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Brief Communication

Different races have different immune microenvironments: comparison of White and Asian patients with liver cancer

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Abstract: Different ethnic groups have different incidence rate of hepatocellular carcinoma (HCC). In addition to lifestyle and environmental factors, genetic susceptibility is also an important reason. In this study, we screened the immune related genes and stromal related genes in White and Asian liver cancer patients cohort of The Cancer Genome Atlas (TCGA) the using ESTIMATE algorithm. Hub genes that significantly associated with overall survival (OS) were selected from White and Asian liver cancer patients, respectively. In addition, we validated the functions of two hub genes, IL-18RAP and GPM6A, *in vivo* and *in vitro*. We confirmed different races have different tumor immune microenvironments. Immune microenvironment can influence and change the efficacy of immunotherapy for liver cancer patients.

Keywords: Hepatocellular carcinoma, tumor microenvironment, IL-18RAP, GPM6A, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is a kind of primary liver cancer with high mortality [1]. It is the most common malignant tumor in the world, especially in Asia, Africa and southern Europe [1]. The main risk factors of HCC include virus infection, alcohol intake and aflatoxin contamination [2]. Compared with European patients, Asian patients have more prevalent viral infection and higher tumor mutation burden (TMB) [3]. There are significant ethnic differences in the pathological process between European and Asian patients [3]. In recent years, more and more attention has been paid to the role of tumor microenvironment (TME) in tumorigenesis and development [4, 5]. However, no previous study showed the difference in TME of European and Asian patients.

TME is the internal environment in the process of tumor development and metastasis [4]. It is a complex mixture of tumor cells, stromal cells and extracellular matrix (ECM) [5]. Immune cells and stromal cells are two main compositions of microenvironment [6]. The environment further enhances the response of tumor cells through the soluble medium secreted by immune cells and stromal cells [6]. These two types of cells play important roles in proliferation, angiogenesis, invasion and metastasis in HCC [7]. At present, multidisciplinary treatment is still an effective treatment strategy to prolong the survival time and improve the quality of life of patients with liver cancer [8]. Compared with the traditional treatment, immunotherapy has the advantages of immune memory and no side effects, so it is necessary to further study the cancer immunity [9].

In this study, we investigated the effect of abnormal immune genome expression on the prognosis of the patients with HCC and the potential regulatory mechanism of these genes. We also compared the differ of the immune related genes in White and Asian patients and hope to provide the targeted genes for liver cancer immunotherapy.

Materials and methods

Data sources

Gene transcripts of liver cancer were downloaded from the public database The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/), including 374 cases of liver cancer tissues and 50 cases of normal liver tissues. The clinical data of liver cancer samples include age, gender, survival time, survival state, clinical stage, tumor grade and TNM stage.

Diferentially expressed genes (DEGs) and functional enrichment analysis

Wilcoxon rank sum test (FDR < 0.05, $\log_2$ |fold change| > 2) was used to screen genes with different expression in HCC and adjacent tissues, and then immune related genes were screened using R software "limma" package. Cluster analysis and heatmap generation were performed using R package "clusterProfiler" and "pheatmap" according to stromal/immune scores.

Cox regression analysis

The immune genes related to overall survival (OS) of HCC patients were obtained by univariate Cox regression analysis. The immune genes that could independently affect the prognosis were selected by multivariate Cox regression analysis. The risk score model was constructed using R package "forestplot".

Gene expression and survival analysis

The expression levels of CLEC5A, GPM6A, SIM1, and IL-18RAP were analyzed in normal and cancer tissues. The Kaplan-Meier (KM) survival analysis were used to compare the survival difference between the patients with the high and low expression levels of these genes using R package "survival" and "survminer".

Evaluation of tumor infiltrating immune cells

The proportion of immune cells was calculated using R package "Cibersport" in liver cancer tissues that divided into high and low expression group according to the expression levels of CLEC5A, GPM6A, SIM1, and IL-18RAP.

Enzyme-linked immunosorbent assay (ELISA)

This study was conducted according to the Helsinki Declaration of 1975 and approved by the Ethics Committee of Jinzhou Medical University. Blood samples were collected from 30 healthy individuals and 76 liver cancer patients before treatment at Center Hospital of Handan between July 2014 and July 2017. Serum IL-18 or IL-18RAP levels were measured using human IL-18 or IL-18RB (IL-18RAP) ELISA Kit as manufacturer's instructions (USCN Business, Wuhan, China).

Silico experiment

As the method in our previous study [10], the structure of IL-18 (PDB code: 1j0s) protein was retrieved from the Protein Data Bank (http://www.rcsb.org). The three-dimensional structure of IL-18RAP protein was predicted using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2). The interaction between IL-18 and IL-18RAP protein was analyzed by HEX 8.0.0 software.

Colony formation assay

Cells (1×10^3) treated with recombinant IL-18 protein, or IL-18RAP protein, or both (Sino Biological, Beijing, China) were plated in 24-well plates. After two weeks, colonies were fixed with 4% formaldehyde and stained with 1% crystal violet. The number of colonies was counted manually.

Transfection

Cells (3×10^5) were seeded in 6-well plates at 70% confluence and transfected with GPM6A overexpression plasmid and mock plasmid (Bio-Techne, Beijing, China) using Lipofectamine 3000 (Invitrogen, Shanghai, China).

Apoptotic assay

Cells were suspended in 400 μl binding buffer with Annexin V-FITC (0.5 μg/ml) and propidium iodide (PI, 50 μg/μl) according to the manufacturer's instructions (KeyGEN, Nanjing, China). After 30 min incubation at room temperature in the dark, the mixture were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Medical Devices, Shanghai, China).

Western blot

Cells were collected and lysed on ice for 30 min in 200 μl lysis buffer (KeyGEN) and then centrifuged at 13,000 g for 15 min. The supernatants were collected from the lysates and the protein concentration was determined. The pro-

teins (30 μg) were electrophoresed using a 8% sodium dodecysulfate-polyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes (Beyotime Biotechnology, Shanghai, China), which were then incubated with primary antibodies. GPM6A (SAB2501507, 1:200) was purchased from Sigma-Aldrich (Shanghai, China). Smad3 (#41181, 1:200), phospho-Smad3 (#9520, 1:200), Smad2 (#12584, 1:200), phospho-Smad2 (#8828, 1:200) and GAPDH (#5174, 1:1000) were purchased from Cell Signaling Technology (Shanghai, China). The nitrocellulose membranes were further incubated with secondary antibodies. Immunostaining was detected using an enhanced chemiluminescence (ECL) system (Beyotime Biotechnology).

Statistical analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Comparisons were made using chi-square tests, the Wilcoxon signed-rank test and the t-test. Overall survival was analyzed using the Kaplan-Meier method, and the significance of differences in survival rates was estimated using the logrank test. Values of $P<0.05$ were considered significant.

Results

Identification of the differ genes in White and Asian HCC patients based on stromal and immune scores

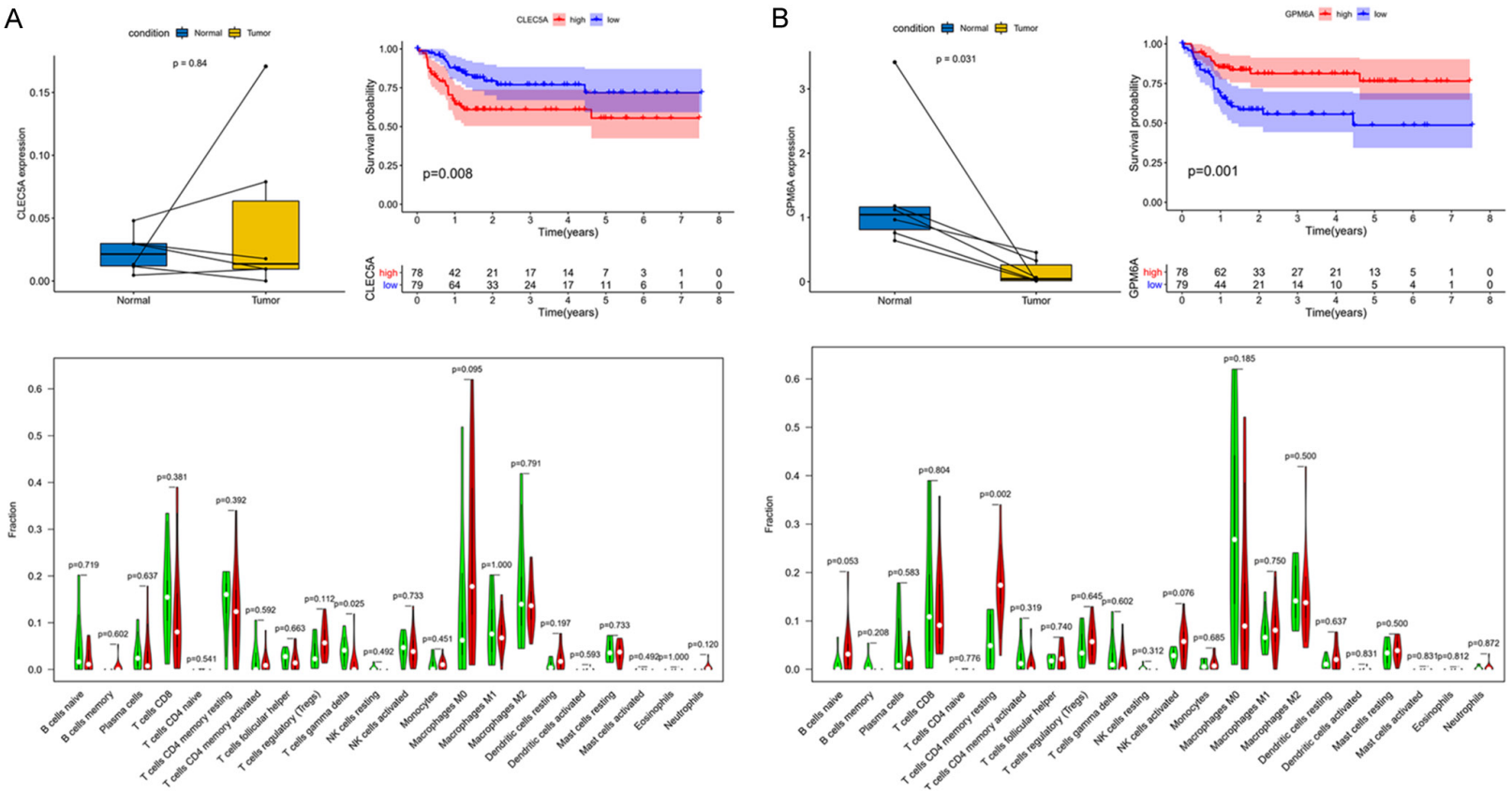
The heatmap demonstrated noticeable gene expression differences in White and Asian HCC patients with different stromal/immune scores. In Asian patients, compared to the low immune/stromal score group, 1259 genes were upregulated, and 48 genes were downregulated in high immune score group, 1748 genes were upregulated, and 62 genes were downregulated in high stromal score group (Supplementary Figure 1, P<0.05). In White patients, 889 genes were upregulated, and 62 genes were downregulated in the high score group, while there were 779 upregulated genes and 126 downregulated genes screened in high vs. low stromal score group (Supplementary Figure 1, P<0.05). The Venn diagrams in **Figure 1** showed the commonly upregulated genes and commonly downregulated genes in the high score groups. GO and KEGG enrichment analyses were performed to figure out the potential function of the genes in White and Asian HCC patients, respectively. In Asian patients, GO analyses revealed that these DEGs were involved in leukocyte cell-cell adhesion and T cell activation (Supplementary Figure 2A), while KEGG pathway analyses showed the DEGs were related with chemokine signaling pathway, cytokine-cytokine receptor interaction, cell adhesion and so on (Supplementary Figure 2B). In White patients, GO enrichment analysis showed that the immune signatures were involved in extracellular matrix organization, T cell activation and lymphocyte differentiation (Supplementary Figure 2C). KEGG pathways analysis showed significantly enriched pathways were cytokine-cytokine receptor interaction, chemokine signaling pathway, and osteoclast differentiation (Supplementary Figure 2D).

Generation of prognostic immune signatures in White and Asian HCC patients

To determine the roles of the DEGs in the overall survival of White and Asian HCC patients, the genes expression profiles and the corresponding survival information were screened using Cox regression analysis. As illustrated in the hazard ratio forest plot, eighteen genes, SEMA3C, ORAI2, HTRA3, NCF4, FMO2, CLEC5A, FCGR2A, NTM, S100A11, COL1A1, ARMCX1, GPR84, LRP8, GPM6A, HLA-DQB2, SYT13, MSC and DOK3 were identified as key prognostic immune related genes in Asian patients (Supplementary Figure 3A), while ten genes, P2RX1, C11orf96, CAMK4, KLRB1, TMIGD2, FLT3, KCNA3, ITK, SIM1 and IL-18RAP were identified in White patients (Supplementary Figure 3B). Kaplan-Meier plots revealed that highly expressed CLEC5A was associated with poor overall survival of Asian patients (**Figure 1A**, P=0.008), whereas the patients with low GPM6A expression will have a good survival compared with those with high expression (**Figure 1B**, P=0.001). IL-18RAP (P=0.001) and SIM1 (P=0.051) were unfavorable prognostic markers for White patients (**Figure 1C** and **1D**).

Influence of IL-18RAP and GPM6A in HCC patients and cells

We found that the serum IL-18RAP and IL-18 protein levels were lower in HCC patients compared with healthy volunteers using ELISA


A
condition Normal Tumor
p = 0.84
CLEC5A expression
Normal
Tumor
CLEC5A high low
Survival probability
p=0.008
Time(years)
high 78 42 21 17 14 7 3 1 0
low 79 64 33 24 17 11 6 1 0
B
condition Normal Tumor
p = 0.031
GPM6A expression
GPM6A high low
p=0.001
high 78 62 33 27 21 13 5 1 0
low 79 44 21 14 10 5 4 1 0
Fraction
B cells naive
B cells memory
Plasma cells
T cells CD8
T cells CD4 naive
T cells CD4 memory resting
T cells CD4 memory activated
T cells follicular helper
T cells regulatory (Tregs)
T cells gamma delta
NK cells resting
NK cells activated
Monocytes
Macrophages M0
Macrophages M1
Macrophages M2
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Dendritic cells activated
Mast cells resting
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Eosinophils
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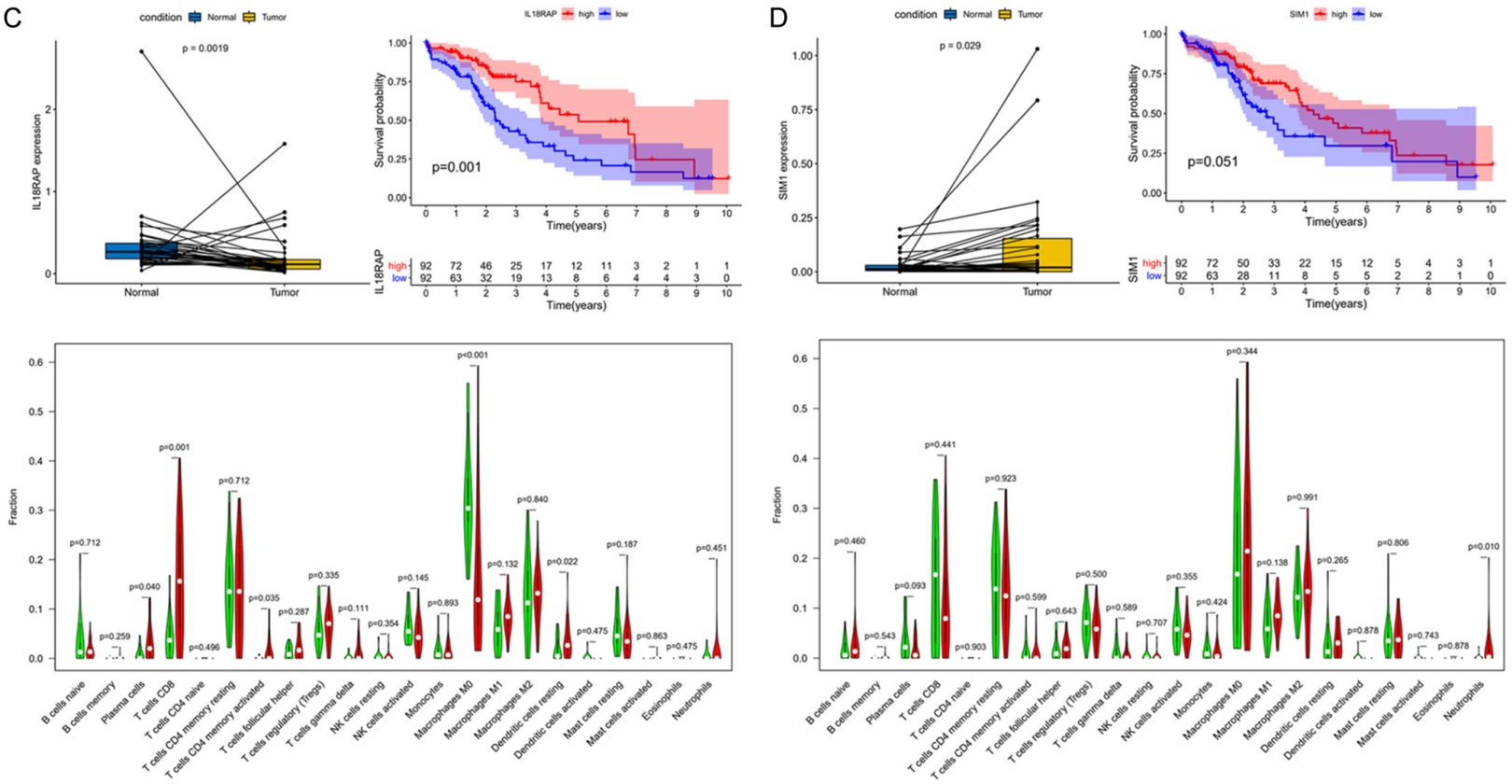

Figure 1. Kaplan-Meier plots for Asian HCC patients based on the expression levels of CLEC5A (A) and GPM6A (B). Kaplan-Meier plots for White HCC patients based on the expression levels of IL-18RAP (C) and SIM1 (D).

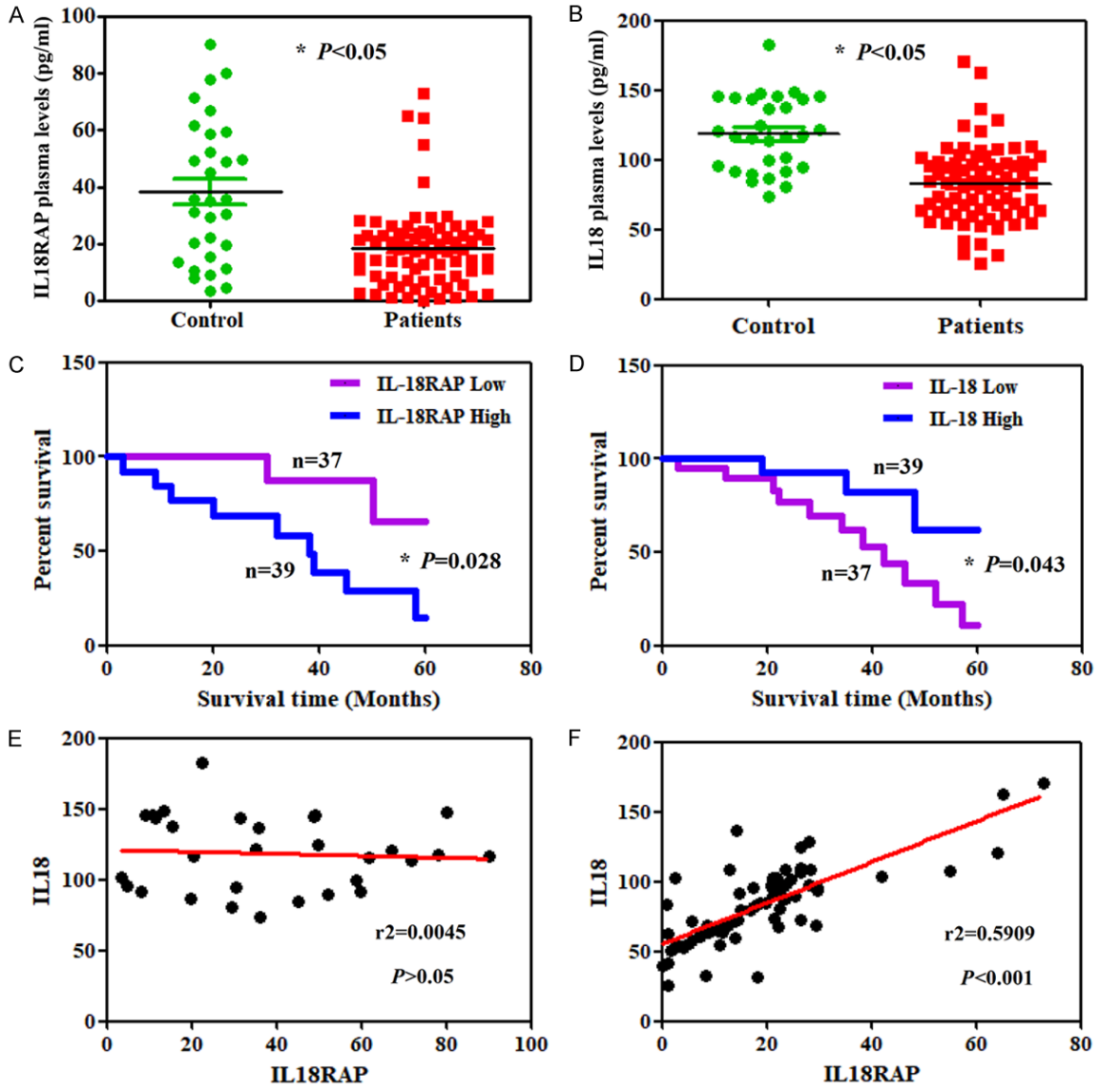

Figure 2. IL-18RAP (A) and IL-18 (B) serum levels from the HCC patients and healthy controls were detected using ELISA. Kaplan-Meier curves of the survival rate of the HCC patients based on their IL-18RAP (C) and IL-18 (D) serum levels. Correlation between IL-18 and IL-18RAP in healthy volunteers (E) and HCC patients (F).

(**Figure 2A** and **2B**, P<0.05). The serum IL-18RAP and IL-18 in 76 patients ranged from 0.1 pg/ml to 72.8 pg/ml with a median of 18.1 pg/ml, and from 26.1 pg/ml to 170.3 pg/ml with a median of 83.45 pg/ml, respectively. Furthermore, we found that serum IL-18RAP and IL-18 were associated with tumor number, tumor differentiation, tumor size, and lymph node metastasis of liver cancer (**Table 1**, P<0.05). Low serum IL-18RAP was correlated with the favorable prognosis, while high serum IL-18 was correlated with the favorable prognosis of the patients with liver cancer, using Kaplan-Meier analysis (**Figure 2C** and **2D**, P<0.05). In healthy volunteers, no correlation between serum IL-18RAP and IL-18 (**Figure 2E**, P>0.05), however, positive correlation between serum IL-18RAP and IL-18 was observed in the patients (**Figure 2F**, r^2=0.5909, P<0.001).

In addition, we validated the functions of IL-18RAP and GPM6A in liver cancer cells. In silico analysis showed the interaction between IL-18RAP and IL-18 (**Figure 3A**). Recombinant IL-18 protein inhibited the proliferation of HepG2 and SMMC-7721 cells using colony for-

Table 1. Serum IL-18RAP and IL-18 associated with the clinicopathological features of 76 hepatocellular carcinoma patients

Clinicopathological features	n (76)	IL-18RAP				IL-18			
		Low (37)	High (39)	χ^2	P	Low (37)	High (39)	χ^2	P
Sex				0.024	0.8769			0.682	0.4088
Female	24	12	12			10	14		
Male	52	25	27			27	25		
Age (years)				0.418	0.5178			0.030	0.8618
<55	28	15	13			14	14		
≥55	48	22	26			23	25		
Tumor number				15.12	0.0001			12.24	0.0005
Multiple	44	13	31			29	15		
Solitary	32	24	8			8	24		
Differentiation				15.002	0.0001			9.027	0.0027
Differentiated	34	25	9			10	24		
Undifferentiated	42	12	30			27	15		
Portal invasion				0.0018	0.9659			0.783	0.3761
-	31	15	16			17	14		
+	45	22	23			20	25		
Lymph node metastasis				8.672	0.0032			4.637	0.0313
-	21	16	5			6	15		
+	55	21	34			31	24		
Tumor size (cm)				7.070	0.0078			8.241	0.0041
< 5	22	16	6			5	17		
≥ 5	54	21	33			32	22		
HBV infection				0.0069	0.9339			1.884	0.1698
-	25	12	13			15	10		
+	51	25	26			22	29		

Abbreviations: χ^2 value, Chi-square distribution.

mation assay (**Figure 3B**, P<0.05). Although, recombinant IL-18RAP protein didn't change the proliferation of these cells, it can reverse the inhibitory effect of IL-18 (**Figure 3B**). Apoptotic ratio of HepG2 and SMMC-7721 cells with GPM6A overexpression was increased using Annexin V-FITC/PI double staining (**Figure 3C**, P<0.05). Compared with parental cells, p-Smad2 and p-Smad3 were upregulated in GPM6A overexpressed HepG2 and SMMC-7721 cells, while total levels of Smad2 and Smad3 didn't change (**Figure 3D**, P<0.05).

Discussion

The immune microenvironment of liver cancer is highly heterogeneous, which is one of the important reasons for drug resistance, recurrence and poor prognosis of liver cancer [4, 5]. Abnormal expression of tumor immune related genomes plays an important role in the process of tumor immune escape [11]. Immune related genes will mutate in the long evolutionary process of human beings [12]. Through the accumulation of generations, a variety of immune systems have been formed in different races [12]. Racial disparities have been considered in recent studies, especially genetic polymorphism and socioeconomic status [13]. In this study, we compared the immune-related genes in White and Asian HCC patients based on TCGA database for the first time. The differ genes were identified in stromal and immune cells of White and Asian HCC patients. After screening, eighteen genes and ten genes were identified as key prognostic immune related genes in Asian patients and White patients, respectively. These evidence showed Asian patients and White patients have huge genetic differences in liver cancer tissues.

We chose four genes, CLEC5A and GPM6A from the Asian HCC patients, and IL-18RAP and SIM1 from the White HCC patients for further analy-

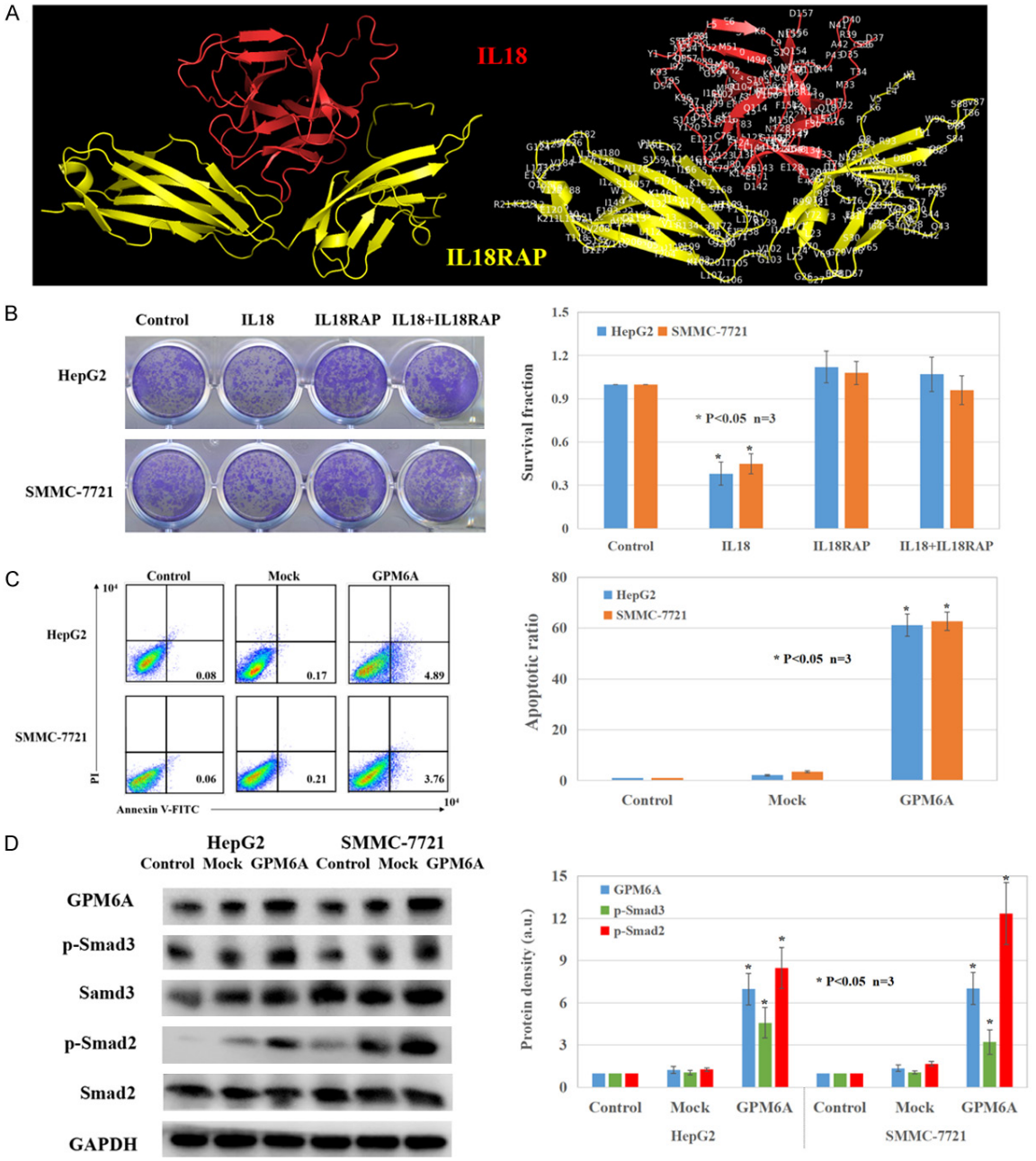

Figure 3. The functions of IL-18RAP and GPM6A in HCC cells. A. The interaction between IL-18 and IL-18RAP was simulated using HEX 8.0.0 software. B. The proliferation ratio was analyzed using colony formation assay. C. The proportion of apoptotic cells was determined by Annexin-V/FITC and PI double staining. D. Western blot analysis of the Smad signaling pathway. GAPDH was used as an internal loading control.

sis. Based on Cox regression analysis, the CLEC5A and SIM1 were hazard factors for HCC patients, while GPM6A and IL-18RAP were protective factors for HCC patients.The expression of these four genes is different in tumor and normal tissues of the patients, and is related to the prognosis of the patients. In our collected blood samples, we found low serum IL-18RAP and IL-18 levels in liver cancer patients. IL-18 is a cytokine produced by activated macrophages [14]. It can play an anti-tumor role by inducing immune cell proliferation and enhancing its activity, promoting the synthesis and secretion of various cytokines, and enhancing Fas-FasL mediated apoptosis [14]. IL-18RAP is a natural antagonist of IL-18, which plays an important

role in immune regulation [15]. Previous studies have demonstrated IL-18 secretion were downregulated in HCC patients in China [16, 17]. Consistent with these studies in China, we also found the low level of IL-18 in the peripheral blood of HCC patients. Interestingly, the studies in other countries, such as Italy [18], Egypt [19], Türkiye [20], and Thailand [21], showed a higher levels of serum IL-18 in HCC patients compared to healthy controls. This is a strong evidence that patients of different races have different immune microenvironments. We also identified the low level of serum IL-18RAP in the HCC patients. In addition, serum IL-18RAP could be used as a diagnostic marker for these patients. However, we didn't find any effect of IL-18RAP alone on HCC cells *in vitro*. The main reason is that IL-18RAP, as an antagonist of IL-18, can reverse the tumor suppressing roles of IL-18, however, it has no direct effect on the tumor cells themself.

GPM6A is involved in the occurrence and progression of liver cancer [22]. Low expression of GPM6A in HCC tissues is associated with poor prognosis of liver cancer [22]. GPM6A overexpression inhibited the proliferation and invasion of HCC cells [23]. In this study, we found that GPM6A induced apoptosis in HCC cells. Mechanically, GPM6A expression was inhibited by miRNA-96 and miR-106b-5p in HCC cells [22, 23]. GPM6A promotes HCC progression by activating the AKT/ERK signaling pathway [23]. We found GPM6A activated Smad signaling pathway in HCC cells for the first time. Smad pathway, as an important negative regulatory pathway of epithelial cell proliferation, regulates tumor cell proliferation and apoptosis [24]. Smad2 and Smad3 are important components of this pathway [24]. In the early stage of tumor, Smad pathway inhibits the proliferation and induces apoptosis of cancer cells [25]. In the late stage of tumor, it plays a role in enhancing tumor progression by promoting tumor cell invasion, participating in immunosuppression and reconstructing microenvironment [25]. Therefore, Smad pathway is a double-edged sword for cancer therapy. Although our data demonstrated GPM6A is a suppressor for liver cancer cells through Smad pathway, the function of GPM6A needs to be validated *in vivo*.

In this study, we screened the immune related genes in different races and confirmed Asian and White HCC patients have different immune microenvironments. In addition, we validated the hub genes that selected from Asian and White HCC patients *in vivo* and *in vitro*. More and more tumor-targeted drugs have been used for the treatment of liver cancer. A comprehensive analysis of the composition of the immune microenvironment of different ethnic groups will greatly improve the effectiveness of targeted therapy for liver cancer.

Acknowledgements

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All patients and volunteers have signed the informed consent form. Their data were anonymized.

Disclosure of conflict of interest

None.

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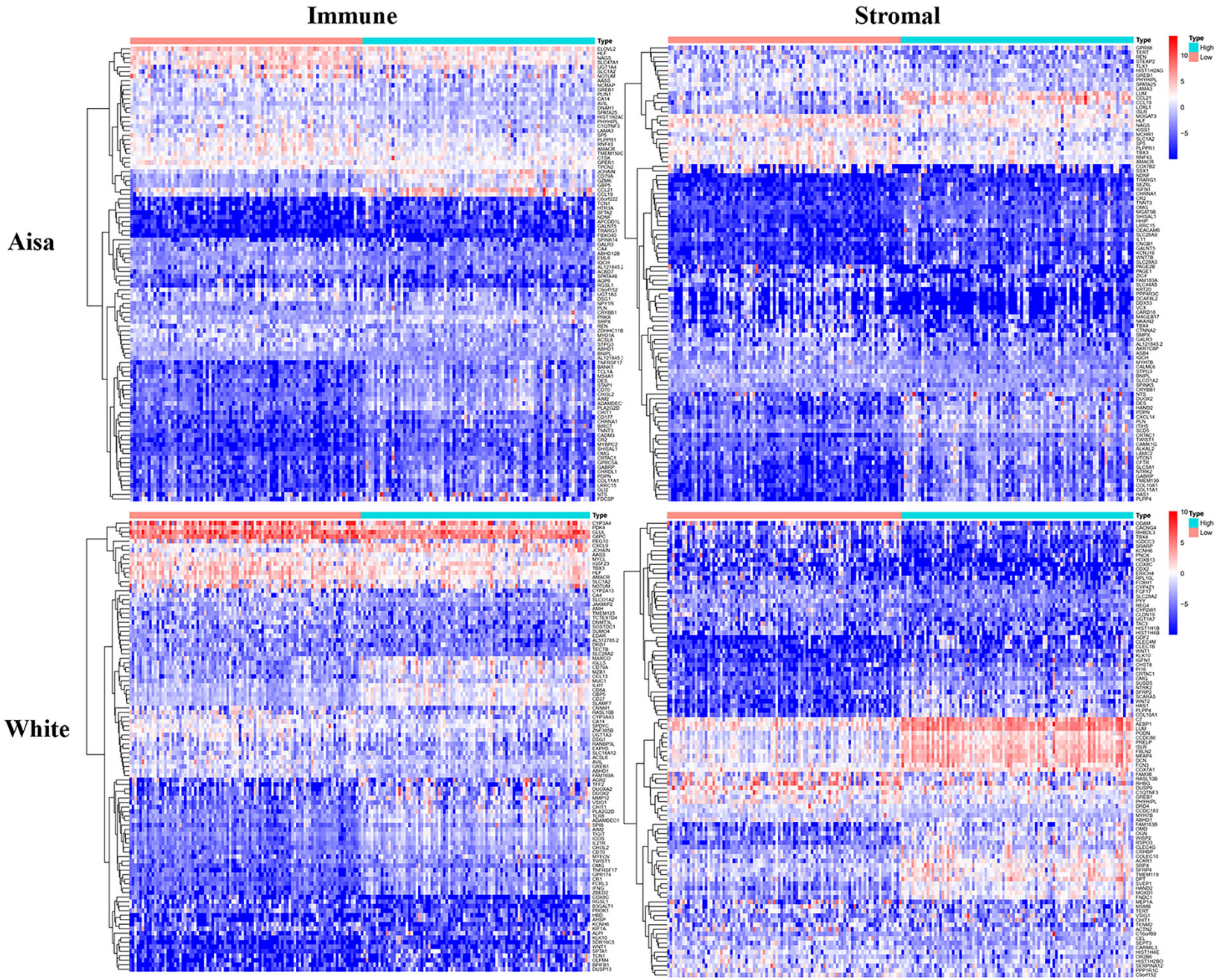
Immune
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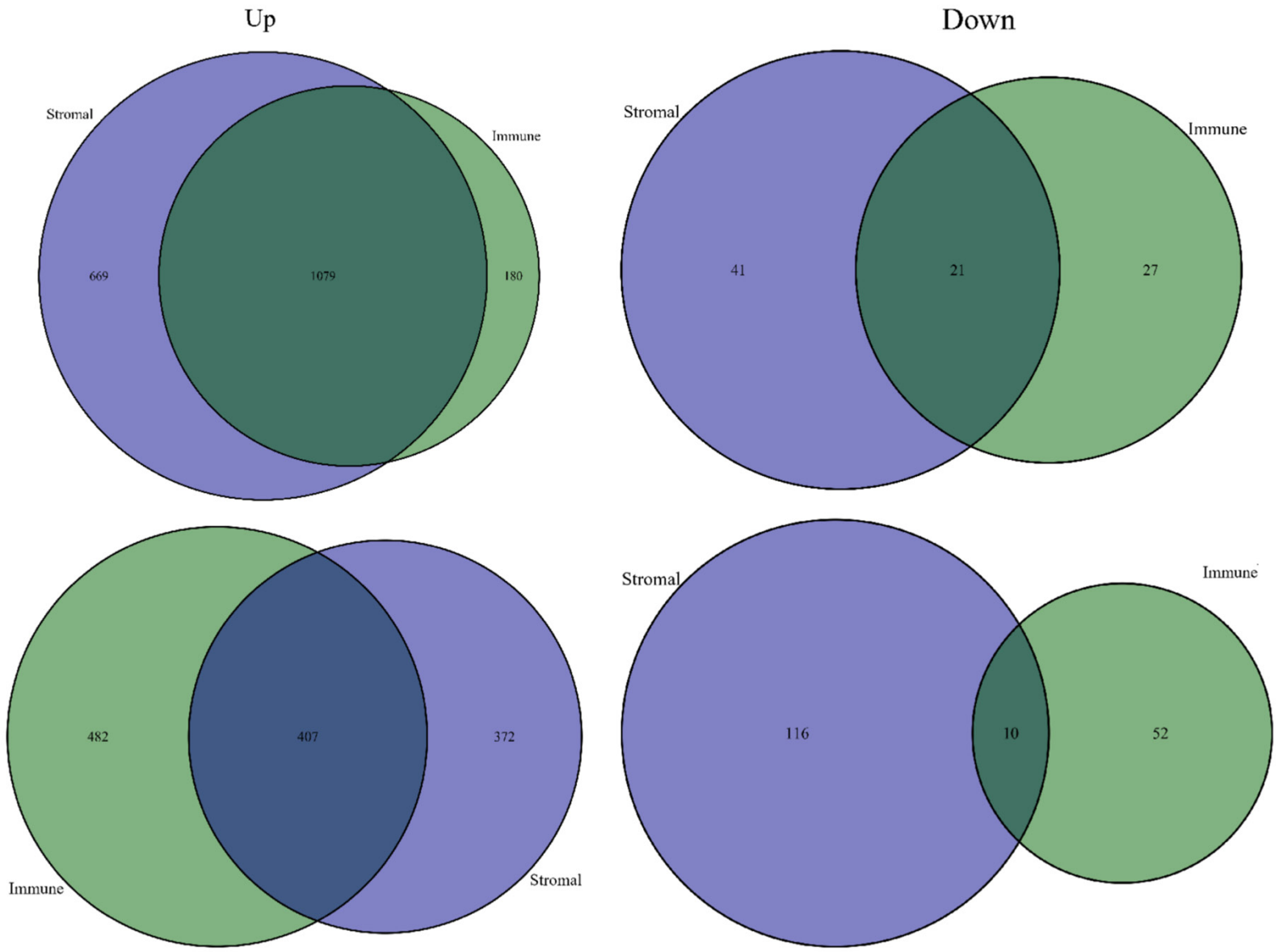
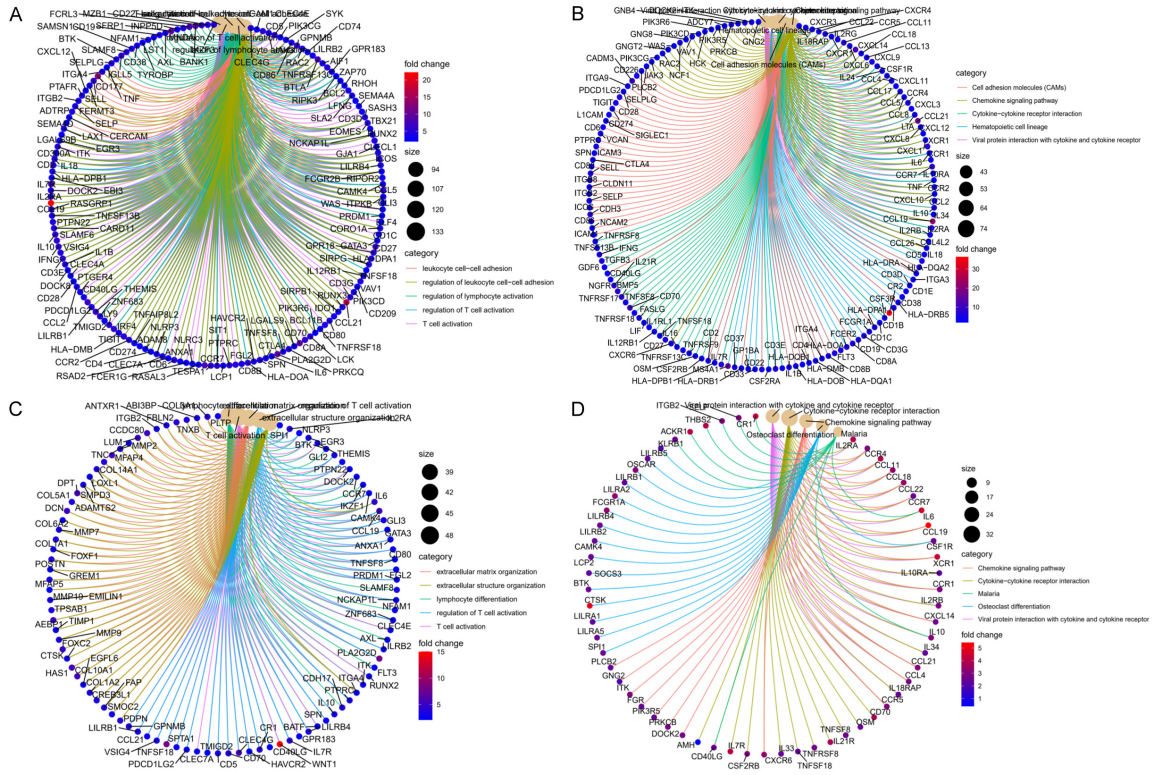
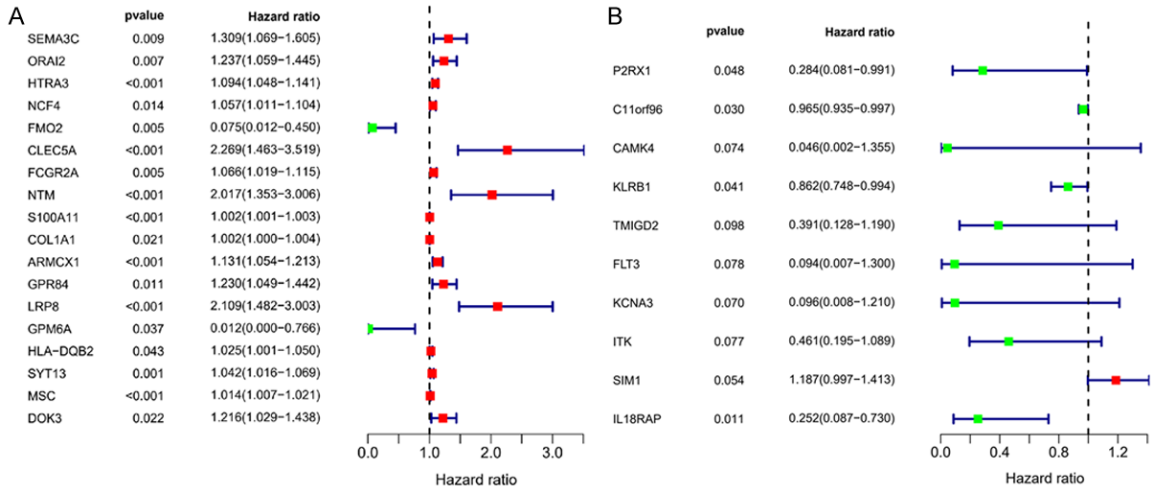


Supplementary Figure 1. Heatmap of the DEGs in Asian and White HCC patients was generated using R software v4.0.3 based on stromal scores and immune scores. Venn diagrams showing the number of commonly upregulated or downregulated DEGs in the different groups.



Supplementary Figure 2. GO enrichment analysis of DEGs in Asian (A) and White (B) HCC patients. KEGG enrichment analysis of DEGs in Asian (C) and White (D) HCC patients.



Supplementary Figure 3. Forest plot of immune-related prognostic genes in Asian (A) and White (B) HCC patients using Cox regression analysis.