Comprehensive analysis of prognostic immune-related genes in the tumor microenvironment of hepatocellular carcinoma (HCC)

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Abstract

Growing evidence supports that the tumor microenvironment plays a key role in the development and progression of tumors. But immune microenvironment of hepatocellular carcinoma (HCC) has not yet been fully explored. In the present investigation, the clinical value and prognostic significance of immune-related genes in HCC were investigated.

The immune and stromal scores of HCC were calculated through the application of Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data Algorithm based on the Cancer Genome Atlas database. Differentially expressed genes were identified using the "edgeR" package of the R software. Functional annotation and pathway enrichment were performed using "ggplots2" and "clusterProfiler" packages in R software. Protein-protein interaction network was constructed using STRING, and the hub genes were identified through the Cytoscape. Survival analysis was performed using Kaplan-Meier methods. Tumor Immune Estimation Resource algorithm was used to view the immune landscape of the microenvironment in HCC.

Firstly, the immune and stromal scores of HCC were calculated and we found that the immune and stromal scores of HCC were closely related to the patients' prognosis. Then the differentially expressed genes were identified respectively stratified by the median value of the immune and stromal scores, and the immune-related genes that related to the prognosis in HCC patients were further identified. Functional enrichment analysis and protein-protein interaction networks further showed that these genes mainly participated in immune-related biological process. In addition, dendritic cells were found to be the most abundant in the microenvironment of HCC through Tumor Immune Estimation Resource algorithm and were significantly associated with the patients' prognosis. To robust the results, the immune-related genes were validated in an independent dataset from the Gene Expression Omnibus database.

We arrived at a more comprehensive understanding of the microenvironment of HCC and extracted 7 immune-related genes that were significantly associated with the recurrence survival of HCC.

Abbreviations: BP = biological process, CC = cellular component, DCs = dendritic cells, DEGs = differentially expressed genes, ECM = extracellular matrix, ESTIMATE = Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data, FCN3 = ficolin-3, GEO = Gene Expression Omnibus, GO = Gene Ontology, HCC = hepatocellular carcinoma, KEGG = Kyoto Encyclopedia of Genes and Genomes, PPI = protein-protein interaction, RFS = recurrence-free survival, TCGA = the Cancer Genome Atlas, TIMER = Tumor Immune Estimation Resource.

Keywords: GEO, hepatocellular carcinoma, immune scores, recurrence, TCGA, tumor microenvironment

1. Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths and consistently ranks among the most aggressive cancers worldwide.[1] Approximately 80% of HCC cases were reported in Asian countries per year, while China alone accounted for 55% of HCC cases in the world.[2] Although successful partial hepatectomy was applied to improve the survival of patients with HCC recent years, the 5-year survival

rate of patients with HCC remains poor due to tumor invasiveness, frequent intra-hepatic spread, and extra-hepatic metastasis.[3,4] These observations demonstrate that liver cancer is a major healthcare problem worldwide, which highlights the critical need for developing novel treatment options for this deadly disease.

The tumor microenvironment, largely constituted by inflammatory cells, is an indispensable participant in the development of tumor, facilitating proliferation, survival, and migration of tumors.[5] Since human HCC is driven by chronic liver inflammation caused by activation of many types of inflammatory cells due to chronic hepatitis, liver cirrhosis, and fatty liver disease, HCCs can be considered the best example for the inflammation-induced cancers.[6] In the last 10 years, because of the pronounced phenotypic and obvious molecular heterogeneity, as well as the high toxicity of the active compounds under clinical evaluation, the development of drugs for patients with advanced liver cancer was greatly restricted.[7] Although, sorafenib, lenvatinib, regorafenib, and cabozantinib have been successfully applied to prolong the survival of HCC patients by a few months, these drugs are costly and are accompanied with significant side effects. Most HCCs evolve in the background of a chronic inflammatory liver damage, so that restoration of the chronically altered hepatic microenvironment becomes particularly important, new approaches considering the cellular and molecular changes/composition involved in oncogenic inflammation are urgently needed for the treatment of HCC.

The tumor microenvironment consists of multiple of immune cells, endothelial cells, mesenchymal cells, along with inflammatory mediators and extracellular matrix (ECM) molecules.[8,9] In the tumor microenvironment, immune and stromal cells are the 2 most important non-tumor components. Many algorithms have been developed to predict tumor purity in various cancers based on the specific gene expression signature from the Cancer Genome Atlas (TCGA) database.[10,11] For instance, Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE) have been widely applied to analyze the tumor microenvironmental in various tumors, such as glioblastoma,[12] cutaneous melanoma,[13] and colon cancer.[14] In this algorithm, through the analysis of specific gene expression signature of immune and stromal cells, immune and stromal scores were calculated to predict the infiltration of non-tumor cells. However, the immune and/or stromal scores of HCC has not been investigated in detail.

In the present study, based on TCGA database, the immune and stromal scores of HCC were calculated through the application of ESTIMATE algorithm. Importantly, we extracted a list of immune-related genes which were closely associated with outcome of HCC.

2. Material and methods

2.1. Gene expression datasets

Level 3 gene expression profile (level 3 data) of melanoma patients were downloaded from the TCGA data portal (https://portal.gdc.cancer.gov/). Clinical characteristics of HCC patients were also downloaded from TCGA data portal, including gender, age, histological grade, T stage, clinical stage, survival time, and survival status. ESTIMATE algorithm was applied to the gene expression matrix to calculate the immune and stromal scores of HCC tissues.[15] For validation, GSE10141 on mRNA expression

of the HCC patients with complete clinical information were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10141), which consists of 85 tumor samples. And the gene expression data were determined using the Illumina HumanHT-12 V4.0 microarray platforms.

2.2. Differential analysis of expressed genes

The differentially expressed genes (DEGs) between high/low immune-score groups and high/low stromal-score groups were identified through the package of "edgeR" in R language.[16] $|\log2FC| > 2$ and adj. $P < .05$ were set as the cutoffs to identify significantly DEGs. Heatmaps were generated using the pheatmap package in R software.

2.3. Kaplan-Meier survival analysis

A univariate Cox model was used to investigate the relationship between patients' recurrence-free survival (RFS) and immune-related genes. Survival analysis was performed using Kaplan-Meier methods. Two-sided log-rank tests were employed to determine the survival differences between high/low immune-score groups and high/low stromal-score groups using the "survival" package in R.

2.4. Functional enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to explore the potential function of immune-related genes using "ggplots2" and "clusterProfiler" packages in R software. $P < .05$ and enrichment > 2 were used as the cutoff values.

2.5. Hub gene identification

In addition, all immune-related genes were uploaded to the STRING database (http://string-db.org/) to construct the protein-protein interaction network (PPI),[17] which was reconstructed and further analyzed via Cytoscape v3.5.1 software.[18] Individual networks with more than 20 nodes were included for further analysis. Significant modules in the PPI network were screened using Molecular Complex Detection plug-in (degree cutoff = 2, max. depth = 100, k-core = 2, and node score cutoff = 0.2).

2.6. TME analysis

Tumor Immune Estimation Resource (TIMER) algorithm was further applied to measure the relationship between the expression of the recurrence-related genes and the abundance of 6 types of infiltrating immune cells (B cells, CD4+ T cells, CD8 + T cells, neutrophils, macrophages, and dendritic cells [DCs]).[19] In order to investigate the possible associations between distinct patterns of immune infiltration and prognosis of HCC patients, we divided those patients into k clusters using the k-means algorithm, and survival analysis was applied to explore the associations between clusters and RFS.

2.7. Statistical analysis

R was used for data collation and analysis. Data were expressed as mean ± SD. Comparisons among different groups were performed by one-way ANOVA using GraphPad Prism 6. P value < .05 was considered statistically significant.

3. Results

3.1. Immune scores and stromal scores are significantly associated with clinical features of HCC

In our study, 269 HCC patients with complete gene expression data and clinical information in TCGA database were included in our analysis. Among them, there are 81 females and 188 males. According to the ESTIMATE algorithm, immune scores were distributed between −866.31 and 3146.06, and stromal scores ranged from −1625.38 to 1171.12, respectively. As Figure 1A shows, the distribution of immune scores did not vary across different clinical stage of HCC, and so did the stromal scores, as is shown in Figure 1B ($P = .191$, $P = .145$). Figure 1C showed that the immune scores were not significantly associated with the histological grade of HCC patients ($P = .916$). While in Figure 1D, the distribution of stromal scores differed in HCC with different levels of histological grade ($P = .012$). The correlations of other clinical information with immune or stromal scores were displayed in Figure S1, Supplemental Digital Content, http://links.lww.com/MD/G410 in detail.

To explore the effects of immune and stromal scores on patients' survival, we divided those patients into the high/low group based on their median values, respectively. As is shown in Figure 1E, the RFS time of patients with lower immune scores was longer than those with higher immune scores. Consistently, we found that stromal scores were significantly positively associated with RFS, patients with high stromal scores had poorer RFS than those with low scores (Fig. 1E). These data suggested that immune and stromal cells in the tumor microenvironment are positive factors for the patients' prognosis.

3.2. Comparison of gene expression profile with immune and stromal scores in HCC

In order to find the prognostic related genes in the tumor microenvironment of HCC, we firstly analyzed the DEGs between high/low immune-score groups and high/low stromal-score groups. In the high-immune score group, 1338 genes were upregulated and 84 genes were downregulated (Fig. 2A). Similarly, in the high-stromal score group, there were 1041 upregulated genes and 81 downregulated genes (Fig. 2B). In addition, Venn diagrams (Fig. 2C, D) showed that 802 genes were commonly upregulated in both high-stromal and high-immune score groups, and 28 genes were commonly downregulated.

Moreover, to further explore the potential function of these DEGs, functional enrichment analysis was applied to the intersection genes (802 upregulated genes and 28 downregulated genes). Top GO terms identified included regulation of leukocyte activation, T cell activation, regulation of lymphocyte (biological process [BP]), ECM, side of membrane, external side of plasma membrane (cellular component [CC]), ECM structural constituent, glycosaminoglycan binding, cytokine receptor activity (molecular function) (Fig. 2E). The result of KEGG analysis showed that these gene were enriched in hematopoietic cell lineage, cytokine-cytokine receptor interaction, and viral protein interaction with cytokine receptor (Fig. 2F). Based on the above results, we found that these genes were positively involved in tumor microenvironment, and were identified as immune related genes.

3.3. Correlation between the expression of immune-related genes and the recurrence-free survival

A univariate Cox model was applied to explore the potential relationship between patients' RFS and immune-related genes. Among the 830 intersection genes, a total of 169 genes were shown to be significantly correlated with the RFS of HCC patients. We just displayed 7 potential genes in Figure 3, which were also confirmed by another independent dataset from GEO database.

3.4. Protein-protein interactions among immune-related genes

By using the STRING database, the PPI network of 830 intersection genes (802 upregulated genes and 28 downregulated genes) was established and consisted of 435 nodes and 123,682 edges. We arranged these genes in the order of degree. A gene with a higher degree is often considered more important in the network (Figure S2, Supplemental Digital Content, http://links.lww.com/MD/G411). With the tool of Cytoscape, we performed a series of deeper analyses on this gene network. Finally, the 3 most significant modules, which contained as least 20 nodes were extracted from PPI network by Molecular Complex Detection for further study. In module A (Fig. 4A), which contained 44 nodes and 1892 edges, PTGER3, CLC19, C5AR1, CCR2, and GPR18 were remarkable for having most connections with other members of the modules (Fig. 4A). In module B with 37 nodes and 1332 edges, GPR84, CD177, CLEC5A, CYBB, and LAIR1 had higher connectivity degree values (Fig. 4B). Module C consisted of 29 nodes and 812 edges (Fig. 4C) was occupied with several immune response critical genes in the center, including COL3A1, COL1A2, COL16A1, COL14A1, COL10A1, and so on.

3.5. Functional enrichment analysis of immune-related genes

The prognostic genes we got from the TCGA database were further analyzed using GO and KEGG methods and the results were shown in Figure 5. With respect to CC, these genes were significantly enriched in T cell activation, regulation of T cell activation, leukocyte cell-cell adhesion (Fig. 5A); For BP, enrichments were focused on ECM, collagen trimer, external side of plasma membrane (Fig. 5B). Additionally, molecular function enrichment indicated that these genes were involved in some BPs such as ECM structural constituent, cytokine receptor activity, integrin binding (Fig. 5C). The results of KEGG pathways suggested that cytokine-cytokine receptor interaction, hematopoietic cell lineage, Th17 cell differentiation may be activated in the tumor microenvironment (Fig. 5D). The above analyses of these genes indicated that immune response and inflammation related pathways play an important role in HCC.

3.6. Validation in the GEO database

To verify the prognostic genes, we downloaded another independent HCC dataset from GEO dataset. The results showed that only 7 genes out of a total of 169 genes were validated to have association with the recurrence survival of HCC patients. Patients with higher expression of ACKR1, CD4, CD6, FCN3,
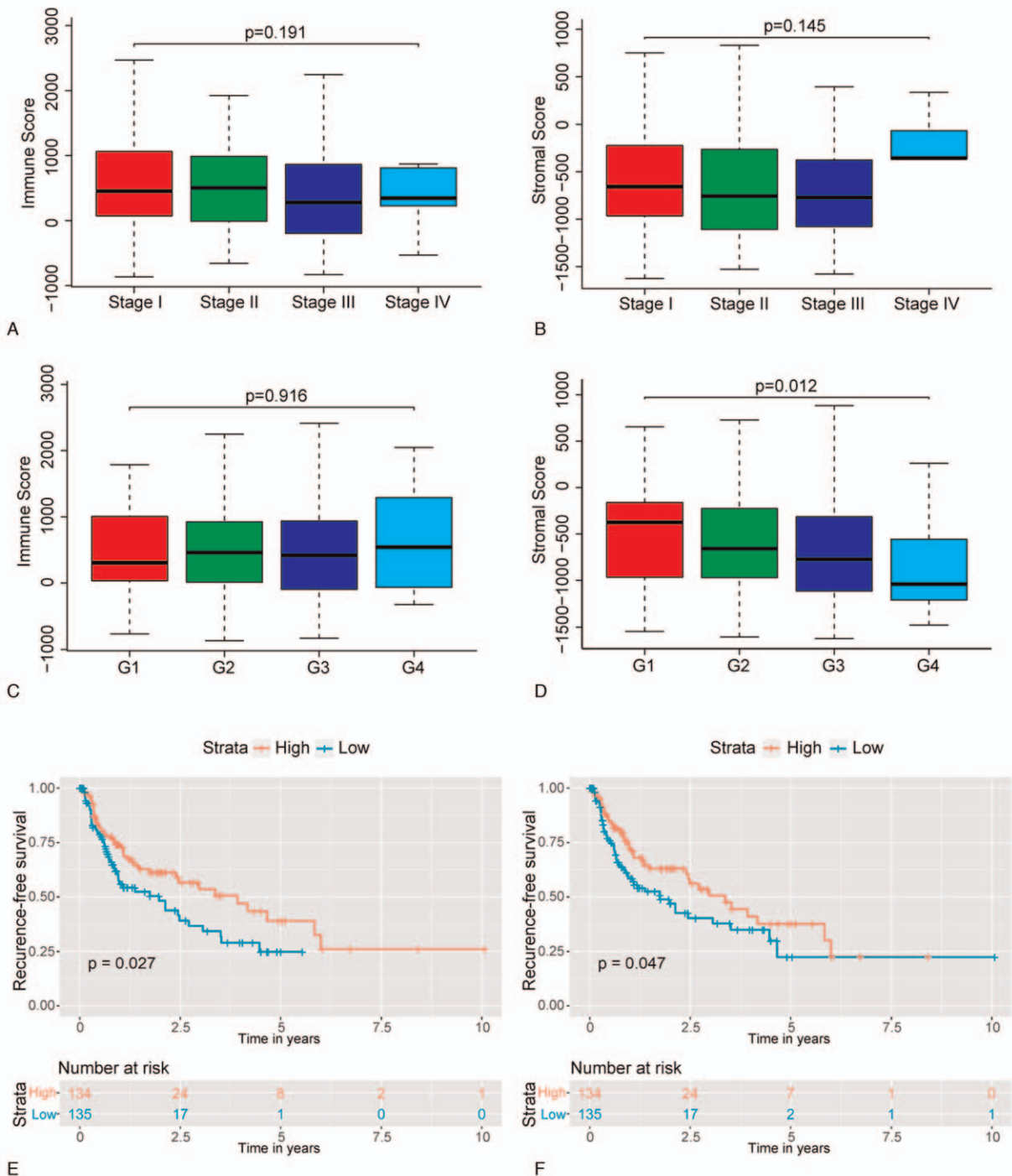
Figure 1. Immune and stromal scores were associated with clinical features and prognosis of HCC. (A,B) The relationship between immune/stromal scores and tumor stage. (C,D) The relationship between immune/stromal scores and histological grade. (E,F) Kaplan-Meier survival curve of recurrence-free survival (RFS) between high-immune/stromal scores group and low-immune/stromal scores group in HCC. HCC = hepatocellular carcinoma.

RYR1, and SUSD5 had significantly longer RFS time except for the gene of PI16 (Fig. 6).

3.7. Correlation between abundance of immune infiltrates and ACKR1, CD4, CD6, FCN3, PI16, RYR1, and SUSD5

Tumor-infiltrating lymphocytes have been shown to possess an independent prognostic utility for cancers,[20,21] so we investi-

gated whether the expression of ACKR1, CD4, CD6, FCN3, PI16, RYR1, and SUSD5 were correlated with immune infiltration levels in HCC. The results showed that the expression of ACKR1, CD4, CD6, FCN3, PI16, RYR1, and SUSD5 had significant correlations with tumor purity. In addition, CD4, CD6, RYR1, and SUSD5 shared the same immune cell profile, showing a clear association with B cell, CD8+ T cells, CD4+ T
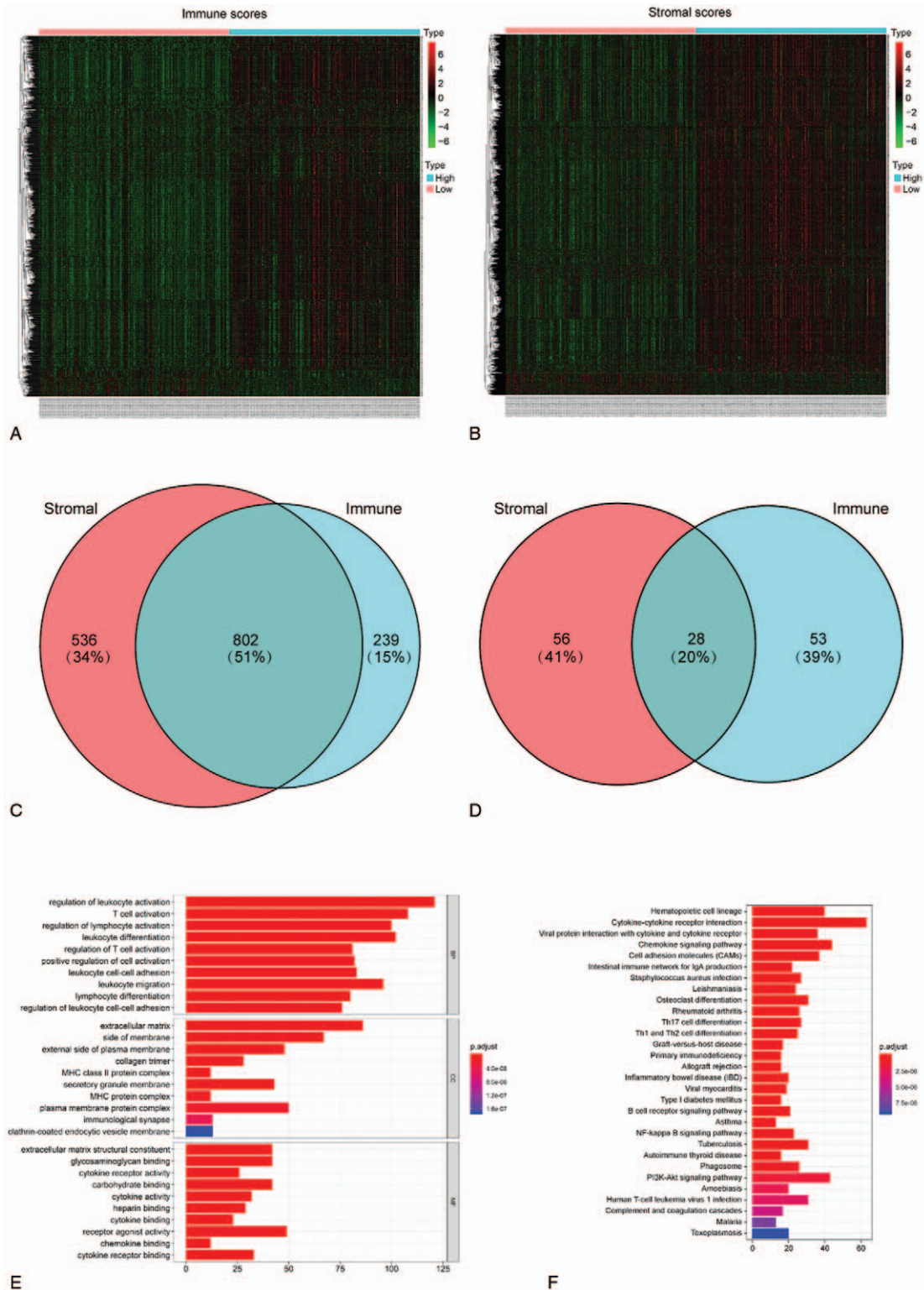
Figure 2. Comparison of gene expression profile with immune and stromal scores in HCC. (A,B) Heatmap of the differentially expressed genes (DEGs) based on immune and stromal scores in HCC. (C,D) A Venn diagram was utilized to screen commonly upregulated (C) or downregulated (D) DEGs based on immune and stromal scores. (E,F) GO and KEGG analysis of commonly upregulated and downregulated DEGs. GO=Gene Ontology, HCC = hepatocellular carcinoma, KEGG=Kyoto Encyclopedia of Genes and Genomes.
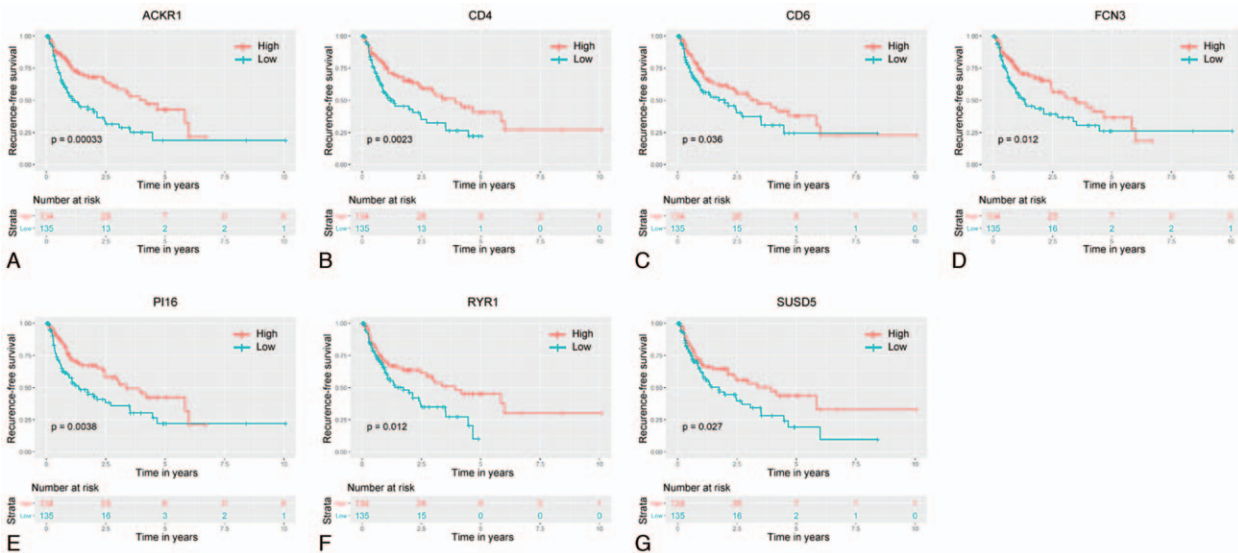
Figure 3. Kaplan-Meier analysis showed the correlation between expression of individual DEGs and RFS in HCC patients. Seven genes that had further validated in GEO dataset were shown. $P < .05$ in Log-rank test. DEGs = differentially expressed genes, GEO = Gene Expression Omnibus, HCC = hepatocellular carcinoma, RFS = recurrence-free survival.

cell, neutrophils, DCs, macrophages populations. However, little or no correlation was found between analyzed immune cell populations and both DARC, FCN3, and PI16 (Fig. 7).

3.8. Immune infiltrates analysis in HCC

In addition, the abundances of 6 immune infiltrates (B cells, CD4 + T cells, CD8+ T cells, neutrophils, macrophages, and DCs) in HCC were estimated through TIMER database. The result showed that DCs was most abundant in the microenvironment of HCC and CD4+ T cell was the second most abundant (Fig. 8B). However, as is known to all, the abundance of tumor-infiltrating immune cells was different for every patient.[22] So, it is necessary for us to discern patterns of immune infiltration through the unsupervised clustering with the k-means algorithm based on the immune-cell proportions of all samples. Eight was set as the optimal number of clusters (k-means = 8, Fig. 8A). The cell proportion with 6 immune cell types for each cluster are shown in Figure 8D. Furthermore, survival analysis was applied for each

cluster and the result was shown in Figure 8C. Although it is not statistically significant, Kaplan-Meier survival curves showed that the cluster 8 which is defined by a high level of DCs and CD4 + T cell was associated with a better prognosis than other clusters. And other clusters such as cluster 1, cluster 2 which were defined by less DCs and more other immune cells were associated with poor prognosis. The findings above showed DCs played a crucial role in progress of HCC.

4. Discussion

Liver cancer is one of the most frequent and deadly cancers in the world with more than 800,000 new cases and 780,000 deaths in 2018.[23] Tumor heterogeneity is the major factor contributing to the refractory nature of HCC. Although many treatments have been developed in the past few decades, the prognosis of advanced liver cancer remains poor. Tumor heterogeneity consists of both tumor cells and heterotypic components, including immune/inflammatory cells, vascular structures, mes-

Figure 4. PPIs among immune-related genes. (A–C) Top 3 modules of PPI networks. (A) Cluster 1 consisted of 44 nodes and 1892 edges. (B) Cluster 2 consisted of 37 nodes and 1332 edges, and (C) Cluster 3 consisted of 29 nodes and 182 edges. PPI = protein-protein interaction.
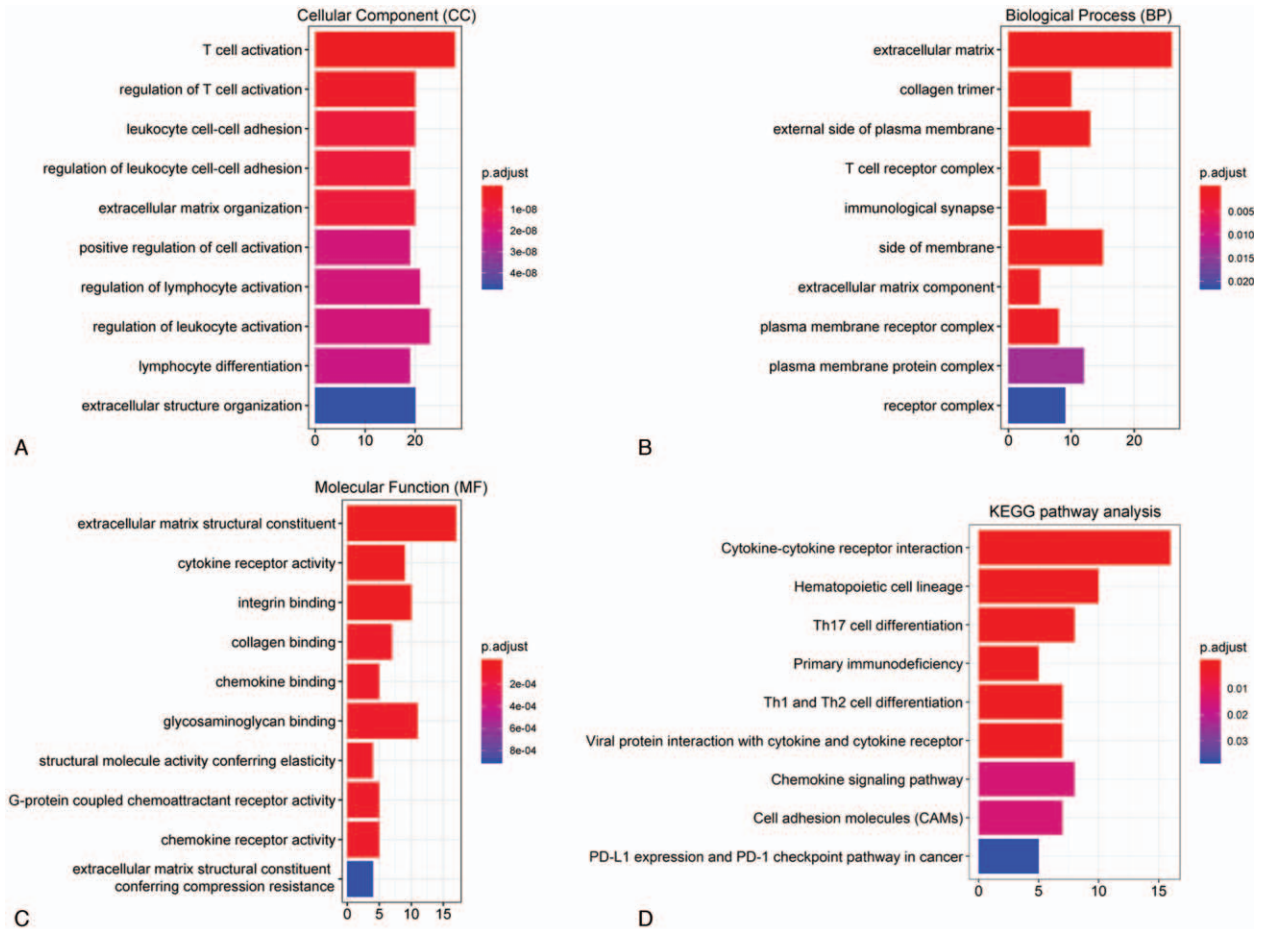
Figure 5. Functional enrichment analysis of immune-related genes. (A–C) GO analysis of the genes in these top 3 modules. (D) KEGG analysis of the genes in these top 3 modules. GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

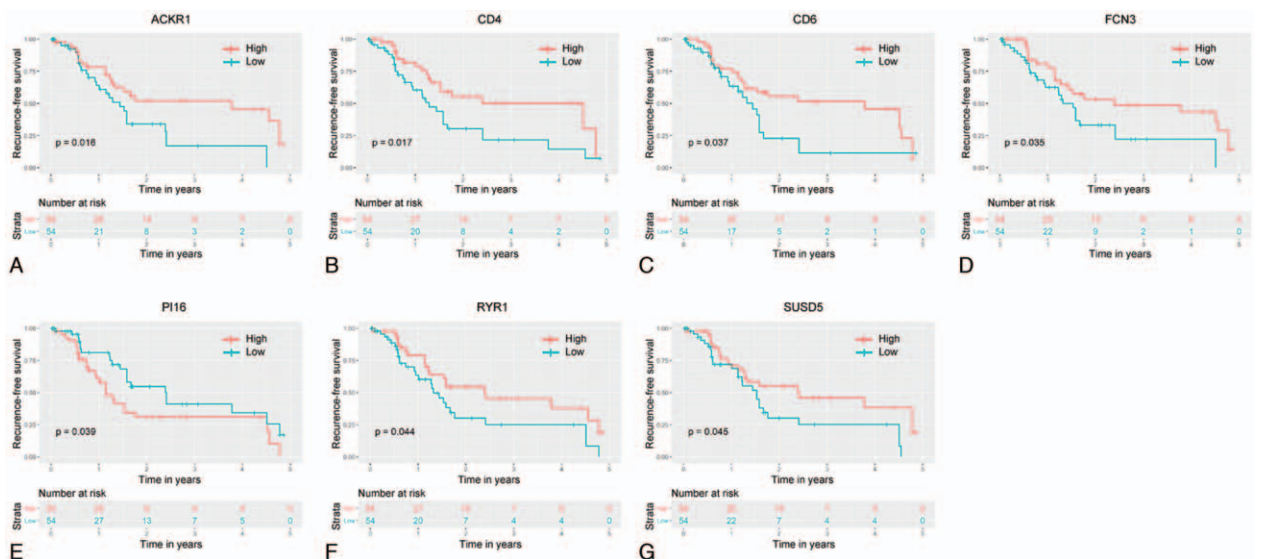
Figure 6. Validation of correlation of DEGs extracted from TCGA database with RFS in GEO dataset. (A–G) Patients with high expression of ACKR1, CD4, CD6, FCN3, PI16, RYR1, and SUSD5 had significantly longer RFS time than patients with low expression of them. DEGs = differentially expressed genes, GEO = Gene Expression Omnibus, RFS = recurrence-free survival, TCGA = the Cancer Genome Atlas.
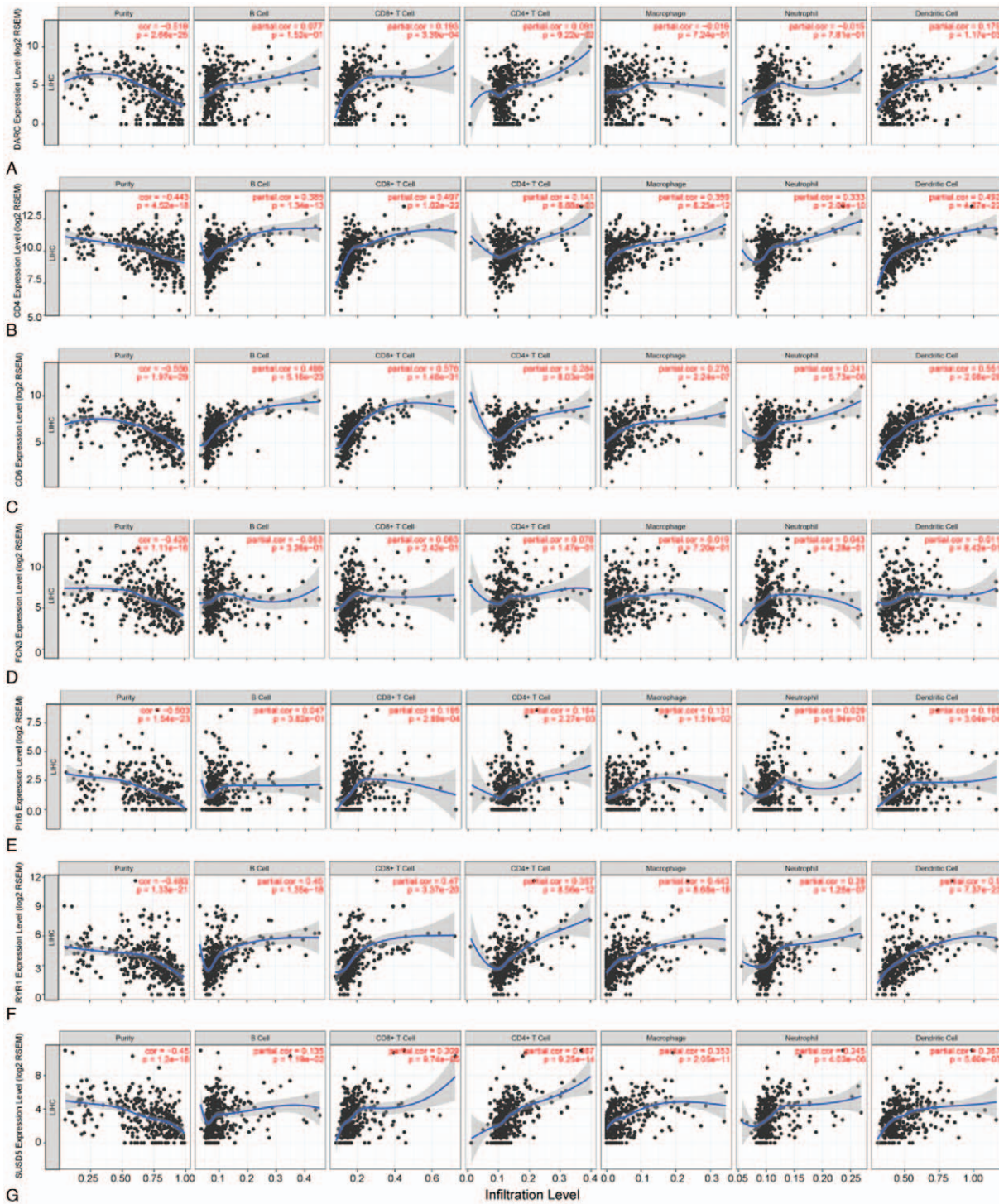
Figure 7. Correlation between abundance of immune infiltrates and ACKR1 (A), CD4 (B), CD6 (C), FCN3 (D), PI16 (E), RYR1 (F), and SUSD5 (G).

enchymal cells, and ECM.[24] In recent years, many researches have focused on the microenvironment of tumor regulating the tumor progression and metastasis.[25–27] As is known to all, liver cancer is a kind of cancer which is mostly induced by inflammation, and the immune cells play an important role in liver cancer initiation and development.[28] Therefore, the goal of our study is to understand the microenvironment and its potential mechanism of HCC.

In our study, ESTIMATE algorithm was applied to calculate the tumor purity of HCC. The results showed that the stromal score was significantly associated with pathological grade of HCC, indicating a significant function of stromal cells in the malignant progression of HCC patients. Previous studies have that the immune/inflammatory cells and stromal cells play a crucial role in tumor progress, and influences therapeutic response and clinical outcome.[29] Therefore, we analyzed the
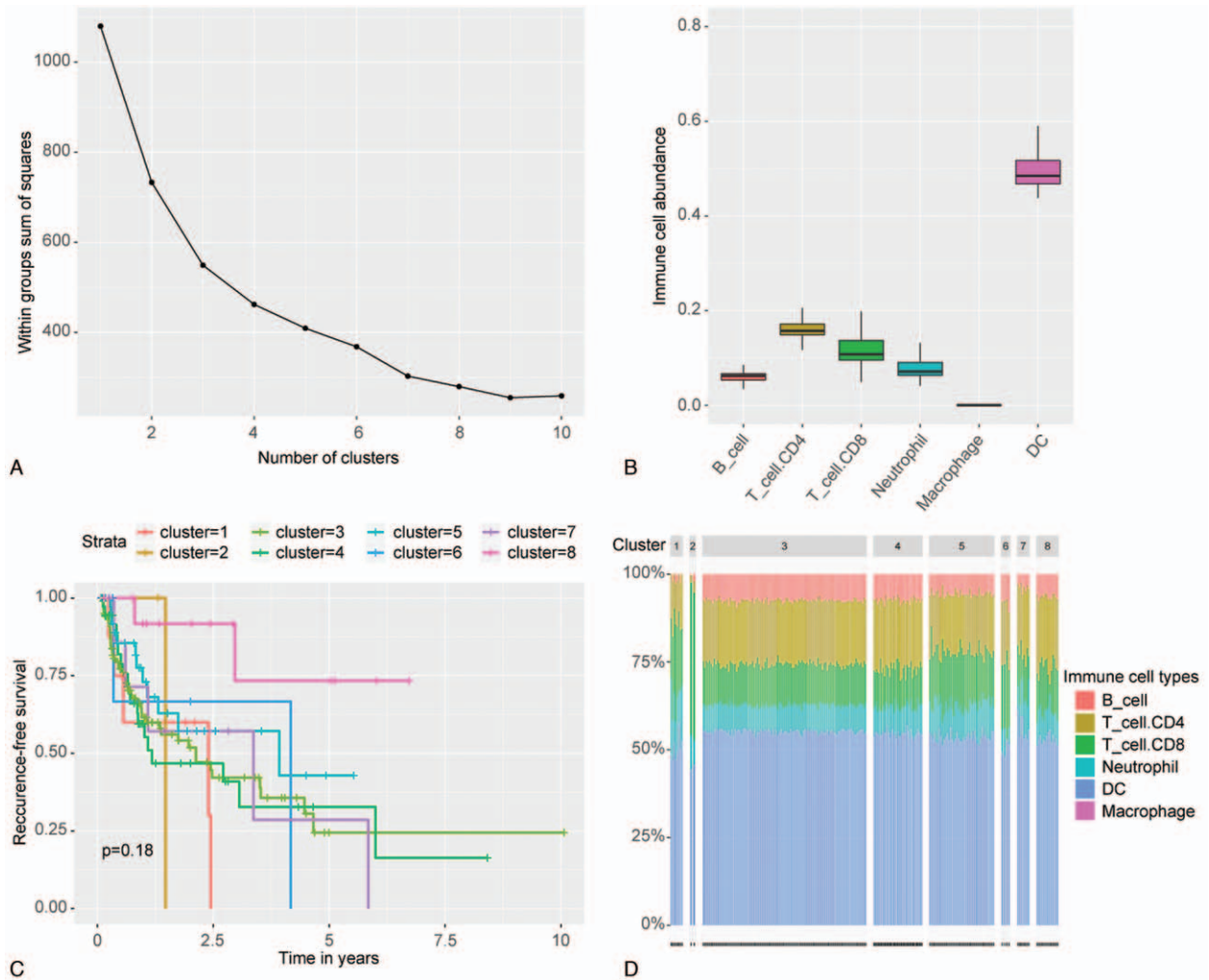
Figure 8. The immune landscape of the tumor microenvironment in HCC. (A) Samples clustering and analysis optimal number of clusters. (B) The immune infiltration cells in the HCC. (C) Survival analysis of HCC patients in different clusters. (D) Unsupervised clustering of all HCC patients based on immune-cell proportions. HCC = hepatocellular carcinoma.

relationship between immune/stroma score and the clinical features of HCC patients. The results showed that the stromal score was associated with histological grade, and patients with high scores (both immune score and stroma score) had a better prognosis than patients with low scores. It indicated that immune/stroma score would have strong potential to be translated into clinical practice, as the current high-throughput gene expression measurement technology has been well developed and been applied in clinic widely recent years.

Next, we analyzed the DEGs between high/low immune-score groups and high/low stromal-score groups. Intersection genes (802 upregulated genes and 28 downregulated genes) were further analyzed though GO and KEGG analysis. The results showed intersection genes were mostly involved in immune-related BP, such as regulation of leukocyte activation, T cell activation, and regulation of lymphocyte. In addition, a total of 830 genes were analyzed with the prognosis of HCC, and finally 169 genes were found to be inversely linked to the RFS of HCC patients. This is consistent with previous reports that the tumor microenvironment coexisted and interacted with immune cells to promote the growth of HCC.[30–32]

In order to further explore the relationship between immune-related genes and the progression of HCC, PPI network, and M-CODE a plugin was applied. The results showed that the genes in the top 3 most significant modules almost enriched in T cell activation, regulation of T cell activation, leukocyte cell-cell adhesion, cytokine-cytokine receptor interaction, hematopoietic cell lineage, and Th17 cell differentiation. Same to the previous findings, the immune-related genes significantly participated in the development of HCC. Moreover, the present study found C5AR stimulated cell invasion and migration in HCC cells, and blocking the expression of C5AR had therapeutic promise to inhibit HCC invasiveness.[33] In patients with liver cancer, CCL2 was overexpressed and associated with the prognosis of HCC, blockade of CCL2/CCR2 signaling inhibited malignant growth and metastasis in murine.[34]

Furthermore, we validated the 830 intersection genes in GEO dataset. The results showed that HCC patients with high expression of ACKR1, CD4, CD6, FCN3, PI16, RYR1, and SUSD5 had significantly longer RFS time than patients with low expression of them. ACKR1/DARC functions as a decoy receptor for many CXC and CC chemokines, such as CCL2 and

CXCL8.[35] ACKR1 was downregulated in thyroid cancer, colorectal cancer, breast cancer, and pancreatic cancer, and have been reported to inhibit the development of several tumor through clearance of angiogenic chemokines.[36–39] CD4 and CD6 both encodes a membrane glycoprotein of T-lymphocytes. Today, inducing the infiltration of cytotoxic T lymphocytes into the tumor has become a common approach to manage cancer.[40,41] It indicated that the recruitment of T-cells in the microenvironment of HCC might be an effective anti-cancer immunotherapy and enhance the survival of patients. It was reported that ficolin-3 (FCN3) was decreased in HCC,[42,43] but there were no more studies on the association between FCN3 and HCC, the role of FCN3 in the development of HCC needs to be further studied. In addition, PI16, RYR1, SUSD5 have not previously been correlated with HCC prognosis, and could serve as novel biomarkers for HCC patients. Consistent with the previous studies, we can conclude that immune-related genes play a crucial role in the progression of tumor, but the real mechanism under these genes still needs further researches and studies.

Finally, we found that DCs were the most abundant in the microenvironment of HCC through TIMER algorithm. Then HCC patients were divided into 8 clusters according to the proportion of immune cells. In addition, the survival analysis between 8 clusters and prognosis HCC were applied. The results showed that DCs did significantly influence prognosis of HCC. DCs are key regulators of the adaptive immune response, and are necessary for inducing anti-tumor T cell responses. However, DCs cannot always induce effective immunity according to the suppressive mechanisms of tumors.[44] Tumor cells have means of suppressing DCs function and lead these DCs to confer immune suppression at the local TME.[45] DCs was reported to possess high anti-tumor and cytotoxic activity against HCC,[46,47] and is widely applied for therapeutic tumor vaccine deliver according to their antigen-presenting ability.[48] In our study, we found the number of DCs was significantly associated with the prognosis of HCC. It implies that DCs could be a potent target in efforts to generate therapeutic immunity against HCC, although the underlying mechanism remains to be studied.

In summary, through various analyses, we had a better understanding of the role of the tumor microenvironment in HCC, and discovered 7 genes closely related to the recurrence survival of HCC, which could be identified as novel biomarkers for prognostic assessment of HCC.

Author contributions

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Writing – review & editing: Mengting Li.

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