Journal of Translational Medicine

RESEARCH

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Multiplex immunohistochemistry to explore the tumor immune microenvironment in HCC patients with different GPC3 expression

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Abstract

Objectives GPC3 has been recognized as a promising target for immunotherapy in hepatocellular carcinoma (HCC). However, the GPC3-targeted immunotherapies have shown limited therapeutic efficacy. The use of anti-PD-1/PD-L1 monoclonal antibodies in HCC treatment is considerably constrained. Furthermore, there is still a notable lack of understanding concerning the immune landscape in HCC, especially regarding varied GPC3 expression levels. Therefore, thorough exploration of the intricate tumor immune microenvironment at different GPC3 expression levels is essential for guiding and improving HCC treatment strategies.

Methods Sixty patients with HCC were enrolled in this study, receiving a first-line treatment that combined anti-angiogenesis targeted drugs and immunotherapy. Immunohistochemistry was used to assess the levels of GPC3 expression. Multiple immunohistochemical markers, such as CD8, PD-1, LAG3, TIGIT, TIM-3, CD103, Claudin18.2, PD-L1, CD4, Foxp3, CD68, CD163, GPC3, CD11C, CD14, CD66b, and HLA-DR, were used to characterize the immune microenvironment and spatial distribution of immune cells in HCC tumors with different levels of GPC3 expression. Cell expression levels and spatial distribution were determined by fluorescence staining and subsequent analysis of fluorescence intensity using the Panoramic Pathology Workstation (Pano ATLAS). This approach facilitated a detailed examination of cell characteristics and spatial information within the samples.

Results Based on the result of GPC3 immunohistochemical analysis, patients with strong positive GPC3 expression were classified as high GPC3 expression, while the others were classified as low GPC3 expression. Patients in the low GPC3 expression group had longer overall survival (OS) than in the high group ($P=0.003$, HR = 2.9240). Further exploration of the immune microenvironment based on different GPC3 expression levels revealed that in high GPC3 expression group, the proportions of $CD8^+$ T cells($P=0.0435$), TIM-3^+ T cells($P=0.0447$), $CD103^+CD8^+$ tissue-resident T cells($P=0.0410$), $CD11C^+CD14^-$DC cells($P=0.0497$), $CD11C^+$HLA-DR^-DC cells($P=0.0309$), $CD11C^+CD14^-$HLA-DR^-DC cells($P=0.0233$), and $CD11C^+CD14^-CD66b^-$DC cells($P=0.0474$) were all higher compared to low expression group.

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At spatial distances of 10 μm, 20 μm, and 30 μm, the levels of $CD8^+$ T cells were higher in the high expression group compared to the low expression group (high vs. low: $P=0.0281$, $P=0.0236$, $P=0.0220$).

Conclusions Multiple immunohistochemistry is a powerful technique for exploring the intricate immune microenvironment of hepatocellular carcinoma, enabling the precise identification of diverse cell subsets and their spatial distribution within the tumor microenvironment. This methodology provides valuable insights into the complex interactions and spatial organization of immune cells in the context of hepatocellular carcinoma progression. Low GPC3 expression in HCC patients indicates potential benefits from combined targeted and immunotherapy. Different levels of GPC3 expression levels can predict the effectiveness of targeted combination immunotherapy in HCC patients. Additionally, different GPC3 expression patterns in HCC patients correspond to unique tumor immune microenvironments, which have implications for guiding HCC treatment approaches.

Keywords HCC, GPC3, Antiangiogenic targeted drugs combined with immunotherapy, Biomarker, Tumor immune microenvironment

Introduction

Cancer is the second most common disease after cardiovascular diseases, responsible for deaths all over the world [1]. Hepatocellular carcinoma (HCC), a primary liver cancer, is the third leading cause of cancer-related deaths globally [2, 3]. Its incidence and mortality rates are increasing worldwide, and HCC constitutes approximately 90% of all primary liver cancers [4, 5]. According to the IMbrave150 study, the combination of anti-angiogenesis-targeted drugs with anti-PD-L1/anti-PD-1 monoclonal antibodies has emerged as the first-line standard of care for patients with unresectable HCC [6]. Compared to monotherapy, the use of this combination strategy has shown notable enhancements in patient overall response rates (ORR) and prolonged overall survival (OS). However, the identification of potential predictive biomarkers for treatment response continues to pose challenges.

Glypican-3 (GPC3) is a heparan sulfate proteoglycan anchored to the cell membrane via glycophosphatidylinositol (GPI) [7].GPC3 is not expressed in normal tissues but is highly expressed in 70% of HCC, with GPC3-positive patients have a worse survival prognosis [8–10]. GPC3 has been widely employed as a diagnostic biomarker for HCC and is also regarded as a promising target for immunotherapy in the treatment of HCC. Immunotherapy specific to GPC3, mainly including immunotoxins, vaccines, bispecific antibodies and chimeric antigen receptor T-cell therapy (CAR-T) [11]. However, the effect of GPC3-targeted immunotherapy in clinical trials is not significant. A phase I clinical trials to assess the chimeric antigen receptor (CAR)-glypican-3 (GPC3) T-cells, the potential for the treatment of HCC [12]. A total of 13 patients received treatment, 3 years, 1 year and 6 months of total survival rate were 10.5%, 42.0% and 50.3%, respectively. Another Phase I clinical study involved 6 patients who underwent GPC3-CAR-T treatment [13]. The objective response rate was 16.7%, with the median progression-free survival (mPFS) of 3.5 months, the median disease control period of 3.2 months, and the median overall survival (mOS) of 7.9 months. Two phase I clinical studies assessed the safety and efficacy of the GPC3 peptide vaccine in HCC treatment [14]. However, no patients achieved a complete or partial response in these trials. GC33, a recombinant humanized anti-glypican-3 antibody, was used to treat HCC patients with different GPC3 expression levels [15]. The median time to progression (TTP) was 26.0 weeks in the high expression group and 7.1 weeks in the low expression group ($P=0.033$). A phase II study investigated codrituzumab, a humanized monoclonal antibody targeting Glypican-3, in patients with advanced HCC following failure of previous systemic therapy. Regrettably, the clinical trials failed to demonstrate favorable outcomes in the context of liver cancer treatment with codrituzumab.

The tumor immune microenvironment (TME) of HCC is characterized by a complex spatial arrangement that includes mesenchymal cells, tumor cells, immune cells, and tumor-associated fibroblasts [16]. These constituents collectively contribute to non-cellular components of the tumor stroma, encompassing a diverse array of growth factors, protein-degrading enzymes, their inhibitors, and inflammatory cytokines. These factors play a crucial role in shaping the therapeutic effectiveness within the HCC TME. Studies have reported the involvement of GPC3 in the Wnt/β-catenin signaling pathway in HCC [17]. The Wnt/β-catenin signaling pathway plays a crucial role in the biology of the liver. In HCC, the Wnt/β-catenin signaling pathway exerts direct or indirect effects on effector T cells, regulatory T cells, helper T cells, dendritic cells, and other cytokine-expressing immune cells, subsequently influencing the regulation of the HCC tumor microenvironment [18]. It was found that the inhibition of the secretion of high levels of soluble programmed death ligand 1 (sPD-L1) in HCC patients reversed GPC3-CAR-NK cell inhibition [19]. Additionally, the anti-PD-1 monoclonal antibody improved the anti-tumor effect of

3p-GPC-3 siRNA and reversed immune failure [20]. This suggests that immunosuppression mediated by the tumor microenvironment is a factor hindering the effectiveness of GPC3-targeted immunotherapy.

Multiple immunohistochemistry, as a robust technique, facilitates the simultaneous detection of up to 8 biomarkers, ensuring the identification of distinct cell populations and their spatial distribution within the tumor microenvironment, all while mitigating concerns regarding cross-reactivity [21]. A study employing mass spectrometry and single-cell RNA sequencing to investigate the correlation between peripheral blood immune cell components and the response to immunotherapy in liver cancer patients revealed a significant association between $CXCR3^+CD8^+$ effector memory T cells and $CD11C^+$ antigen-presenting cells in the peripheral blood of patients undergoing immunotherapy and the treatment response rate [22]. However, these methodologies are limited in capturing the spatial distribution of immune cells within the liver cancer tissue in situ. In a foundational study of early HCC recurrence mechanisms, single-cell RNA sequencing illuminated that tumor cells in early recurrent HCC possess the capability to suppress dendritic cell activation of $CD8^+$T cells and exhibit heightened immune evasion characteristics. Moreover, the utilization of multiple immunofluorescence staining (mIF) revealed the proximity of $CD80^+CD1C^+$ macrophages to PD-$L1^+$ tumor cells within recurrent HCC tissues, shedding light on the underlying mechanisms of recurrence [23]. Hence, multiple immunohistochemistry staining technology enables the precise detection of cell phenotypes and spatial tissue morphology information in situ, effectively overcoming the challenges encountered by conventional methods that frequently struggle to simultaneously capture both phenotypic and morphological characteristics.

In summary, the predictive role of GPC3 in targeted combination immunotherapy remains uncertain, and the association between GPC3 expression and the tumor immune microenvironment is still ambiguous. Hence, the primary objective of this study was to investigate the HCC tumor microenvironment across varying levels of GPC3 expression using multiplex immunohistochemistry, improve the therapeutic outcomes and offer personalized guidance on drug usage for patients with HCC.

Methods

Patients

This study included 60 HCC patients who received anti-angiogenesis targeted drugs combined with immunotherapy at the Comprehensive Cancer Center of Drum Tower Hospital of Nanjing University from 2018 to 2023. Paraffin specimens of tumor tissues before receiving treatment were available in each case, and the patients' basic clinical information and follow-up information were complete. The study was approved by the Ethics Committee of Drum Tower Hospital of Nanjing University.

Immunohistochemistry

The patients' paraffin blocks were collected and cut into 4–5 μm thick serial slices using a slicer, with at least 2 slices per patient. One sheet was used for HE staining and the other for GPC3 immunohistochemical staining. Sections were subjected to antigen repair, primary antibody incubation, secondary antibody incubation and sealing before being placed under a light microscope to interpret the staining results. Immunohistochemical staining scores were calculated based on the percentage of positive cells and the intensity of staining of positive cells, with a corresponding value of staining intensity × percentage of positive cells of 0–1 defined as negative (-), 2–4 defined as weakly positive (+), 5–8 defined as moderately positive (++), and 9–12 defined as strongly positive (+++).

Multiple immunohistochemistry

Patient paraffin tissue sections were obtained and stained according to the instructions of Multiplex immunohistochemistry Staining Kit after deparaffinization, fixation, antigen repair, and primary/secondary antibody incubation, including: panel 1: CD8, PD-1, LAG3, TIGIT, TIM3; panel 2: CD8, CD103, Claudin18.2, PD-L1, PANCK; panel 3: CD4, Foxp3, CD68, CD163, GPC3; panel 4: CD11C, CD14, CD66b, HLA-DR, PANCK; each fluorescent dye was reacted for 10–15 min. At the end of the staining, DAPI was stained with DAPI staining solution; at last, the slices were quenched and sealed with antifluorescent quenching, and stored away from light.

Multiple immunohistochemistry imaging and region selection

Fluorescence images of sections were acquired by a high-throughput panoramic scanner (Pano VIEW VS200). Two pathologists observed the scanned fluorescence section images through OlyVIA software to delineate the tumor region and mesenchymal region. The two pathologists were responsible for controlling the quality of the stained areas to ensure that the fluorescence of all sections was within the appropriate signal intensity range. Finally, the images processed by the pathologists were transferred to a panoramic pathology workstation (Pano ATLAS) for image and statistical analysis.

Recognition of cell morphology and spatial distribution

Multiple immunohistochemistry image features were extracted by Panoramic Pathology Workstation (Pano ATLAS). Target proteins were labelled with special antibodies and presented as fluorescent carriers on single-stained sections. Spectral libraries were created based

on these fluorophores. Autofluorescence spectra of tissues were extracted from unstained sections. Based on the single-stained slides of each fluorophore, a spectral library was created to provide a reference for cell phenotyping. Based on the images of fluorescent signals in the spectral library, cell phenotypic features were extracted to identify each cell. Through the above steps, the positivity rate (percentage of target cells to the total number of cells in the region), cell density (ratio of target cells to the area of the region within the region), and spatial distribution characteristics of each cell type in the tissue microenvironment were calculated and analyzed. Quantify the effective scoring of central tumor cells and surrounding immune cells in forming spatial networks within a defined radius, defined as follows: $\text{Effective score} = \frac{\text{Number of immune cells in radius}}{\text{Central tumor cell count}}$。

Statistical analyses

Survival analyses were performed using the Kaplan-Meier method, and comparisons between groups were performed using t-tests (continuous data) and χ^2 tests (categorical data). All analyses were achieved by R studio version 4.3.2, and all tests were two-sided, with statistical significance at $P < 0.05$.

Results

Patient clinical information

A total of 60 patients with HCC were included in this study, and the first-line treatment regimen for all patients was a combination of antiangiogenic targeted agents and immunotherapy (Fig. 1a). The mean age of the patients was 58 years and 95% of the patients were male (Table 1). Among the cohort, 1 patient (2%) had an efficacy evaluation of CR, 18 (30%) patients were PR patients, 30 (50%) patients were SD, and 11 (18%) patients were PD. GPC3 expression in patients was detected by immunohistochemistry and was negative in 20% (12/60) patients, weakly positive in 30% (18/60) patients, moderately positive in 22% (13/60) patients and strongly positive in 28% (17/60) patients.

Association of GPC3 expression with clinical features and immunotherapy

To further explore the relationship between GPC3 expression and HCC immunotherapy, we analyzed the survival curves of different GPC3 expression strata. It has found that OS was longer in patients with negative and weak/moderate GPC3 positivity than in strong positivity patients ($P = 0.0033$, HR = 2.9240), none of the other survival curves of GPC3 expression strata were statistically significant (Fig. 2). Therefore, we defined patients with strong positive GPC3 immunohistochemical scores as patients with high GPC3 expression, and negative immunohistochemical scores and weak/moderate positivity as patients with low GPC3 expression (Fig. 1c). Examining the relationship between patients with different GPC3 expression and other clinical features, we found that GPC3 expression was associated with AFP expression ($P = 0.0059$) (Table 2).

In addition, the proportion of GPC3 positive cells and cell density in different GPC3 expression tissues were detected by immunohistochemistry. It was found that both the proportion of GPC3-positive cells and cell density in tissues with high GPC3 expression were higher than those in tissues with low GPC3 expression ($P = 0.04950$, $P = 0.0497$) (Fig. 3).The proportion and cell density of GPC3-positive cells in patients with AFP ≥ 400 were higher than those in AFP < 400 patients were high (high vs. low: $P = 0.0312$, $P = 0.0297$). No statistical differences in the proportion of GPC3-positive cells and cell density were reached in gender, age, ECOG score, history of hepatitis B, and HCC differentiation (Fig. 3).

Multiple immunohistochemistry to explore different GPC3 expression tumor microenvironments

GPC3 is an important target for the treatment of HCC, and various immunotherapies have been developed based on the GPC3 target, but with poor efficacy. One of the main reasons for this is the complex immune microenvironment of HCC, therefore, we explored the different GPC3-expressing HCC tumor microenvironment by multiplex immunohistochemistry (Fig. 1b) and analyzed the expression of different GPC3 expression immune cell subtypes.

The results showed that throughout the HCC tissues with high expression of GPC3, $CD8^+$ T cells($P = 0.0435$), $TIM3^+$ T cells($P = 0447$), $CD103^+CD8^+$ tissue-resident memory T cells($P = 0.0410$), $CD11C^+CD14^-$DC cells($P = 0.0497$), $CD11C^+$HLA-DR^-DC cells($P = 0.0309$), $CD11C^+$CD14-HLA^-DR^-DC cells($P = 0.0233$), and $CD11C^+CD14^-CD66b^-$DC cells($P = 0.0474$) all had a higher proportion of cells than in low expression tissues (Fig. 4). In high GPC3 expression tissues, $CD8^+$ T cells($P = 0.0406$), $TIM3^+$ T cells($P = 0.0409$), $CD8^+$PD-$L1^-$T cells($P = 0.0471$), $CD103^+CD8^+$ tissue-resident T cells($P = 0.0453$), $CD11C^+$DC cells($P = 0.0392$), $CD11C^+CD14^-$DC cells($P = 0.0376$), $CD11C^+CD66b^-$DC cells($P = 0.0356$), $CD11C^+$HLA-DR^-DC cells($P = 0.0197$), $CD11C^+CD14^-CD66b^-$DC cells($P = 0.0337$,), $CD11C^+CD14^-$HLA-DR^-DC cells($P = 0.0185$), had higher cell densities than in tissues with low expression of GPC3 (Fig S1).

Spatial analysis of HCC different GPC3 expression tumor cells and immune cells

Multiple immunohistochemistry was able to accurately characterize the location of individual tumor cells and

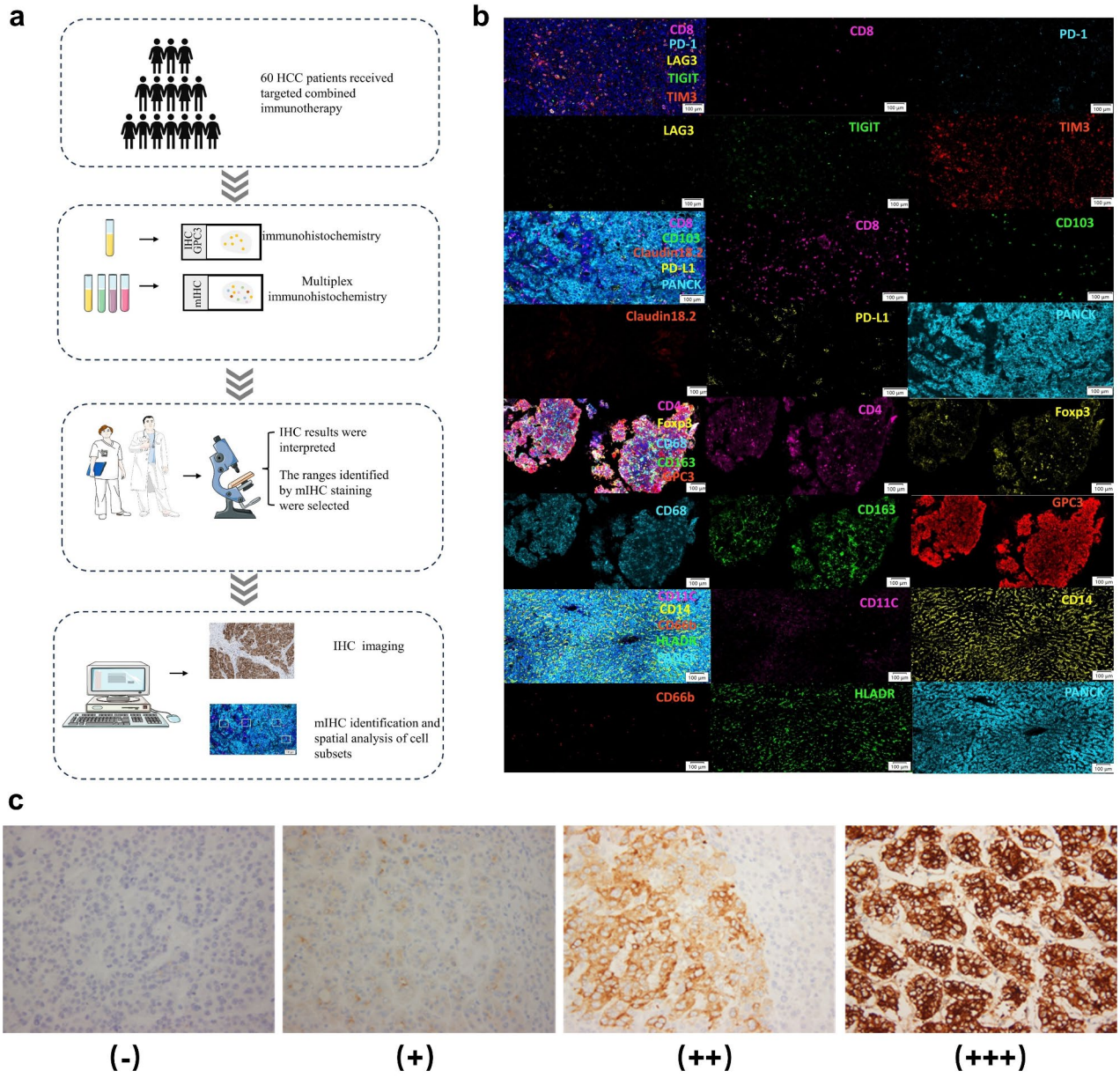


Fig. 1 (**a**) Flowchart of the study; (**b**) Representative image of 4 panel multiple immunohistochemistry; (**c**) Representative image of GPC3 immunohistochemical staining(40×)

immune cells. Therefore, we also assessed the spatial distribution of various types of immune cells in HCC.

We analyzed the spatial distribution of cells within 3 radii of 10μm, 20 μm and 30 μm (radius is the distance from tumor cells to immune cells) (Fig. 5a). The results showed that the effective proportions of $CD68^+CD163^+$ macrophages, $Foxp3^+$ regulatory T cells, $CD8^+$ T cells, $CD11C^+$ DC cells, $CD14^+$ monocytes, $CD66b^+$ neutrophils, and $CD103^+$ memory T cells were all higher in tissues with high expression of GPC3 than those with low expression of GPC3, although only the $CD8^+$ T cells reached statistical significance (high vs. low: $P = 0.0281$, $P = 0.0236$, and $P = 0.0220$, respectively) (Fig. 5b-d). These results indicate that the HCC tumor microenvironment is different for different GPC3 expression. In addition, we also analyzed the spatial analysis of $CD8^+PD\text{-}L1^+$ cells within these 3 radii, and the $CD8^+PD\text{-}L1^+$ cells were all higher in the high GPC expression group than in the low GPC3 expression group, but did not reach statistical significance (Fig S2).

Table 1 Basic clinical information of HCC patients

Clinical feature	All(*N* = 60)
Age	58(32–76)
Gender	
57(95%)	3 (5%)
ECOG	
0	42(70%)
1	18(30%)
HBV	
Yes	45(75%)
No	15(25%)
AFP	
≥ 400	18(30%)
< 400	42(70%)
Differentiation	
Moderate	18(30%)
Moderate-poor	18(30%)
Poor	24(40%)
Effect	
CR	1 (2%)
PR	18(30%)
SD	30(50%)
PD	11(18%)
GPC3 expression	
Negative	12(20%)
Weakly positive	18(30%)
Moderate positive	13(22%)
Strongly positive	17(28%)

Discussion

Anti-angiogenesis targeted drugs combined with anti-PD-1/PD-L1 monoclonal antibodies have been a routine treatment option for unresectable HCC, but the number of patients who benefit is still very limited, with objective remission rates of only 20–30% [6, 24]. While GPC3 has been established as a diagnostic marker and therapeutic target for HCC, its utility as a predictive biomarker for the efficacy of targeted combination immunotherapies in HCC remains uncertain [25].Furthermore, current clinical studies have demonstrated limited success with immunotherapies targeting GPC3. Hence, this study aimed to assess the potential of GPC3 as a biomarker for predicting efficacy and to explore the HCC tumor microenvironments across varying levels of GPC3 expression.

In the current study, we found that GPC3 expression correlated with AFP. The proportion and cell density of GPC3-positive cells were also higher in tissues with high levels of AFP compared to those with low levels. Alpha-fetoprotein (AFP) is a 70 kDa glycoprotein synthesized by the fetal liver and yolk sac in the initial stages of pregnancy. AFP serves as a widely employed biomarker for early detection, monitoring, and diagnosis [26]. Elevated serum AFP levels have been associated with a poor prognosis. One study has already reported that baseline AFP < 400 μg/L levels prior to immunotherapy correlate with objective remission rates [27]. In HCC, the subtyping of liver cancer based on AFP and GPC3 serves as a meaningful prognostic marker for liver cancer [28]. Our study also found that GPC3 expression correlated with the prognosis of anti-angiogenic drugs combined with immunotherapy. In a retrospective study evaluating the combination of anti-PD-L1 antibodies with anti-angiogenesis targeted agents, the expression of oncogenes such as GPC3 and AFP was linked to reduced clinical benefit [29]. This finding aligns with the outcomes of our investigation. Moreover, the proportions of GPC3-positive cells and cell densities in various clinical characteristic subgroups exhibited differences. Nevertheless, it is essential to acknowledge that these distinctions did not reach statistical significance, likely due to the limited sample size inherent in this study.

In diverse tumor types, including hepatocellular carcinoma (HCC), the molecular and cellular attributes within the tumor microenvironment play a pivotal role in promoting cancer cell proliferation and establishing a supportive milieu that modulates immune system activation [30]. The presence of inflammatory (hot) or non-inflammatory (cold) HCC tumors, along with their distinct genomic features, is closely linked to the response to immune checkpoint inhibitors. In HCC, $CD8^+$ T cells are enriched in the intertumoral and peritumoral regions, respectively [31]. In this study, it was found that the positivity rate of $CD8^+$ T cells and TIM-3^+ T cells as well as the cell density were higher in the high GPC3 expression group than in the low GPC3 expression group. T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) are immunosuppressive molecules that are expressed on the surface of T lymphocytes [32]. TIM-3 expression in TILs is negatively associated with disease-free survival in HCC patients [33]. $CD103^+CD8^+$ tissue-resident memory T cells represent a crucial effector cell population for anti-tumor immune responses [34]. The assessment of $CD103^+CD8^+$ T cell expression in hepatocellular carcinoma tissues is valuable for evaluating the effectiveness of hepatocellular carcinoma treatments. In our study, we found that $CD103^+CD8^+$ T was higher in the high GPC3 expression group than in the low GPC3 expression group.

Dendritic cells (DCs), specialized antigen-presenting cells in the body, critically contribute to antigens presentation to T cells and deliver co-stimulatory signals essential for T cell activation [35]. Dendritic cell (DC) populations exhibit significant heterogeneity in their developmental spectrum, differentiation stage, and the physiological and pathological microenvironment. They are typically categorized as conventional dendritic cells (cDCs), plasma cell-like dendritic cells (pDCs), and inflammatory dendritic cells (infDCs) [36]. Enhancing our understanding of dendritic cell (DC) subpopulations provides valuable insights into

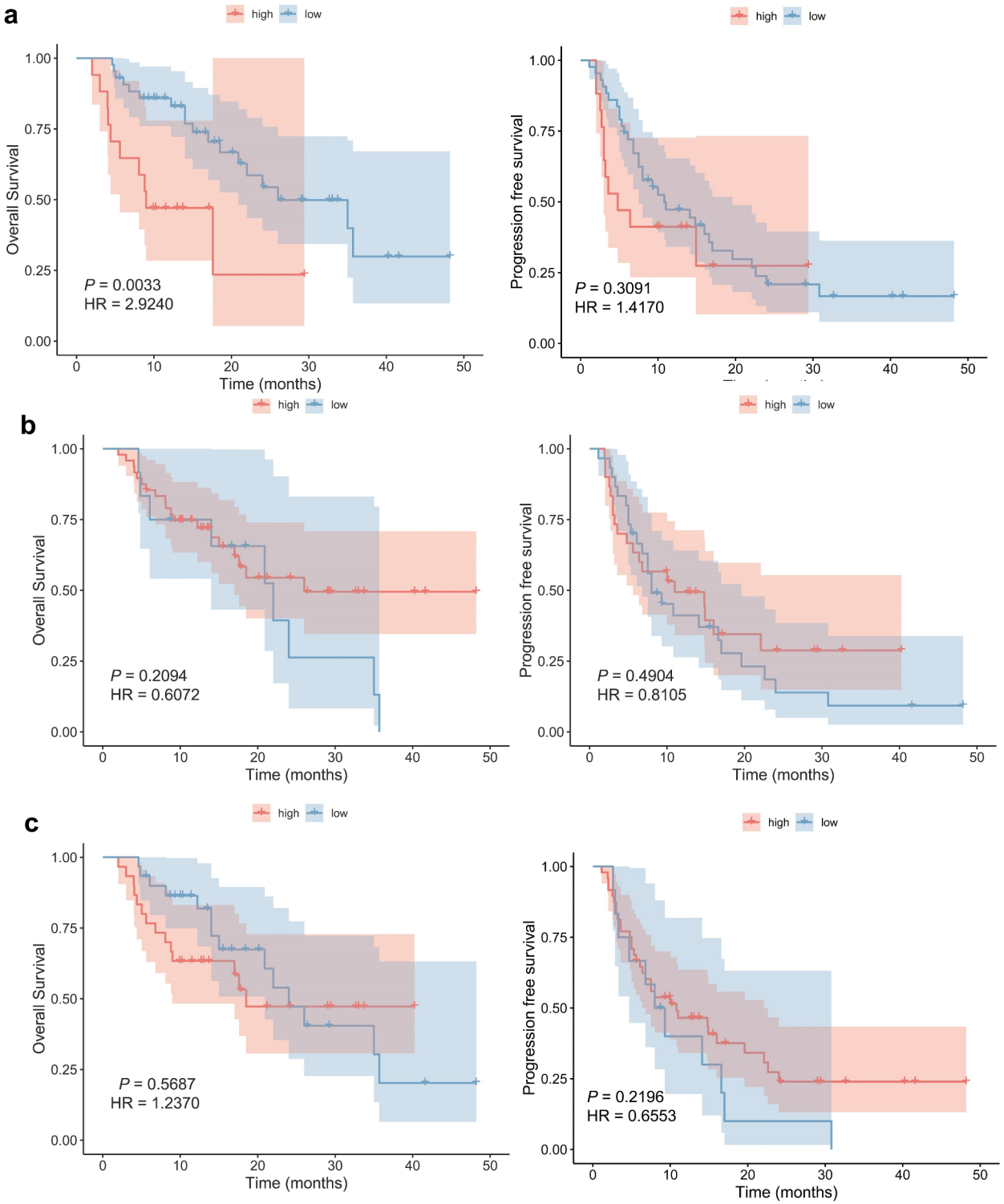


Fig. 2 Survival curves for different GPC3 expressions. (**a**) Immunohistochemical scores of strong positive were defined as high expression, and the rest as low expression for OS and PFS; (**b**) Immunohistochemical staining scores of negatives were defined as low expression, and the rest as high expression for OS and PFS; (**c**) Immunohistochemical staining scores of negative and weakly positive were defined as low expression, and the rest as high expression for OS and PFS

Table 2 Clinical characteristics of HCC patients with different GPC3 expression

Clinical feature	High_ expression	Low _ expression	χ^2	*P* Value
Gender			0.2117	0.6455
Male	17	40		
Female	0	3		
Age			1.5273	0.2174
≥58	7	27		
<58	10	16		
ECOG			0.7761	0.3814
1	7	11		
0	10	32		
HBV			<0.0000	1.0000
Yes	13	32		
No	4	11		
AFP			7.5669	**0.0059**
≥400	10	8		
<400	7	35		
Differentiation			3.6389	0.1621
Moderate	4	14		
Moderate-poor	4	15		
Poor	10	14		

the immune microenvironment associated with different levels of GPC3 expression in HCC. In our study, we observed higher proportions of $CD11C^+CD14^-$DC cells, $CD11C^+$HLA-DR^-DC cells, $CD11C^+CD14^-$HLA-DR^-DC cells, and $CD11C^+CD14^-CD66b^-$DC cells in the high GPC3 expression group compared to the low GPC3 expression group. These findings shed light on the distinct immune cell composition within the tumor microenvironment in relation to GPC3 expression levels.

The incomplete understanding of the intricate mechanisms through which HCC cells evade the body's immune response contributes to the suboptimal effectiveness of immunotherapy in HCC patients. Tumor progression is regulated by interactions between cancer cells and the surrounding microenvironment, and the immune system plays a dual role in malignant tumor development and progression [37]. The spatial distribution of immune cells in the HCC tumor microenvironment has been reported to be heterogeneous [38]. In our study, we found that the spatial distribution of $CD8^+$ T cells was more in high GPC3 expression tissues in different spatial distances. However, the prognosis of patients with high GPC3 expression receiving targeted combination immunotherapy was poorer than that of patients with low GPC3 expression, which may be due to a higher distribution of depleted $CD8^+$ T cells, which requires further exploratory studies.

GPC3 is regarded as a promising target for immunotherapy in the realm of liver cancer. Nonetheless, clinical research has not demonstrated a substantial anti-tumor effect. Our study has revealed that HCC patients with low GPC3 expression benefit from a combination of anti-angiogenic targeted drugs and immune therapy. Preclinical investigations have demonstrated that PD-1 knockout enhances the in vivo anti-tumor activity of GPC3-CAR-T cells against liver cancer [39]. Furthermore, study has reported that bispecific GPC3/PD-1 CAR-T cells exhibit superior tumor inhibition compared to GPC3-CAR-T cells targeting a single antigen [40]. Our research also indicates that HCC patients with different levels of GPC3 expression exhibit distinct tumor immune microenvironments. Therefore, targeting GPC3 expression could present a viable strategy to boost the effectiveness of anti-PD-1/PD-L1 monoclonal antibody treatment in HCC patients exhibiting high GPC3 expression. Moreover, the inhibition of immunosuppressive checkpoints like PD-1, PD-L1, and TIM-3 shows potential in enhancing the efficacy of GPC3-CAR-T cell therapy for treating HCC.

Multiplex immunohistochemistry, widely utilized in drug development, scientific research, and clinical settings, plays a crucial role in evaluating the immune status of lesion tissue microenvironments, especially in complex diagnoses like tumor immunotherapy. A systematic review and meta-analysis published in JAMA Oncology in 2019 compared various diagnostic tests for anti-PD-1 and PD-L1 drugs. The results highlighted that panoramic pathological testing, exemplified by mIHC/IF, emerges as the most accurate scheme for predicting the efficacy of these drugs. Its predictive model outperforms traditional methods such as PD-L1 IHC testing, tumor mutation burden assessment (TMB), and gene expression profiling (GEP) [41].

In conclusion, this study aims to use multiple immunohistochemistry to delve into the intricacies of the immune microenvironment within liver cancer tumor tissues. This systematic approach seeks to shed light on the complex interplay of immune cells within the tumor, potentially informing novel insights into therapeutic strategies for liver cancer. The study found a correlation between different levels of GPC3 expression and overall survival (OS) in HCC patients receiving combination therapy of anti-angiogenic drugs and immunotherapy. HCC patients with varying GPC3 expression exhibited unique tumor immune microenvironments, which holds guiding significance for improving HCC treatment.

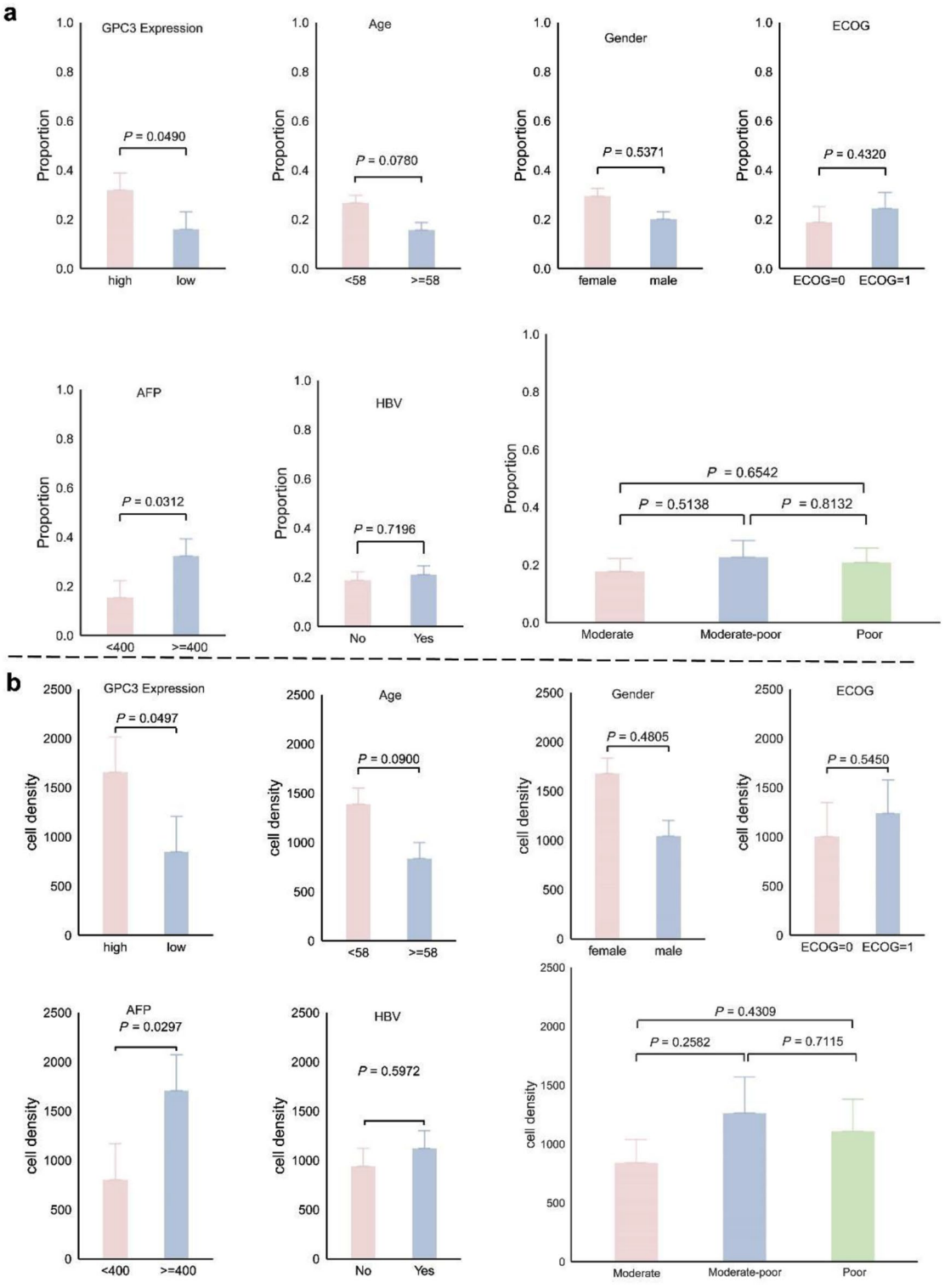


Fig. 3 Relationship between GPC3 expression and clinical features. (**a**) Comparison of GPC3-positive cells in each clinical feature subgroup, proportion: number of GPC3-positive cells/total number of cells; (**b**) Comparison of GPC3 cell density in each clinical feature subgroup, cell density: number of GPC3-positive cells/total area

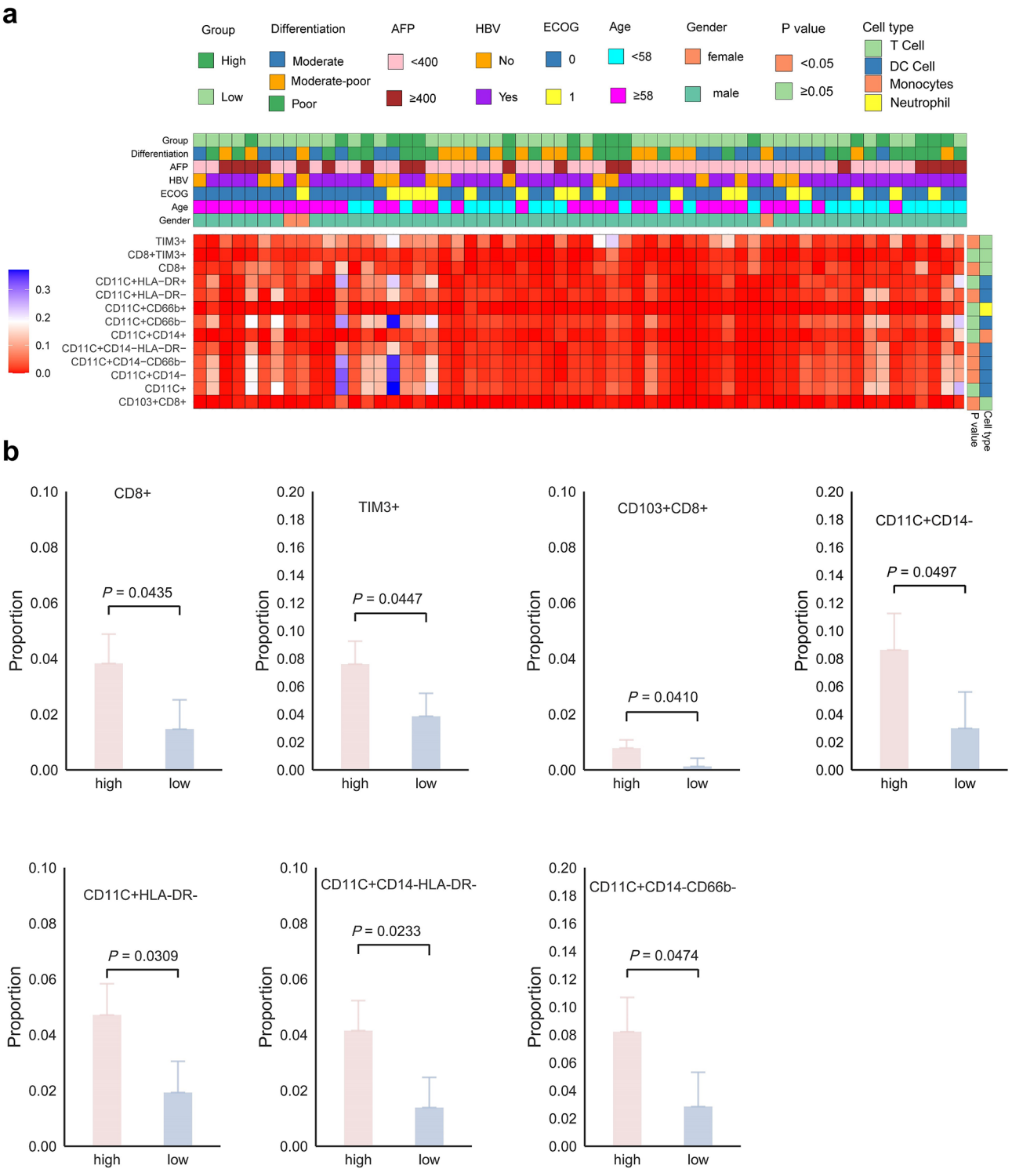
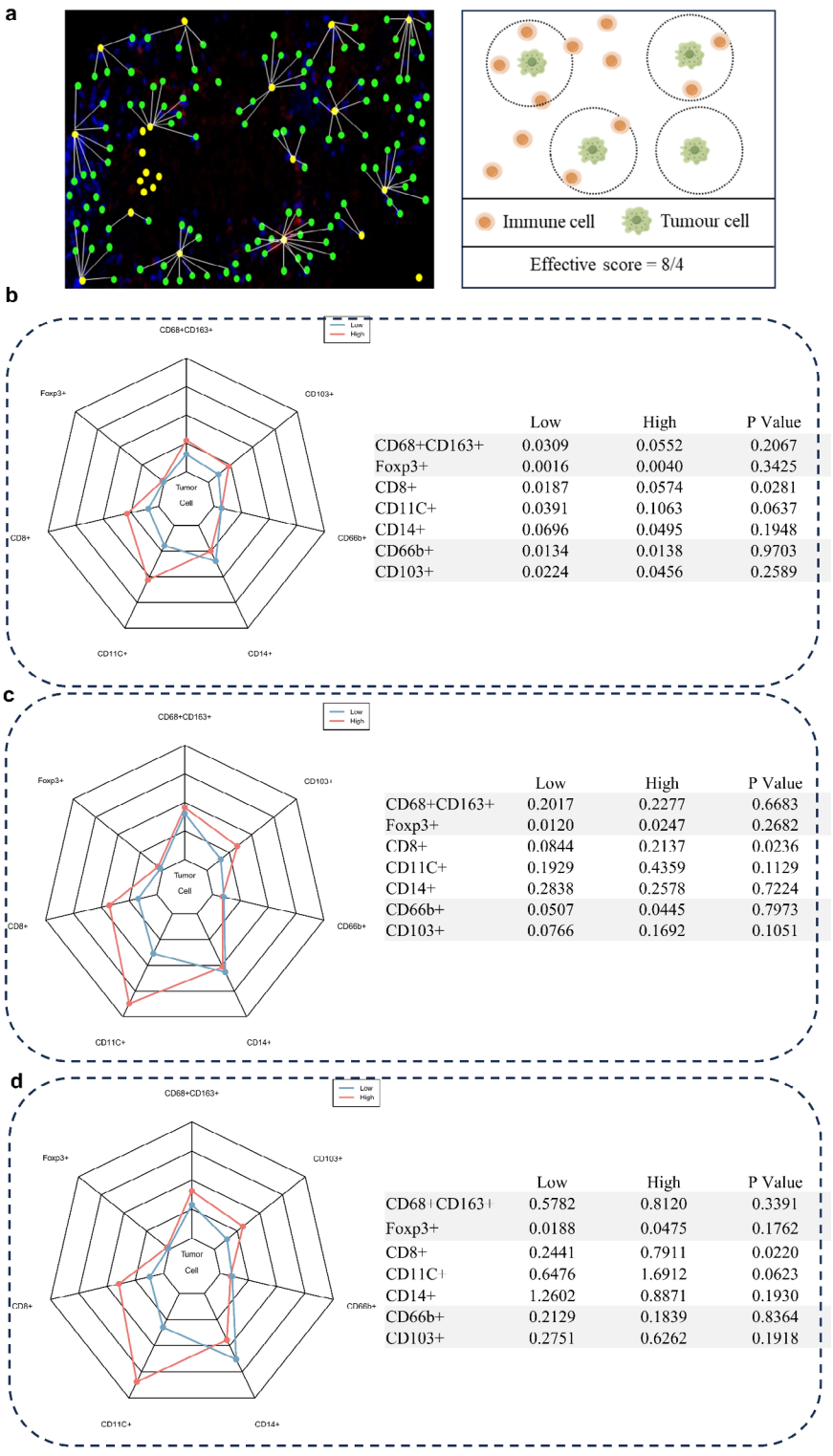


Fig. 4 Immune cell subpopulations with different GPC3 expression. (**a**) Clinical characteristics and immune cell subpopulations based on GPC3 expression grouping. (**b**) Expression of immune cells in the high and low GPC3 expression groups



b

	Low	High	P Value
CD68+CD163+	0.0309	0.0552	0.2067
Foxp3+	0.0016	0.0040	0.3425
CD8+	0.0187	0.0574	0.0281
CD11C+	0.0391	0.1063	0.0637
CD14+	0.0696	0.0495	0.1948
CD66b+	0.0134	0.0138	0.9703
CD103+	0.0224	0.0456	0.2589

c

	Low	High	P Value
CD68+CD163+	0.2017	0.2277	0.6683
Foxp3+	0.0120	0.0247	0.2682
CD8+	0.0844	0.2137	0.0236
CD11C+	0.1929	0.4359	0.1129
CD14+	0.2838	0.2578	0.7224
CD66b+	0.0507	0.0445	0.7973
CD103+	0.0766	0.1692	0.1051

d

	Low	High	P Value
CD68+CD163+	0.5782	0.8120	0.3391
Foxp3+	0.0188	0.0475	0.1762
CD8+	0.2441	0.7911	0.0220
CD11C+	0.6476	1.6912	0.0623
CD14+	1.2602	0.8871	0.1930
CD66b+	0.2129	0.1839	0.8364
CD103+	0.2751	0.6262	0.1918

Fig. 5 Spatial distribution of different GPC3 expression immune cell subpopulations. (**a**) Illustration of the spatial analysis of tumor cells and immune cells, yellow dots and green dots represent tumor cells and immune cells, respectively, and the white line connecting the yellow dots and the green dots indicates the distance between the two cells; effective score: the number of immune cells/number of tumor cells in the radius; (**b**) Radius of 10 μm, the spatial distribution of the cells of each immune cell (**c**) radius of 20 μm, spatial distribution of each immune cell; (**d**) radius of 30 μm, spatial distribution of each immune cell

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06106-0.

Supplementary Material 1

Author contributions

Jie Shen and Jun Chen conceived and designed the experiments. Mingzhen Zhou, Ziyan Zhou, Lina Hu, Sidong Chen and Fanyan Meng collected and analyzed data. Mingzhen Zhou and Jie Shen composed and wrote the manuscript. Jie Shen and Jun Chen reviewed and edited the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Funding

The study was supported by National Natural Science Foundation of Nanjing University of Chinese Medicine (No. XZR2023075); The National Health Commission Health Development Research Center (No. WKZX2023CX020001); and Medical Research project of Jiangsu Provincial Health Commission (No. H2023068).

Data availability

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

This study protocol was reviewed and approved by ethics committee of Drum Tower Hospital of Nanjing University. The patients/participants provided their written informed consent to participate in this study.

Consent for publication

The patients/participants provided their written informed consent to participate in this study.

Competing interests

The authors declare that they have no conflict of interests.

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Received: 23 August 2024 / Accepted: 8 January 2025
Published online: 21 January 2025

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