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Identification of immune cell-related prognostic genes characterized by a distinct microenvironment in hepatocellular carcinoma

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Abstract

BACKGROUND

The development and progression of hepatocellular carcinoma (HCC) have been reported to be associated with immune-related genes and the tumor microenvironment. Nevertheless, there are not enough prognostic biomarkers and models available for clinical use. Using seven prognostic genes, the study calculated overall survival in patients with HCC using a prognostic survival model and revealing the immune status of the tumor microenvironment (TME).

AIM

To develop a novel immune cell-related prognostic model of HCC and depict the basic profile of the immune response of it.

METHODS

We obtained clinical information and gene expression of HCC from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) datasets. TCGA and ICGC datasets were used for screening prognostic genes along with developing and validating a 7-gene prognostic survival model by weighted gene coexpression network analysis and LASSO with Cox regression. The relative analysis of

tumor burden mutation (TBM), TME cell infiltration, immune checkpoints, immune therapy and functional pathways was also performed based on prognostic genes.

RESULTS

Seven prognostic genes were identified for signature construction. Survival receiver operating characteristic analysis showed the good performance of survival prediction. TBM could be regarded as an independent factor in HCC survival prediction. There was a significant difference in stromal scores, immune scores and estimate scores between groups. Several immune checkpoints, including VTCN1 and TNFSF9, were found to be associated with the 7 genes and risk scores. Different combinations of checkpoint blockade targeting inhibitory CTLA4 and PD1 receptors and potential chemotherapy drugs hold great promise for specific HCC therapies. Potential pathways, such as cell cycle regulation and metabolism of some amino acids, were also identified and analyzed.

CONCLUSION

A novel 7-gene (CYTH3, ENG, HTRA3, PDZD4, SAMD14, PGF, and PLN) prognostic model showed high predictive efficiency. The TBM analysis based on the 7 genes could depict the basic profile of the immune response in HCC, which might be worthy of clinical application.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer that has the highest prevalence among liver cancer subtypes. It is well known that the mortality of HCC has increased gradually and is likely to break through the millions[1]. Although rapid technological and medical advances in new tests and treatments have been applied to HCC patients, the overall five-year survival rate is less than 5%[2]. In addition, since HCC had no specific clinical manifestations in patients with HCC, patients might have been

diagnosed in the late stages of HCC. In this regard, it is necessary to explore novel prognostic biomarkers and risk models to help forecast overall survival (OS) with greater accuracy of HCC patients.

The immune system and immune cells are both considered to be essential for developing various cancers, including HCC[3]. A previous study reported that an imbalance in immune regulation affected the cancer microenvironment and contributed to tumor progression and metastasis[4]. The altered crosstalk between tumor cells and the immune system represents a new hallmark in the diagnosis and treatment of HCC. For instance, CD8+CXCR5+ T cells invade supernatants from primary HCC (HCC-SN) cells and cause patients with HCC to have a worse prognosis[5]. Theactivation of tumor-associated macrophages could contribute to increasing the number of HCC cells *via* destabilization of adherens junctions[6]. Likewise, increasing the number and activity of mature dendritic cells could improve the prognosis of HCC patients[7]. To date, the specific status of different types of cell subtypes and programmed cell death have been explored in the tumor microenvironment (TME) of HCC[8,9]. However, few researchers have focused on exploring the specific association or mechanism between immune infiltration and liver cancer through the genes of all immune cells. Moreover, potential clinical applications along with the mechanism of different types of immune cell-related genes regulating the TME for HCC progression remain unclear and need further research.

In this research, we first identified immune cell-related genes (ICRGs) through weighted gene coexpression network analysis (WGCNA) in The Cancer Genome Atlas (TCGA) datasets to explore the potential prognostic immune-related genes that were most enriched and correlated with HCC. After LASSO regression with Cox regression analysis, we found 7 ICRGs to establish and validate a well-worked prognostic model in the TCGA and International Cancer Genome Consortium (ICGC) datasets. Meanwhile, a series of bioinformatic analyses, including protein–protein interaction, immune checkpoint analysis, immune checkpoint blockade, chemotherapy drug analysis, gene set variation analysis (GSVA) and gene set enrichment analysis, were also introduced in

to meet the goal of determining how all immune cell-related genes may impact HCC's TME in this study. The results of this scenario also helped determine potential biomarkers that might be novel therapeutic targets in HCC. In this scenario, our studies also published biomarkers that help determine the best therapeutic strategy for each patient.

MATERIALS AND METHODS

Datasets

The clinical information of HCC patients and corresponding cancer tissue RNA-seq data were identified and retrieved from the TCGA (https://portal.gdc.cancer.gov/repository) for model construction and ICGC (https://dcc.icgc.org/projects/LIRI-JP/) database for validation. There were 374 HCC tissues and 50 normal tissues in TCGA datasets, and 265 tumor samples in ICGC datasets.

ICRGs

First, we identified the hub immune-cell-related genes by method of WGCNA. A co-expression network of ICRGs was built by performing the "WGCNA v1.68" package on the R platform. After a topological overlapping matrix was performed to detect modules, 405 genes in black module were chosen and regarded as ICRGs. Next, we identified 338 differentially expressed ICRGs with screening standards set as P value < 0.05. In addition, String (Protein–Protein Interaction Networks, V: 10.5) database (https://string-db.org/) on 338 differentially expressed ICRGs were performed to obtain the interactions between proteins-targeted genes. Then, 51 hub ICRGs were filtered out by performing the univariate cox regression analysis ($P < 0.05$).

Construction and validation of the prognostic model

A penalized shrunken regression method named the least absolute shrinkage and selection operator (Lasso) was conducted on the β-coefficients to minimize potential

overfitting. Based on the results of a cox regression analysis, 7 prognostic ICRGs were identified and took part in constructing the prognostic model. We calculated and obtained each patient's risk score according to the formula below: Risk score = esum (each gene's expression × corresponding coefficient). Then, a model for immune-cell-related prognostic indicator was developed as follows: Risk score = $\sum I = 1\ N$ (Explg*Coef). The median risk score could be used to divide HCC samples into low-risk (< median) and high-risk (≥ median) group.

Prognostic and clinical implication

Overall survival probability analysis and the time-dependent receiver operating characteristic (ROC) curve analysis in risk model and 7 prognostic ICRGs were performed using "survival", "survminer", "survivalROC" or "timeROC" in the R platform. Using the survival R package of "survival", we performed multivariate and univariate independent prognostic analyses in TCGA and ICGC group, respectively. In addition, the areas under the curve (AUC) at 1-, 3-,5-year and various clinical factors were calculated.

The available clinical factors, like age, gender, tumor grade and stage, were compared to assess the clinical implication of the model and depicted by R packages of "ggpubr". In order to improve the possibility of usage in clinical practice, nomogram was constructed and the calibration curves were plotted based on 7 prognostic ICRGs with the help of the "rms" R package.

Analysis of mutational load and TME

We assessed the status of TMB and somatic mutations in HCC in both two risk groups with the help of "maftools" in R package. The K-M curves were also obtained after HCC samples were divided into high-TMB or low-TMB.

Next, we further depicted the TME of HCC in the risk model by assessing the immune cells' immune-related functions through single-sample gene set enrichment analysis (ssGSEA) in "gsva" of R. For assessing immune infiltration subtype between

high- and low-risk groups, we performed two-way statistical ANOVA analysis here. The relationship between tumor stemness and risk score were obtained after spearman correlation analysis. ESTIMATE algorithm helped to explore the levels of cell infiltration by calculating the immune and stromal scores.

Immune-check points analysis and immune checkpoint blockade

Immune-check points (ICP) analysis were conducted here to determine and visualise the correlations among genes related to ICP, 7 ICRGs and risk scores by using "limma, BiocManager, reshape2 and ggplot2" packages. The immunophenoscore (IPS) of HCC samples were collected from the cancer-immune group atlas, whose specific website was "https://tcia.at/home". The higher the IPS scores of risk groups were, the higher positive correlation occurred to the increased immunogenicity. The tumor immune dysfunction and exclusion (TIDE) algorithm (http://tide.dfci.harvard.edu) was used to predict the response to immune checkpoint blockade (ICB) treatment between two risk groups.

Chemotherapy drug analysis

To predict the half-maximal inhibitory concentration (IC50) of chemotherapeutic agents for each patient between two risk groups, the R package "pRRophetic" were downloaded from "https://github.com/paulgeeleher/pRRophetic". However, based on risk model, a great number of chemotherapeutic drug was found. In order to conduct further research on HCC, we selected four commonly used drug that had been used in clinical trials.

Meanwhile, NCI-60 human cancer cell lines from a publicly available dataset named CellMiner project page (https://discover.nci.nih.gov/cellminer) were also downloaded to determine the connection between 7 ICRGs and drug sensitivity, respectively.

Functional enrichment analysis

We determined the potential functional pathway that might play an essential role in HCC progression and TME regulation by GSVA and Gene set enrichment analysis (GSEA) with the help of the R package of "BiocManager, limma, GSEABase, GSEV". The edition of GSEA was "GSEA 4.0.1 software".

RESULTS

Construction of WGCNA and identification of hub modules

To screen the hub genes related to the TME characteristic index in HCC patients, the RNA seq and relevant clinical data of samples (50: 374 = Normal: Tumor) were downloaded from TCGA. The samples were conducted to identify outliers with the help of hierarchical clustering analysis and no samples were found to be outliers (Figure 1A). Next, we set the β-value to 20 to ensure that the network was scale-free (Figure 1B). After the method of dynamic cutting, separate gene co-expression modules was divided; Then, similar modules was combined with a difference < 25% (Figure 1C and D). Modules of similar sizes were screened and merged using the height parameter of 0.25, deep split parameter of 4, and minModuleSize parameter of 60. As a result of using WGCNA, we were able to obtain 6 modules for futher research (Figure 1E). In order to ascertain the hub module, a Pearson test ($P < 0.05$) was employed to compute the correlation between the characteristic genes of said module and immune-infiltrated cells. Then, based on the criteria of correlation > 0.1 and $P < 0.05$, we calculated the numbers of immune cells in each module to ascertain the appropriate immune-related module. The 405 genes in the black module were selected for the subsequent research because they had 13 immune cells that met the criteria, compared with 10 in red, 8 in lightcyan, 8 in tan, 11 in purple and 12 in gray.

The identification of ICRGs

We next identified ICRGs from the 405 genes in the black module. A total of 338 ICRGs were identified and expressed differentially between normal and HCC patients (Figure 2A). The protein–protein interaction network was constructed to show that the genes

could be roughly divided into four categories: laminin family members, collagen family members, ADAMTS family members and the Notch signaling network (Figure 2B). Then, we used univariate Cox regression to identify the hub ICRGs that played an essential role in model construction, and 51 hub ICRGs were obtained here (Figure 2C).

Establishment of a prognostic signature based on 7 prognostic ICRGs

Seven prognostic ICRGs (CYTH3, ENG, HTRA3, PDZD4, SAMD14, PGF, and PLN) were screened out; The objective was to develop a prognostic signature through the utilization of LASSO Cox regression analysis (Figure 3A and B). According to the formula of risk score = 0.369451059941312 * CYTH3 + -0.287762938289728 * ENG + 0.221566259099006 * HTRA3 + -1.00820639333083 * PDZD4 + 0.542684310219352 * SAMD14 + 0.526196082946206 * PGF + -0.393399578080273 * PLN, the risk score of each patient was calculated. All patients could be divided into high- and low-risk groups based on 7 prognostic ICRGs in the two datasets (Figure 3C and F). Using the median cutoff value of risk scores for patents in the TCGA and ICGC datasets, two groups with high-risk and low-risk scores were also classified (Figure 3D and G). Moreover, as supported by the higher density of red dots observed in the high-risk area within both cohorts, the patients who belonged to high-risk groups might have a worse outcome (Figure 3E and H).

Prognostic implication of the 7-ICRG prognostic model

In comparison to their counterparts, patients with higher risk scores exhibited a notably diminished OS in the TCGA dataset., which was further verified in the ICGC dataset (Figure 4A and B). Simultaneously, the potential significance of the risk score as a variable factor in prognostic prediction was also confirmed through univariate Cox regression analysis. In this study, we conducted multivariate survival analyses in both TCGA and ICGC datasets to demonstrate the efficacy of the model. The results revealed a hazard ratio (HR) of 1.375 (95%CI: 1.256-1.504; $P < 0.001$) in TCGA and an HR of 1.123 (95%CI: 1.025-1.229; $P = 0.012$) in ICGC (Figure 4C and D). The area under the receiver

operating characteristic (ROC) curve (AUC) demonstrated the highest predictable values in TCGA (AUC = 0.762) and ICGC (AUC = 0.754) compared to other traditional features such as stage, age, sex, and grade (Figure 4E and F). The AUC values obtained from the time-dependent ROC analysis were 0.762, 0.748, and 0.757 at time points of 1-, 3-, and 5-year in TCGA and 0.794, 0.778, and 0.776 at 1, 3 and 5 in ICGC, respectively (Figure 4G and H).

The survival probability of 7 ICRGs and nomogram construction

Then, the patients diagnosed with HCC were categorized into separate subgroups based on ICRGs of CYTH3, ENG, HTRA3, PDZD4, SAMD14, PGF and PLN to conduct a more comprehensive investigation into the prognostic significance of individual genes within the signature. In this study, we have provided evidence indicating that the survival probabilities of CYTH3, HTRA3, PGF, and SAMD14 were significantly lower in the high-risk group compared to the low-risk group, while ENG, PDZD4 and PLN showed the opposite trend (Figure 5A-G).

In addition, according to the fundamental prognostic variables from the multivariate Cox regression analysis, we have developed a nomogram to accurately forecast the OS rates for HCC patients over a span of 1 year, 3 years, and 5 years. Initially, patients have the opportunity to accrue points by summing the values of all variables within the nomogram. Subsequently, the cumulative points for each patient can be computed to derive the 1-, 3-, and 5-year OS rates, employing a vertical line extending from the prognostic factor axis to the points axis (Figure 5H). Figure 5I exhibited a notable level of accuracy in predicting the OS of HCC patients at the time of 1 year, 3 years, and 5 years.

Clinical traits of 7 ICRG prognostic models

In each dataset, we evaluated the clinical implications of clinical factors such as age, sex, grade and stage as explanatory variables in 7 ICRG prognostic models. Here, we noticed that patients with high grade and stage III−IV were more likely to have a higher

risk score in TCGA, and a similar trend occurred in stage III−IV in ICGC datasets (Figure 6C, D, and G). However, no significant differences were observed in age or sex (Figure 6A and B, E-F).

Genomic features of the 7-ICRG prognostic model

According to the results of somatic mutation analysis, in the low-risk group, mutations in CTNNB1 (26%), TTN (21%), TP53 (17%), and MUC16 (13%) were highly enriched. Concurrently, the high-risk group exhibited a significant enrichment of mutations in TP53 (38%), TTN (26%), CTNNB1 (25%), and MUC16 (16%). Missense mutation was the main type of genetic variation (Figure 7A and B). As the risk score of each patient increased, we also observed increased tumor burden mutation (TBM) in tumor samples (Figure 7C). The data presented in Figure 7D indicated that patients belonging to the low-TBM group exhibited a significantly elevated probability of survival, while patients in the high-TBM group displayed a notably diminished probability of survival ($P < 0.001$). When combining risk and TBM classification, the lowest survival probability occurred in patients in the high-risk + TBM group, while the highest survival probability occurred in the low-risk + TBM group ($P < 0.001$, Figure 7E).

Tumor microenvironment analysis

In this study, we investigated the potential correlation between different immune cell types and the risk scores of patients within TCGA dataset. Based on the outcomes of the analyses conducted using seven software applications (XCELL, TIMER, QUANTISEQ, MCPCOUNTER, EPIC, CIBERSORT-ABS, and CIBERSORT), our observations were that CD8+ T cells, mast cells, *etc.*, were consistent with the decrease in patient risk, while CD4+ T cells, B cells, M0 macrophages and regulatory T cells (Tregs) were associated with the increase in patient risk scores (Figure 8A). Subsequently, we performed ssGSEA to assess and contrast the ssGSEA scores across distinct risk groups, thereby elucidating the disparities in immune infiltration between the high- and low-risk cohorts. Here, it was observed that macrophages exhibited a more pronounced

association with the high-risk group, whereas B cells, CD8+ T cells, dendritic cells, mast cells, neutrophils, natural killer cells, plasmacytoid dendritic cells, T helper cells, and tumor infiltrating lymphocytes were found to be linked with the low-risk group (Figure 8B, $P < 0.05$). Then, we conducted a comparative analysis of the immune functions in both groups in order to investigate the potential involvement of immune pathways in their differentiation. Our research revealed that the high-risk group exhibited greater activity of major histocompatibility complex class I, while antigen presenting cell coinhibition, cytolytic activity, human leukocyte antigen, inflammation promotion, and type I and type II IFN responses in the low-risk group exhibited higher levels of activity (Figure 8C, $P < 0.05$).

Next, we introduced four distinct immune infiltration types, namely wound healing (C1), interferon gamma (IFN-γ) dominant (C2), inflammatory (C3), and lymphocyte depleted (C4), in order to ascertain their association with the signature. The findings revealed a strong correlation between C1 and patients classified as high-risk, whereas C2, C3, and C4 were more likely to manifest in individuals with low-risk scores (Figure 8D).

Then, the RNA stemness score (RNAs) and DNA methylation pattern (DNAss) were used to evaluate tumor stemness. As presented in Figure 8E-F, no significant correlation was observed between DNAss and Risk score, while a more positive association was identified between RNAss and Risk score ($r = 0.24$, $P < 0.05$). In addition, the risk score was significantly associated with the stromal score, immune score and estimate score of patients with HCC in the low-risk group, implying a more essential role of the TME in the low-risk group than in the high-risk group (Figure 8G).

Immune checkpoint (ICP) analysis and ICB therapy

The expression association between 47 immune checkpoints and the risk score or 7 ICRGs was also studied. The results showed that the levels of most immune checkpoints, such as VTCN1, TNFSF9, TNFSF4, TNFSF18, TNFSF15, TNFRSF9, TNFRSF4 and TNFRSF18, were positively related to the risk score. However, TMIGD2,

PDCD1LG2, and IDO2 were negatively correlated with the risk score (Figure 9A). IPS analysis was also conducted here for the prediction of patient responsiveness to CTLA-4 and PD-1. The results showed that the IPS, IPS-CTLA4, IPS-PD1 and IPS-PD1-CTLA4 scores were higher in the low-risk group ($P < 0.05$, Figure 9B-E).

In order to conduct a comprehensive assessment of the risk score's efficacy as a prognostic indicator for patients undergoing ICB therapy, a TIDE analysis was conducted on HCC patients from TCGA dataset. Here, it was observed that patients classified within the high-risk group exhibited lower TIDE scores, indicating a potential heightened responsiveness to ICB therapy in comparison to the low-risk group (Figure 9F).

Chemotherapy drug analysis

After performing the algorithm provided from the R package "pRRophetic", the IC_{50} analysis of 4 relatively frequently used chemotherapeutic drugs for HCC was conducted in two groups. In this research, axitinib, dasatinib, and erlotinib were observed to have significant differences in estimated IC_{50} between the two groups. In addition, we noticed that patients in the high-risk group had higher IC_{50} values, implying that patients in the low-risk group were more sensitive to these 3 drugs (Figure 10 A-C). Meanwhile, the IC_{50} values of gemcitabine were found to be higher in the low-risk group compared to the high-risk group, indicating a greater likelihood of gemcitabine sensitivity among patients in the high-risk group (Figure 10D).

We also explored the top 16 correlation analyses between prognostic genes and drug sensitivity according to NCI-60. Figure 10E demonstrates that *SAMD14* was sensitive to bleomycin. However, *CYTH3* was insensitive to palbociclib, dexrazoxane, crizotinib, oxaliplatin, LDK−37, valrubicin, teniposide, nitrogen mustard, LEE−011, DAUNORUBICIN, raloxifene, etoposide, epirubicin and daunorubicin. In addition, the expression of *PDZD4* was insensitive to palbociclib (Figure 10E).

Identification of the 7 ICRGs and risk score-associated biological pathways

The predictive ability of 7 ICRGs and the risk score can be attributed to their key roles in tumorigenesis and metastasis. Thus, we further explored the richness differences by GSVA in 7 ICRGs and risk scores. As GSEA enrichment depicted in Figure 11A, we observed that several pathways correlated with tumorigenesis and T cells were positively related to risk score, including WNT_BETA_CATENIN_SIGNALING, TGF_BETA_SIGNALING, PI3K_AKT_MTOR_SIGNALING,MYC_TARGETS, MTORC1_SIGNALING, MITOTIC_SPINDLE, GLYCOLYSIS, G2M_CHECKPOINT,E2F_TARGETS, and DNA_REPAIR. Some pathways were negatively related to risk score, such as XENOBIOTIC_METABOLISM, PEROXISOME, and KRAS_SIGNALING.

Meanwhile, GSEA method was employed to investigate the potential involvement of signaling pathways in HCC patients between the two groups. We selected the ten pathways with the highest enrichment scores, and the findings indicated that PYRIMIDINE_METABOLISM, BASE_EXCISION_REPAIR, RNA_DEGRADATION, NUCLEOTIDE_EXCISION_REPAIR and CELL_CYCLE were more related to the high-risk group, while TRYPTOPHAN_METABOLISM, VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION, FATTY_ACID_METABOLISM, GLYCINE_SERINE_AND_THREONINE_METABOLISM and BUTANOATE_METABOLISM were more related to the lower group (Figure 11B).

DISCUSSION

In this research, we identified 7 prognostic ICRGs from all the immune cell-related genes in TCGA and established and validated a well-worked prognostic model for HCC patients in TCGA and ICGC datasets. To the best of our knowledge, this was the first time we screened the prognostic genes of HCC from all the immune cell-related genes by WGCNA. To date, plenty of research has explored the associations between specific subtypes of immune cells and HCC to construct a prognostic model based on them. For example, a 4-gene signature and nomogram related to macrophages were constructed,

and the immune landscape with abnormal infiltration of macrophages was depicted as well[10]. The infiltration of CD8+ T cells and the expression of MMP9 were closely related to the overall survival of HCC patients[11]. However, these studies, which only explored the infiltration of a single subtype of immune cells, ignored the fact that immune cells could cooperate or counter each other to promote the progression and metastasis of different cancers, including HCC. Innate immune cells, such as dendritic cells, Kupffer cells and natural killer T cells, interact with the T-cell response to regulate the TME of HCC[12]. Macrophage-lymphocyte interactions were also observed to be a key element of cancer-related inflammation[13]. In addition, cross-talk between Tregs and mast cells was allowed by a positive feedback system that functioned through TGFβ1 and IL-9 in gastric cancer development[14]. This evidence suggests that we should also explore the mechanism and TME of HCC from larger immune cell subtypes rather than just a single subtype of immune cells.

With this conjecture, we screened 7 prognostic genes in the black module, named CYTH3, ENG, HTRA3, PDZD4, SAMD14, PGF, and PLN, from the entire immune cell gene pool through WGCNA, LASSO regression and Cox regression analysis. Interestingly, what we found proved the correctness of our conjecture. Here, we observed that the prognostic model based on 7 ICRGs had a good predictive ability in the TCGA and ICGC datasets, which was verified by the results that patients belonging to higher risk scores were associated with shorter OS. The AUC of the various clinical features indicated that the risk score exhibited the highest level of predictability. Through time-dependent ROC analysis, the AUC values were determined to be 0.762, 0.748, and 0.757 at 1, 3, and 5 years in the TCGA dataset; And it was 0.794, 0.778, and 0.776 at 1, 3 and 5 in ICGC, respectively, which all indicated the signature worked well in prediction of HCC patients' survival. Then, we noticed that the 7 ICRGs in the black module had a strong correlation with the T-cell population, which consisted of CD8+ T cells, resting memoryCD4 T cells, activated memory CD4 T cells, follicular helper T cells and regulatory T cells (Tregs) in HCC[15]. Thus, we mainly focused on the subtypes of T cells here. After thorough literature research, the expression of ENG, HTRA3, PDZD4,

PGF and PLN was reported to be associated with HCC. HTRA3, PDZD4 and PLN were only reported to have participated in the prognostic model of HCC[16,17]. High endoglin (ENG) expression in endothelial cells might activateHCC cell adhesion and endothelial functions[18]. ENG is also regarded as a mediator that stimulates T-cell activation, proliferation, and Th1 cytokine secretion to promote T-cell-mediated cytolysis in cancer treatment[19]. PGF (placental growth factor) induced from cancer-associated fibroblasts facilitates neoangiogenesis in HCC[20]. PGF can be secreted by the Th17 subset of helper T cells to promote angiogenesis and take part in Th17 differentiation in return in inflammation[21]. For CYTH3 and PDZD4, no new discovery has been reported to have a connection with either HCC or any subtypes of T cells until now.

Next, the genomic features of 7 ICRG prognostic models were explored. As shown by Shuo Wang et al., TP53 and CTNNB1 are two of the most commonly mutated genes in Chinese HCC patients[22]. In our research, we found that CTNNB1 (26%) was most enriched in the low-risk group, while TP53 (38%) was the most enriched. CTNNB1 activation promoted immune escape, including consequently impaired T-cell activity in HCC[23]. Somatic mutations in CTNNB1 were found to combat increasing levels of T-cell cytotoxicity or immunological shifts toward cytotoxic CD8+ T cells, leading to the failure of chemotherapy for HCC[24]. TP53 (tumor protein p53), the most commonly mutated cancer driver gene, might cooperate with EGF domain-specific O-linked N-acetylglucosamine transferase (EOGT) to reduce the proportion of CD8+ T cells and impair regulatory T-cell differentiation in HCC[25]. Here, we noticed that the lowest survival probability occurred in patients in the high-risk and TBM groups, which indicated that that TBM had the potential to serve as a standalone variable in survival analysis, owing to its capacity to modulate immune status.

Then, the TME of HCC was analyzed based on the risk model. CD8+ T cells were more related to a decrease in patientrisk, while CD4+ T cells and Tregs were associated with higher risk scores. It is well known that elevated levels of cytotoxic CD8+ T cells are associated with stronger antitumor effects, and low levels of CD8+ T cells are correlated with poor HCC outcomes[26]. The presence of CD4+ T cells helped the

proliferation and activation of CD8+ T cells to avoid CD8+ T-cell exhaustion. CD4+ T cells are indispensable for the secondary expansion and memory of CD8+ T lymphocytes[27]. In fact, CD4+ T cells often become exhausted as HCC progresses. Furthermore, we conducted ssGSEA and found that CD8+ T cells and cytolytic activity were enriched in the low-risk group. No significant difference was observed between the two risk groups in CD4+ T cells, which suggested that the collapse of CD8+ T cells might contribute to insufficient CD4+ T cells. In addition, a further detailed analysis of the immune infiltration of HCC was performed to identify the role of the risk score in immune infiltration. The results showed that the enrichment of C1 (wound healing) mainly occurred in patients belonging to the high-risk group in the analysis of the TME. As previously reported, the wound-healing response could participate in promoting the development of HCC[28]. T cells coordinate with other proinflammatory cytokines and chemokines to take part in hepatic fibrosis, which is regarded as a wound-healing response in HCC[29]. In addition, significant differences were detected between the two risk scores with stromal score, immune score, estimate score and RNAss, which again emphasized that the 7 ICRG-related TMEs might facilitate the development of HCC and that RNAss could be involved.

A comparison between the risk score, ICP and ICB was conducted to find the beneficial target for immunotherapy and evaluate the efficacy of immunotherapy. Immune checkpoints, such as VTCN1, TNFSF9, TNFSF4, TNFSF18, TNFSF15, TNFRSF9, TNFRSF4 and TNFRSF18, were positively related to the risk score, while others were weakly related, which indicated that targeted and specific treatments should be carried out for the two risk groups. Moreover, the IPS, IPS-CTLA4, IPS-PD1 and IPS-PD1-CTLA4 immune checkpoint scores were higher in the low-risk group, suggesting that patients in the low-risk group might have better response to immunotherapy. PD-1 and CTLA-4 are checkpoint receptors that can be targeted to relieve the exhaustion of CD8+ T cells and were enriched in the low-risk group[30]. PD1 inhibitors might have directly acted on CD8+ T-cell cells to enhance the antitumor effect in this risk model.

In recent studies, immune checkpoint inhibitors (ICIs) plus tyrosine kinase inhibitors (TKIs), which are antiangiogenic drugs, seemed more effective in antitumor therapy, especially in reversing the immunosuppressive profile of the TME and improving the efficacy, OS and safety profile[31]. We must be more cautious and precise in the utilization of ICIs for HCC treatment, given the escalating incidence of HCC cases attributed to "metabolic" and "metabolic+alcohol" etiologies, as well as the intricate interplay between liver metabolism and immune system regulation[32,33]. In addition, Tregs are the most prevalent suppressor cells in the TME and express immune checkpoints such as PD-1 and CTLA-4, showing a potential therapeutic role of targeting Tregs in HCC treatment[34]. In our research, we observed an association between Tregs and patients at higher risk, suggesting that PD-1 and CTLA-4 might have the potential to alleviate patients by specifically targeting Tregs. Furthermore, the utilization of transarterial chemoembolization (TACE) has the potential to stimulate the generation of antigen-specific CD4+ and CD8+ T cells in patients with HCC, thus rendering the combination of TACE and ICI treatment highly promising in terms of its application prospects[35]. Radiogenomics research associated with TME heterogeneity has also made progress in recent years. Dandan Wang established a CT-derived radiomics signature based on 7 hub genes and revealed infiltration of CD4+ T cells, plasma cells and macrophages among different risk groups[36]. It is noteworthy that the Austrian team had devised an ART score for assessing the potential efficacy of repeated TACE treatment in patients, although this score had not yet undergone clinical validation[37]. This result implied that additional clinical validation is needed for the exploration of the TME and ICI in our research.

HCC exhibits significant resistance to various chemotherapy agents, whether administered as monotherapy or in combination. Therefore, it is imperative to select suitable chemotherapy medications that have obtained approval from the Food and Drug Administration for varying levels of patient risk. In the course of our investigation, it was observed that patients classified within the high-risk category exhibited heightened sensitivity to gemcitabine, whereas axitinib, dasatinib, and

erlotinib may potentially yield more favorable outcomes in patients categorized as low-risk. Then, the data from a total of 60 distinct cell lines was scrutinized in order to identify additional chemotherapy agents that could augment the efficacy of gene-targeted drugs while mitigating the risk of drug resistance. For instance, an increased expression of *SAMD14* genes was observed to render cancer cells more susceptible to the effects of bleomycin. Based on the aforementioned findings, it is possible that future advancements in precision strategies could unveil novel directions for drug treatment in HCC.

In the final analysis, we hoped to identify some representative functional pathways involved in the immune-related progression of HCC, especially in T-cell regulation. Several pathways, such as the Wnt/β-catenin pathway and the TGF-β signaling pathway, have been reported to correlate with T-cell regulation in the TME of HCC. Inhibition of the Wnt/β-catenin pathway has been reported to boost the infiltration and IFN-γ production of TIL CD8+ T cells and reduce the number of Tregs[38]. Inhibition of TGF-β promoted the activity of CD8+ T cells and reduced the action of CD4+ Treg cells by interrupting the differentiation of M2-type macrophages in HCC[39]. Tregs were induced under stimulation with lactate, *via* the PI3K/Akt/mTOR signaling pathway, to construct immunosuppression in HCC[40]. Meanwhile, GSEA between the high- and low-risk groups showed that cell cycle regulation (PYRIMIDINE_METABOLISM, BASE_EXCISION_REPAIR, RNA_DEGRADATION, NUCLEOTIDE_EXCISION_REPAIR and CELL_CYCLE) might be an important pathway in the TME of HCC. Although T-cell proliferation rarely occurred in infiltration analysis of HCC, Tregs were elevated and in proliferation status to fight against the antitumor immunity of HCC patients[41,42]. The metabolism of some amino acids (tryptophan, alanine, leucine, glycine, threonine, *etc.*) was related to the low-risk group. The metabolism of various amino acids in shaping tumor immunity and therapeutic efficacy in patients with cancer has long been investigated[43]. For example, alanine deprivation delays naive and memory T-cell activation to impact the TME[44]. By adding tryptophan, IDO(+) MΦ-suppressed T-cell responses could be reversed in the

TME of HCC[45]. T cells showed a defective response to antigen stimulation in SLC7A5-deficient mice because of leucine transportation failure[46]. Targeting the metabolism of various amino acids might be worthy of clinical application in the treatment of HCC.

CONCLUSION

In conclusion, we identified 7 prognostic genes that were closely related to the immune-related TME in HCC. A novel prognostic model based on 7 ICRGs was constructed to predict the OS and prognosis of HCC patients. The related potential functional information and TME profile of the 7 ICRGs were explored and depicted in HCC as well. The model and several potential chemotherapy drugs could provide useful insights into the potential clinical treatment of HCC.

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