**
SLC2A2	-0.5073	0.60212	0.13326	-3.807	0.000141	***
SNRPB	-0.24875	0.77978	0.15415	-1.614	0.106596	
SPINK4	0.26322	1.30112	0.06985	3.768	0.000164	***

SPP2	-0.32324	0.7238	0.11778	-2.744	0.006061	**
TKT	0.35073	1.4201	0.12487	2.809	0.004972	**
TUBA1C	0.20245	1.22439	0.1289	1.571	0.116289	
UBE2C	0.38277	1.46634	0.13926	2.749	0.005984	**
UGT2B15	-0.27107	0.76256	0.10102	-2.683	0.007286	**

Supplementary Table 1: The coef of 28 HDRGs which were involved in the HDRG-ps.

Subsequently, the risk score for each patient, along with their corresponding survival data, was computed, leading to the classification of patients into high- and low-risk groups using the R package "surviminer." As depicted in Figure 4 B-D, patients in the low-risk group exhibited notably improved OS compared to the high-risk group in both the TCGA-LIHC training cohort and the other two validation cohorts (all $P < 0.05$). The predictive accuracy of our signature was evaluated using ROC analysis, yielding 1-, 3-, and 5-year AUCs of 0.806, 0.716, and 0.715, respectively, in TCGA-LIHC. In GSE542336 (which lacked five-year survival data), the AUCs were 0.803 for 1-year and 0.845 for 3-year survival. The meta-cohort analysis resulted in AUCs of 0.803 for 1-year, 0.703 for 3-year, and 0.64 for 5-year survival (Figure 4E-G).

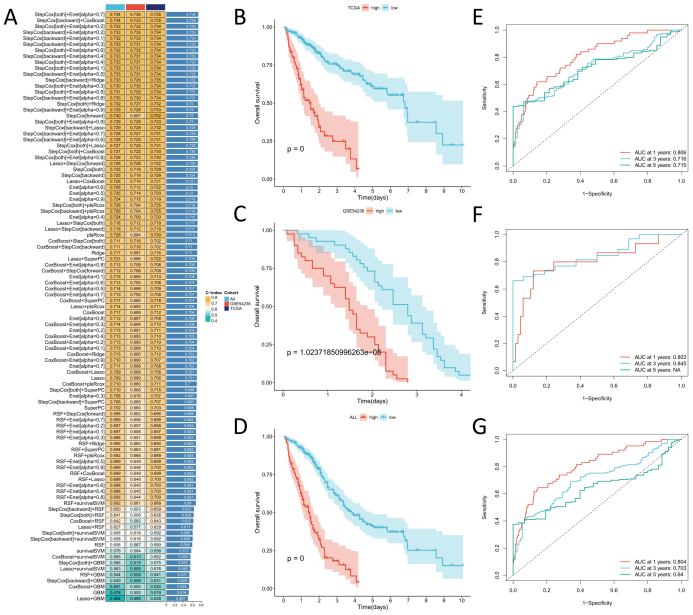


Figure 4

Then we made a raise of published signatures to compare the accuracy with HDRG-ps. Incorporated into the study were a total of 91 signatures, which encompassed diverse biological processes, including autophagy, ferroptosis, immune response, hypoxia, and M6-methyladenosine. As expected, the C-index of HDRG-ps was higher than almost all signatures in every dataset (Figure 5).

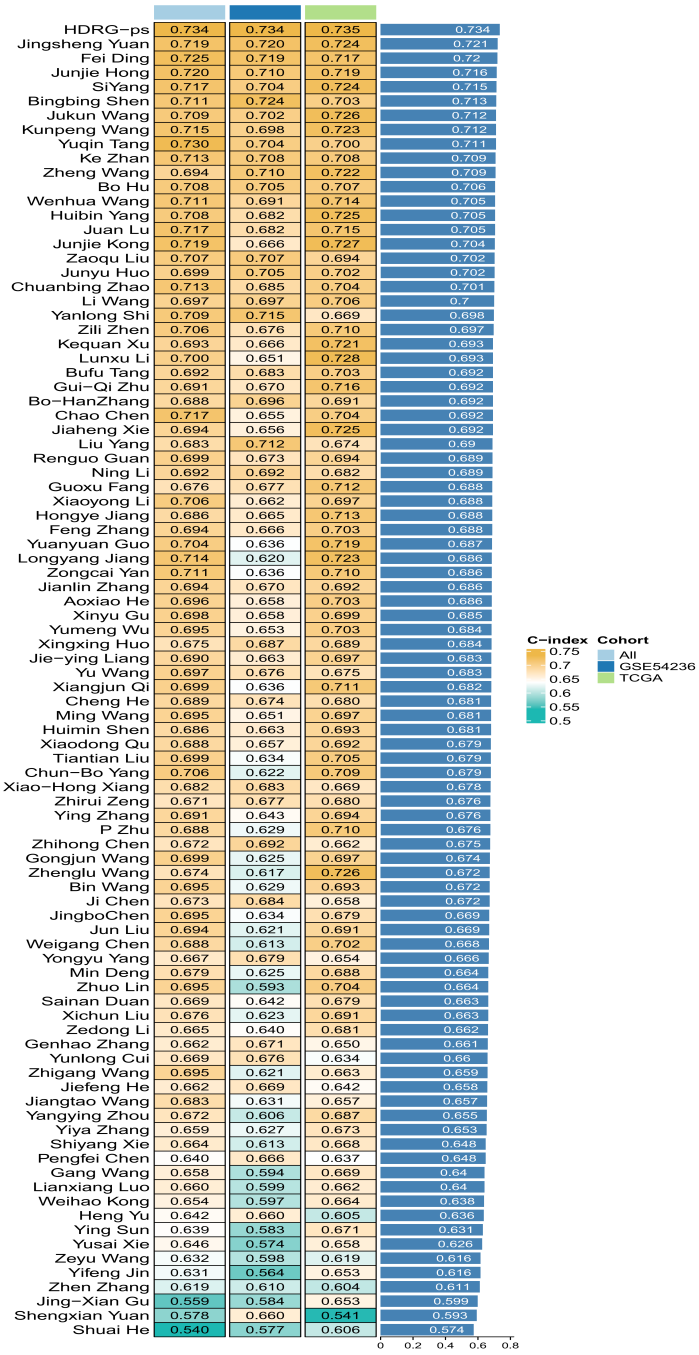


Figure 5

Then, to verify if the HDRG-ps is an independent predictive factor for OS in patients with HCC compared with other clinicopathological factors, Cox regression analysis was performed .The result showed that risk score and tumor TNM stage were negatively correlated with the survival duration (Figure 6A).However, after the multivariate Cox analysis, the risk score was the only survival predictive factor, with an HRs (95% CI) of 3.346(2.400−4.667)(Figure6 B). The DCA indicated a better net benefit of decision based on HDRGs signature compared to clinical predictive factors (Figure 6C).

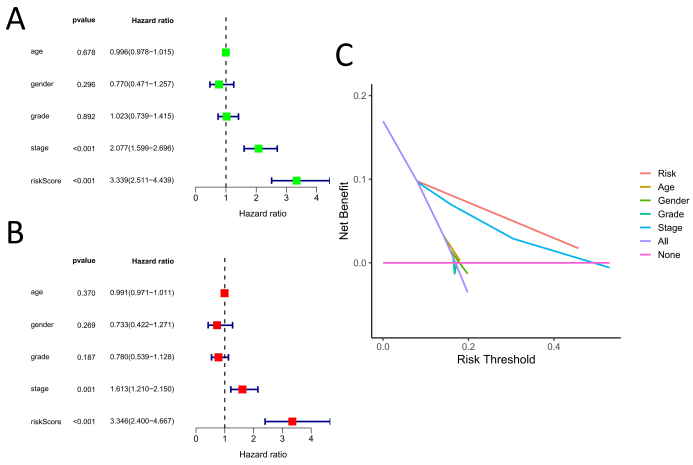


Figure 6

Functional Enrichment in GO and KEGG

Differential analysis between the two risk groups was applied. Low-risk group had a total of 110 up-related genes and 903 down-related genes relative to the high-risk group. To better understand the difference between these two risk groups at the molecular and cellular functional levels, GO and KEGG functional enrichment analyses were applied and the results were shown in Figure7. The top 3 enriched biological process by GO analysis were nuclear division, organelle fission and chromosome segregation. In the cellular component those were chromosomal region, spindle and apical part of cell. Signaling receptor activator activity and receptor ligand activity were most enriched in molecular function(Figure 7A-B). Then we could see that in the KEGG pathway enrichment analysis, these genes were bioenriched in PI3K-Akt-signaling pathway, Proteoglycans in cancer, HIF−1-signaling pathway and Retinol metabolism(Figure 7C-D).

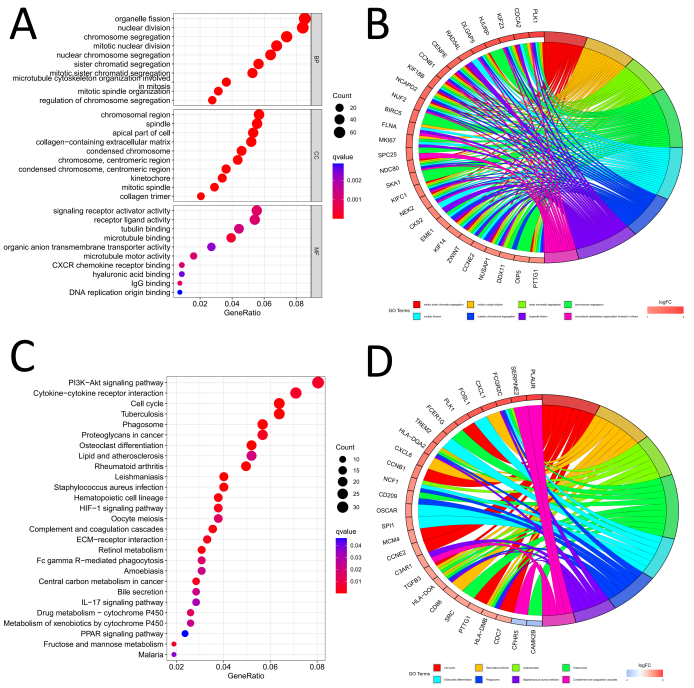
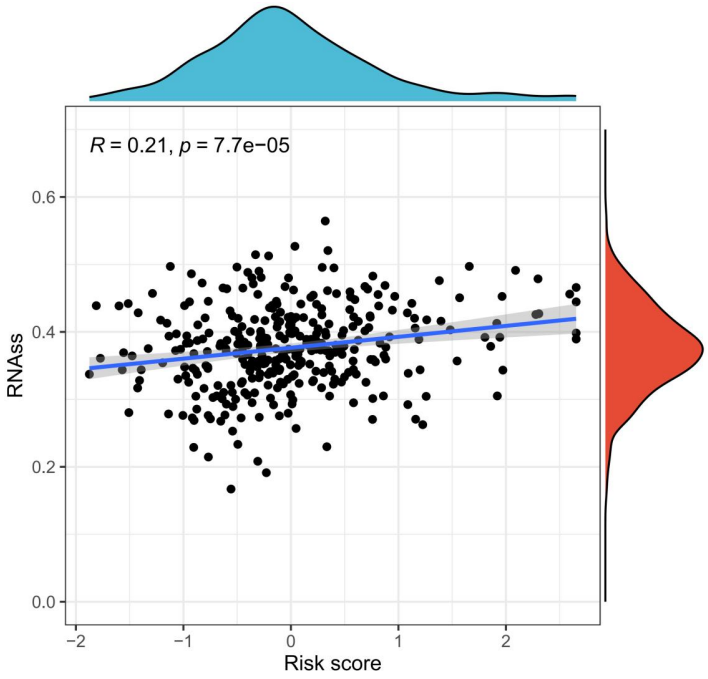


Figure 7

Most of these pathways were related to cellular proliferation and cellular apoptosis, which is consistent with the differentiation characteristics of tumor cells in cancer progression : poorly differentiated tumor cells always have a high velocity of propagation and apoptosis resistance. Additionally, we observed a positive correlation between the risk score and the RNA stem cell score (RNAss), confirming our previous finding (Figure S1).



Supplementary Figure 1

Comprehensive Analysis of Immune Landscape by the HDRGs Predictive Signature

The R package "maftools" was performed to calculate the TMB value for each risk group. The results showed that gene mutations were more likely to occur in high-risk patients, of which TP53 mutation was the most common, about 41% of the patients had this mutation (Figure 8A). In the low-risk group, the overall gene mutation rate is much lower, for example, the TP53 gene mutation rate is 19% lower than that in the high-risk group(Figure 8B).

Immune checkpoint stoppage therapy has been proven to be effective for many types of cancer and the key point of these therapies is to curb high-expressed immune checkpoint inhibitor genes(ICIs). So,we further investigate the correlation between the expression levels of 48 ICIs and two risk groups identified by HDRGs. In conclusion,we found 30 differently expressed ICI genes including CTLA4 and PDCD1 and except for IDO2 , other ICIs were all highly expressed in the high-risk group and may lead to tumor cell immune escape (Figure 8C). Differences between immune-related pathways and immune cells were further investigated in ssGSEA . Figures 8D and 8E demonstrate notable distinctions between the high-risk and low-risk groups. Specifically, in the high--risk group, scores for Macrophages M0, Dendritic cells resting, Neutrophils, APC costimulation, and MHC_I were significantly elevated, while scores for Plasma cells, Mast cells resting, Type I and II IFN Response were significantly reduced compared to the low-risk group.

We then implemented immune infiltration estimations by TIMER, CIBERSORT, and other five different algorithms with the Timer 2.0 website (http://timer.comp-genomics.org/) and obtained a heatmap showing the results of all algorithms. According to CIBERSORT algorithm, in HCC patients, the proportions of T cell CD4+, T cell regulatory (Tregs) and Neutrophil granulocyte were positively related to the risk score(Figure 8F). The risk score was not only a predictive factor of survival time but also can reflect the tumor immune microenvironment characterized by different infiltration levels of immune cells which may help guide patients' immunotherapy directions.

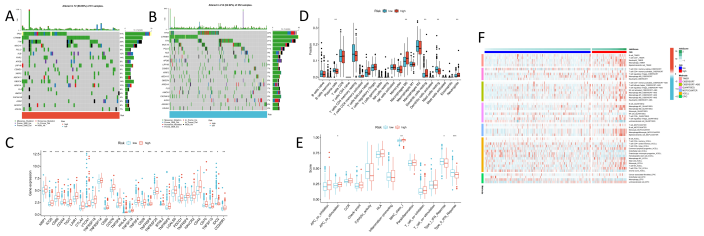


Figure 8

Prediction of Drug Sensitivity by the Risk Signature

In order to screen for potential drugs targeting the HDRGs model to treat HCC patients, we used the R package "pRRophetic" to evaluate the IC50 based on the GDSC database. Results showed that Sorafenib, Sunitinib, Paclitaxel, Crizotinib and AZ628 had good effects on high-risk patients (Figure 9A-E). Sorafenib has been widely used in liver cancer; while Sunitinib, Paclitaxel and Crizotinib were mainly used in other cancers; however, AZ628 is only used in scientific research for now. Patients with low risk score were more susceptible to Erlotinib, which was one of the Growth Factor Receptor (EGFR) inhibitors and recommended as a third-line anti-tumor drug for lung cancer. (Figure 9F)

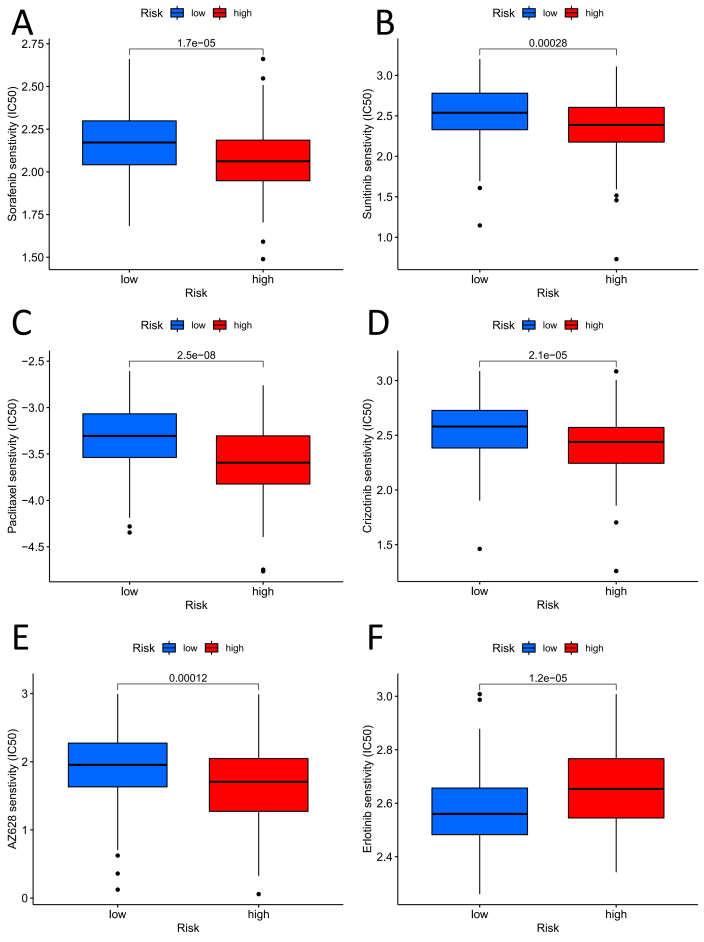


Figure 9

Discussion

HCC has been characterized by high intratumoral heterogeneity in previous studies [17-20]. At the same time, malignant tumors with various molecular types always lead to different prognoses. Previous studies have identified that the genetic landscape undergoes specific changes during the progress of HCC. TERT promoter mutations can be found in preneoplastic lesions and are often positively correlated with good differentiation and low aggressiveness. Then, as the increase of TP53 and CTNNB1 mutations, tumor tissues are gradually characterized by chromosomal instability, poor histological differentiation and high aggressiveness [21-24]. However, we still lack a reliable molecular typing method in clinical practice [25].

In this study, we delved deeper into HCC heterogeneity by investigating the cell differentiation trajectory of HCC cells. Based on the scRNA-seq data, we identified five subsets exhibiting distinct differentiation characteristics. Subset I genes showed a positive correlation with multiple metabolic disorders, while subset II genes were associated with the synthesis of immunoglobulins, those genes of subset III were positively correlated with epithelial cell differentiation and endothelial cell proliferation, those of subset IV were involved in blood circulation and metabolism and those of subset V were closely related to a non-dynamic status of immune response in cancer cells. Subsequently, we utilized machine learning techniques to construct a prognostic RS signature based on HDRG, which was subsequently validated in two independent datasets. Compared with other clinicopathological factors, the HDRG-related RS signature had a better prediction ability of the OS in HCC patients. Hence, these findings confirmed the strong correlation between cell differentiation disruption and intratumoral heterogeneity in HCC at the genetic level, suggesting a potentially significant role of cell differentiation disorder in the development and progression of HCC.

Consequently, the high-risk group exhibited a significant association with abnormal activation of the PI3K-Akt signaling pathway, which is known to be involved in HCC progression and sorafenib resistance. However, apatinib demonstrated the capability to inhibit this pathway [26-27]. Proteoglycans (PG) are one of the fundamental compositions of the extracellular matrix (ECM) and PG expression is often significantly modified in the tumor microenvironment [28]. In HCC, previous studies have found that loss of Syndecas (a cell surface proteoglycans family) expression is correlated with poorly differentiated HCC and higher metastatic potential [29]. HIF−1 induce hypoxic HCC cells scavenging ECM which could induce distant metastasis [30-31]. Low serum retinol levels have been linked to liver cirrhosis, non-alcoholic fatty liver disease, and an elevated risk of HCC [32-34]. Patients with downregulation of retinol binding protein 5 tend to have poor prognoses because of the low histological differentiation and high aggressiveness of tumor cells [35]. Mitochondria is one of the most important organelles. Srinivasan et al. have revealed that mtDNA depletion could induce higher levels of mitochondrial fission and promote Tumorigenesis [36].Huang et al. found that increased mitochondrial fission could inhibit the apoptosis of HCC cells and promote HCC cell survival [37]. m6A modifications are very common in HCC cells because of chromosomal instability. Rong et al. have demonstrated that the m6A modification of circHPS5 could regulate cytoplasmic output and HMGA2 expression level to accelerate HCC progression [38].

Tumor mutation burden (TMB) often reflects the instability of tumor cytogenetics. A number of previous researches have pointed out that TMB is associated with prognosis and reactiveness to immunotherapy [39-41].As expected, in our study, TMB was significantly elevated in the high-risk group.

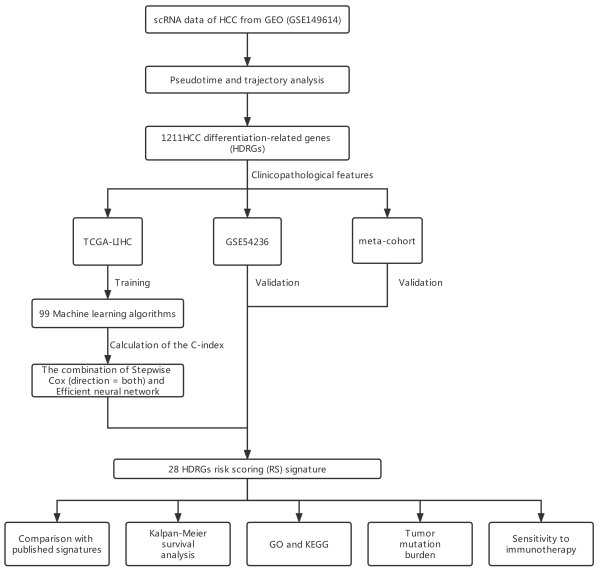
Furthermore, this present study possesses several limitations. First, the HDRG-ps was developed using data from public databases (TCGA and GEO), and further external validation and prospective clinical studies are needed. Second, many potential prognostic factors, such as drinking, radiotherapy and family history were not contained in our study because of the limitation of TCGA database. Therefore, the HDRG-based RS signature should be further improved.

Conclusion

In summary, we constructed a reliable prognostic risk model through Machine learning and Cell differentiation trajectory, which performed a good predictive value in prognosis and immunotherapy response in HCC patients. Moreover, we also revealed potential targets that are waiting for further relevant experimental verification.

Graphical Abstract

HCC differentiation-related prognostic signature (HDRG-ps) provides a promising prospect as a prognostic indicator. Comparison with other HCC prognostic models highlights the superiority of our model. We found that the two risk groups showed a marked difference in immune status and may be beneficial for the precise treatment of patients.



Graphical Figure 1

Data Availability Statement

The datasets utilized in this study are available in online repositories. The article or Supplementary Material contains the names of the specific repository or repositories, along with the corresponding accession number(s).

Author Contribution

All authors were involved in the conception and design of the study. Sihao Du and Ke Cao conducted the material preparation, data collection, and analysis. The initial draft of the manuscript was written by Sihao Du and Ke Cao. Yadong Yan, Yupeng Wang, Zhenshun Wang, and Dongdong Lin provided critical feedback and made revisions to the manuscript. All authors carefully reviewed and approved the final version of the manuscript.

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Conflict of Interest

This study has no conflict of interest.

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