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PROZ is a Biomarker for Progression of Early Hepatocellular Carcinoma and Correlated with Tumor-Infiltrating Immune Cells

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Abstract

Protein Z (*PROZ*) is a liver vitamin K-dependent glycoprotein, which has been associated with some cancer types; however, its role and mechanism in Hepatocellular Carcinoma (HCC) development and progression remain unclear. We used public datasets of cancer tissues and cell lines from the Oncomine and Human Protein Atlas to compare the expression of *PROZ* in various cancer types. The *PROZ* expression level was the highest in HCC. *PROZ* expression was decreased in HCC compared with normal tissues. A higher *PROZ* level was positively correlated with favorable overall survival, disease-free survival, progression-free survival, relapse-free survival, and disease-specific survival in patients with HCC. These results were validated in clinical HCC patient's survival information. Moreover, the expression levels of microRNAs predicted to target *PROZ* were inversely correlated with overall survival in HCC patients. Notably, a higher expression level of *PROZ* was associated with a lower risk of progression in HCC patients at stage I or without vascular invasion. The correlations of *PROZ* expression with the numbers of tumor-infiltrating cells and their marker genes were further analyzed using tumor immune estimation resource and GEPIA tools. *PROZ* expression was negatively correlated with the numbers of tumor-infiltrating CD8+ T cells, CD4+ T cells, macrophages, and neutrophils in HCC, but was positively correlated with the level of INOS, a marker of M1 macrophages. Overall, these results indicate that *PROZ* is a tumor-suppressor gene, and could serve as a biomarker for early HCC progression, which may be attributed to its influence on tumor-infiltrating cells.

Keywords: *PROZ*; Hepatocellular carcinoma; RNA expression; Prognosis; Tumor-infiltrating cells

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Introduction

Hepatocellular Carcinoma (HCC) is one of the leading causes of cancer-related mortality [1]. Since there is no effective method for the treatment of HCC, more basic research is needed to understand the molecular mechanisms contributing to HCC development and progression. Timely diagnosis of HCC at an early stage followed by appropriate and effective treatment is the best chance to improve the prognosis of patients. Thus, it is of great significance to find a biomarker that can predict the progression of early HCC.

The Protein Z gene (*PROZ*) encodes a liver vitamin K-dependent glycoprotein that regulates blood coagulation by forming a complex with *PROZ*-dependent protease inhibitor to directly inhibit activated factor X [2]. A low level of *PROZ* did not have an influence on bleeding diatheses or thrombophilia, but was found to be associated with diseases of the reproductive system [3,4]. Abnormal expression of *PROZ* has also been detected in some cancers, including acute leukemia, multiple myeloma, lung cancer, gastric cancer, breast cancer, ovarian cancer, and plasma cell neoplasms, suggesting that *PROZ* might be a prognostic or diagnostic biomarker for these cancers

[5-10]. However, the role of *PROZ* in HCC development and progression has not been assessed to date.

In this study, we compared the expression levels of *PROZ* in HCC and other cancer types based on public databases and patients, and determined the association of variations in *PROZ* expression with patient prognosis. Moreover, the clinical outcome and therapeutic response to anticancer treatments are influenced by the tumor microenvironment, which includes both cellular and non-cellular components [11]. Immune cells, tumor-associated fibroblasts, hepatic stellate cells, and endothelial cells are the main cellular components of the tumor microenvironment in HCC [12], and component reprogramming of immune cells has been associated with the prognosis of patients with HCC. Therefore, we further assessed the correlations of *PROZ* expression and tumor-infiltrating cells in the tumor environment of HCC. These results can provide a basis for further development of *PROZ* as a prognostic biomarker in HCC.

Materials and Methods

Expression analysis

The expression levels of *PROZ* in various types of cancers and cell lines were obtained from the Oncomine [13] and Human Protein Atlas (HPA) [14] databases, respectively. The Oncomine database includes *PROZ* mRNA expression levels in tumor and adjacent normal tissues of patients. The HPA database comprises several distinct atlases, including the Pathology Atlas based on data from The Cancer Genome Atlas (TCGA).

Quantitative real-time PCR (qPCR) analysis

Sixty-four pairs of liver tumors tissues and adjacent normal tissues cDNA was obtained from patients diagnosed with HCC. Followed qPCR was administrated using FastFire SYBR Green qPCR PreMix (TianGen, Beijing, China). The expression levels of *PROZ* genes were normalized to GAPDH. Relative mRNA expression levels were analyzed by the $2^{-\Delta\Delta}$ Cycle Threshold (CT) method. The qPCR primer sequences were as follows: GAPDH forward: 5'-G C C C T C A A C G A C C A C T T T G T-3' reverse: 5'-T G G T G G T C C A G G G G T C T T A C-3'; *PROZ* forward: 5'-A G G C G T C C A G G A A A G C T T A T T-3'; reverse: 5'-C A G A A G A T A G G A G C C C G C A C-3'.

Survival analysis

The Kaplan-Meier plotter [15] and Gene Expression Profiling Interactive Analysis (GEPIA) [16] databases were used to analyze the effect of variations in *PROZ* expression on survival in patients with several types of cancer based on available RNA-seq data from TCGA cohorts. The Kaplan-Meier database generates four types of survival curves, including Overall Survival (OS), Progression-Free Survival (PFS), Relapse-Free Survival (RFS), and Disease-Specific Survival (DSS). The GEPIA database generates only OS and Disease-Free Survival (DFS) curves. Furthermore, 64 HCC patients were also used for survival analysis, patients were divided into high- and low-expression groups according to the cut-off value, and survival curves were plotted using Kaplan-Meier analysis to explore the correlation between the *PROZ* expression and the OS for patients with HCC. The associations with survival were based on P-values from log-rank analysis and the hazard ratio.

Tumor-Infiltrating cells analysis

Tumor Immune Estimation Resource (TIMER) is a publicly accessible tool for correlation analysis between genes and tumor-infiltrating immune cells for pan-cancer datasets based on a deconvolution approach [17,18]. We used TIMER to analyze the correlations of *PROZ* expression with the numbers of tumor-infiltrating immune cells, including CD4+ T cells, CD8+ T cells, neutrophils, and macrophages, in HCC *via* the gene modules. Moreover, the correlations between *PROZ* expression and genetic markers of tumor-infiltrating cells were explored *via* the correlation modules. Since purity of the tumor sample (the proportion of tumor cells in the mixture) could introduce bias in the analysis of immune cell infiltration [19,20], we determined the correlation between *PROZ* expression and infiltrating immune cells using tumor purity-corrected statistical analysis.

Cell analysis

Given the importance of tumor-infiltrating immune cells in the tumor microenvironment, we used xCell (a webtool can performs cell type enrichment analysis from gene expression data) to determine the fractions of 64 immune cells of HCC cases in the TCGA. The risk score was calculated based on the expression levels of *PROZ*. According to the median risk score, HCC patients were divided into high-risk group and low-risk group. The correlation between risk scores and immune infiltration was calculated by Pearson correlation. We illustrated the differential density of immune cells in two risk groups using a pheatmap package, where the colors ranging from red to blue represented the low to high infiltrating levels. We showed the results as a violin plot, and the various immune cells were labeled under the legend. Furthermore, we conducted a Wilcoxon rank-sum test to compare the differential abundance of immune cells in the two risk groups.

Statistical analysis

The correlation analysis was statistically evaluated by Spearman's correlation coefficient; a P-value <0.05 was considered statistically significant.

Results

PROZ exhibits higher expression in HCC compared with other cancers

Analysis of data in the Oncomine database revealed that the *PROZ* expression level was lower in bladder, kidney, and liver tumors compared with those of corresponding normal tissues (Figure 1A). In addition, higher expression levels of *PROZ* were found in breast and colorectal cancers in some datasets. To further evaluate *PROZ* expression in human cancers, we used TCGA RNA-seq data. Compared to adjacent normal control tissues, *PROZ* was more highly expressed in tumors of Bladder Urothelial Carcinoma (BLCA), Breast Invasive Carcinoma (BRCA), Colon Adenocarcinoma (COAD), Head and Neck Squamous Cell Carcinoma (HNSC), Lung Adenocarcinoma (LUAD), Lung Squamous Cell Carcinoma (LUSC), Prostate Adenocarcinoma (PRAD), Rectum Adenocarcinoma (READ), Stomach Adenocarcinoma (STAD), and Uterine Corpus Endometrial Carcinoma (UCEC), and showed significantly lower expression in Cholangiocarcinoma (CHOL), Kidney Renal Clear Cell Carcinoma (KIRC), Kidney Renal Papillary Cell Carcinoma (KIRP), Liver Hepatocellular Carcinoma (LIHC/HCC), and Thyroid Carcinoma (THCA) compared with adjacent normal tissues (Figure 1B). Overall, *PROZ* expression was significantly the highest in HCC compared to that of all other cancers. Consistently, among cancer cell lines, *PROZ* showed the highest expression in the HCC cell line HepG2 (Supplementary Figure 1).

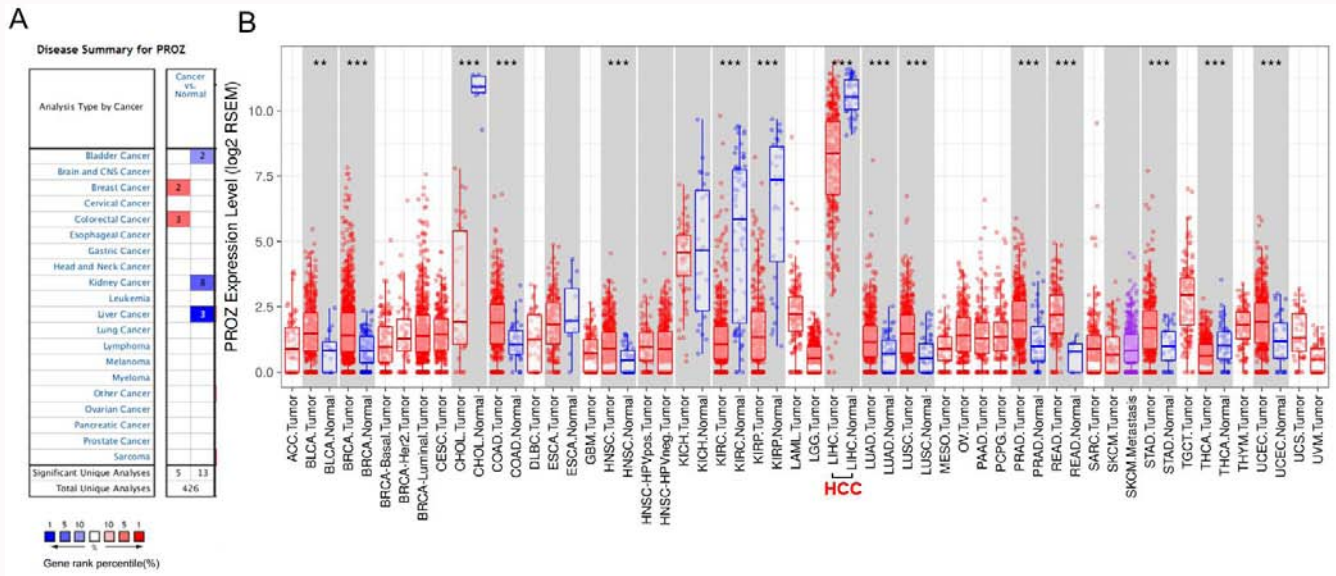

Figure 1: *PROZ* expression levels in different types of human tissues and cancers. (A) Increased or decreased *PROZ* expression in cancer and adjacent normal tissues from the ONCOMINE database. (B) *PROZ* expression levels in different tumor types from the TCGA database determined by TIMER (**P<0.01, ***P<0.001).

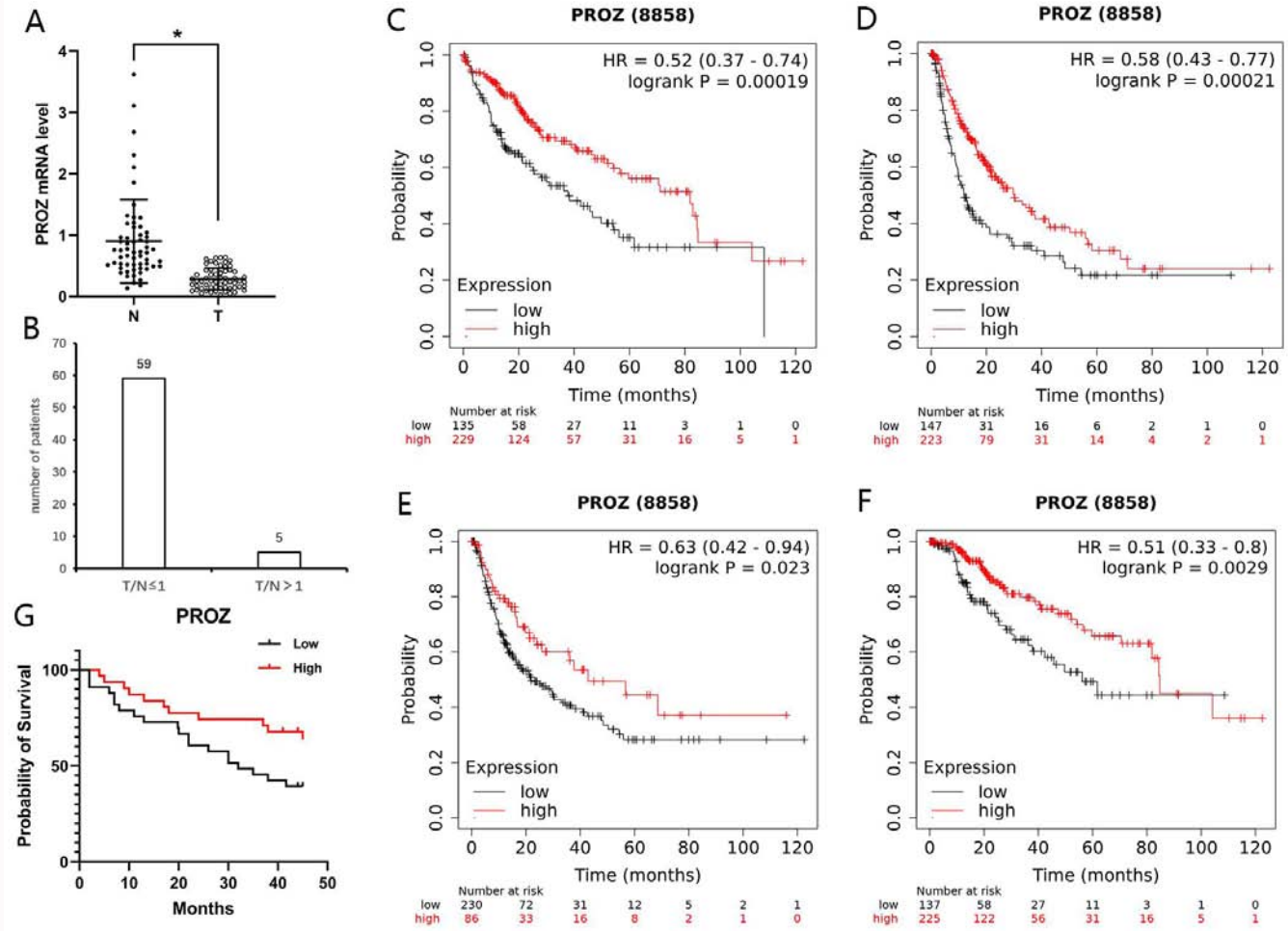

Figure 2: *PROZ* mRNA expression in HCC and adjacent normal tissues and Kaplan-Meier survival curves comparing HCC patients with high and low expression of *PROZ* in the Kaplan-Meier plotter database and clinical survival information's. (A) The differential *PROZ* mRNA expression in HCC and adjacent normal tissues, (B) Number of patients with different T/N in 64 paired tumor and normal tissues, (C) overall survival, (D) progression-free survival, (E) relapse-free survival, (F) disease-specific survival, (G) Survival curves suggested that *PROZ* mRNA levels was positive correlated with OS in 64 HCC patients.

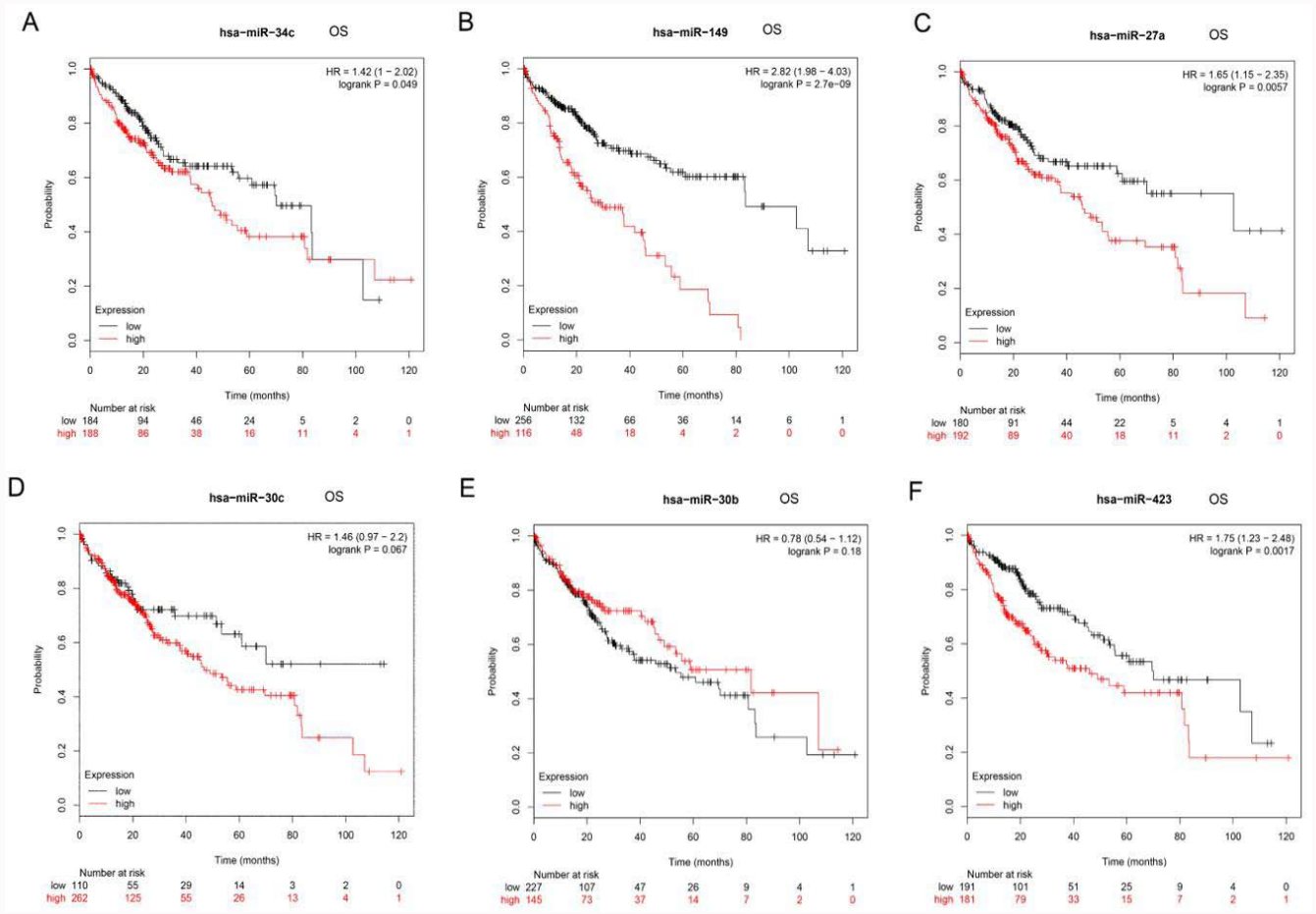

Figure 3: Kaplan-Meier survival curves comparing high and low expression of microRNAs targeting *PROZ* in patients with HCC. (A) hsa−miR−34c, (B) hsa−miR−149, (C) hsa−miR−27a, (D) hsa−miR−30c, (E) hsa−miR−30b and (F) hsa−miR−423.

The mRNA expression levels of *PROZ* in HCC is decreased compared with normal tissues

To explore *PROZ* mRNA expression in HCC tissues, we performed qPCR in 64 paired tumor and normal samples with available clinical follow-up data. We found significantly decreased *PROZ* mRNA expression in HCC samples compared with normal tissues: 90.7% of HCC patients has lower mRNA expression levels of *PROZ* than normal tissues (Figure 2A, 2B).

Prognostic potential of *PROZ* in HCC

We next investigated whether alterations of *PROZ* expression between tumor and adjacent normal tissues was correlated with prognosis in patients with the 15 types of cancers mentioned above (Supplementary Figure 2). High *PROZ* expression levels were negatively but weakly associated with OS in COAD (P=0.046) and KIRC (P=0.049). However, strong positive correlations were found between *PROZ* and OS in patients with BLCA and HCC. DFS in patients with HCC and PRAD was strongly positively correlated with *PROZ* expression. Both OS and DFS were only significantly associated with *PROZ* expression for patients with HCC among all cancer types, suggesting the *PROZ* may play a very import role in HCC. Furthermore, the Kaplan-Meier plotter database confirmed the positive correlation between prognosis of patients with HCC and *PROZ* expression with respect to OS (P=0.00019), PFS (P=0.00021), RFS (P=0.023), and DFS (P=0.0029) (Figure 2). To further validate correlation between *PROZ* expression levels and OS in HCC, 64 clinical HCC patients survival information's were used, we divided *PROZ* expression levels into high- and low-expression groups according to cut-off value. As expected, an expression level of PROZ was significantly positively correlated with OS in patients with HCC. We further assessed the correlation of OS in HCC patients with the expression of six microRNAs targeting *PROZ* to negatively regulate its expression. Higher expression levels of hsa−miR−34c, hsa−miR−149, hsa−miR−27a, and hsa−miR−423 were associated with a poor OS of HCC patients (P<0.05; Figures 3A–3C, 3F). However, the expression of hsa−miR−30c and hsa−miR−30b had no influence on OS (Figure 3D, 3E). These results indicated that *PROZ* is a tumor-suppressor gene with an impact on the prognosis of HCC patients.

PROZ mainly affects the progression of early-stage HCC

We further used the Kaplan-Meier plotter database to evaluate the relationship between *PROZ* expression and clinical characteristics of HCC patients, revealing significant effects on PFS in patients at stage I, but not at stages II and III (Table 1). Moreover, *PROZ* showed the highest expression levels in HCC stage I samples, which decreased gradually with advanced stages (Supplementary Figure 3). Similarly, the PFS of HCC patients without vascular invasion was positively associated with *PROZ* expression, but this association was not detected for patients with vascular invasion of HCC. These results indicated that *PROZ* is a potential biomarker for predicting HCC progression at an early stage.

Table 1: Correlation of *PROZ* expression with different clinicopathological factors by Kaplan-Meier plotter.

Clinicopathological characteristics	Progression-free survival (n=370)		
	N	Hazard ratio	P-value
Stage			
I	170	0.57 (0.33-0.98)	**0.04**
II	84	0.64 (0.35-1.17)	0.14
III	83	0.65 (0.36-1.16)	0.4
Vascular invasion			
None	204	0.48 (0.28-0.84)	**0.0082**
Micro	91	0.61 (0.34-1.07)	0.081

PROZ expression is correlated with tumor-infiltrating cells in HCC

Since the tumor microenvironment is related to the progression of HCC, we investigated whether *PROZ* expression was correlated with infiltration levels of immune cells using TIMER and xCell. The tumor purity-corrected analysis showed significant negative correlations between *PROZ* expression levels and the numbers of infiltrating T cells (CD8+ and CD4+), macrophages, and neutrophils in HCC (Figure 4). By applying the CIBERSORT algorithm to TCGA data, we found that the low-risk group had significantly higher aDC, Astrocytes, Basophils, B-cells, CD4+ memory T-cells, CD4+ naive T-cells, CD8+ T-cells, CD8+ Tcm, memory B-cells, CLP, DC, Epithelial cells, Keratinocytes, Mast cells, Mesangial cells, Monocytes, naive B-cells, Neurons, NKT, Plasma cells, pro B-cells, Sebocytes, Smooth muscle, Th1 cells, Th2 cells, but lower Adipocytes, Endothelial cells, GMP, Hepatocytes, HSC, ly Endothelial cells, Macrophages M2, Megakaryocytes, mv Endothelial cells, Preadipocytes (Figures 5A-5I).

Moreover, the *PROZ* expression level was significantly correlated with most immune marker sets in HCC, including monocytes, Tumor-Associated Macrophages (TAMs), M1 macrophages, neutrophils, and Tregs, as well as T cell exhaustion, although there were no associations with markers of total T cells, natural killer cells, and M2 macrophages (Table 2). Specifically, there were significant negative correlations between *PROZ* and marker genes of Tregs and T cell exhaustion such as CCR8, TGFβ, PD-1, CTLA4, and TIM-3. With respect to innate immune cells, the expression levels of most markers of monocytes showed strong correlations with *PROZ* expression in HCC. The level of CCL2 (TAMs marker) was negatively correlated with *PROZ* expression, whereas the level of INOS (M1 macrophage marker) was positively correlated with *PROZ* expression. These findings suggested that *PROZ* might regulate macrophage polarization in HCC.

Table 2: Correlation between *PROZ* and marker genes of tumor-infiltrating cells in TIMER analysis.

Description	Gene marker	Cor	P
T cell	CD3D	-0.079	1.43E-01
	CD3E	-0.058	2.84E-01
	CD2	-0.081	1.34E-01
Treg	FOXP3	0.037	4.92E-01
	CCR8	-0.232	**1.32E-05**
	STAT5B	0.072	1.85E-01
	TGFβ (TGFB1)	-0.265	**5.66E-07**
T cell exhaustion	PD-1 (PDCD1)	-0.14	**9.09E-03**
	CTLA4	-0.159	**3.15E-03**
	LAG3	0.008	8.82E-01
	TIM-3 (HAVCR2)	-0.209	**9.06E-05**
	GZMB	-0.002	9.72E-01
NK cell	KIR2DL1	0.093	8.60E-02
	KIR2DL3	-0.084	1.18E-01
	KIR2DL4	-0.062	2.53E-01
	KIR3DL1	-0.054	3.17E-01
	KIR3DL2	-0.025	6.64E-01
	KIR3DL3	0.019	7.31E-01
	KIR2DS4	0.069	1.99E-01
Monocyte	CD86	-0.202	**1.63E-04**
	CD115 (CSF1R)	-0.158	**3.29E-03**
TAM	CCL2	-0.129	**1.68E-02**
	CD68	-0.066	2.22E-01
	IL10	-0.104	5.28E-02
M1 Macrophage	INOS (NOS2)	0.157	**3.54E-03**
	IRF5	-0.092	8.85E-02
	COX2(PTGS2)	-0.085	1.16E-01
M2 Macrophage	CD163	-0.003	9.63E-01
	VSIG4	-0.053	3.23E-01
	MS4A4A	-0.041	4.53E-01
Neutrophils	CD66b (CEACAM8)	-0.025	6.48E-01
	CD11b (ITGAM)	-0.217	**4.64E-05**
	CCR7	0.054	3.20E-01

Treg: Regulatory T Cell; NK: Natural Killer; TAM: Tumor-Associated Macrophage; Cor: R value of Spearman's Correlation. The correlation is adjusted by tumor purity.

Discussion

PROZ encodes a vitamin K-dependent glycoprotein that is synthesized in the liver and secreted into the plasma, which has been found to be abnormally expressed in some cancer types. Here, we demonstrated significant differences in *PROZ* expression levels between tumor tissues and adjacent normal tissues in 15 types of cancers. Increased *PROZ* expression was detected in BLCA, BRCA, COAD, HNSC, LUAD, LUSC, PRAD, READ, STAD, and UCEC, whereas CHOL, KIRC, KIRP, HCC, and THCA showed decreased *PROZ* expression. We further explored *PROZ* expression in HCC, using qPCR to compare the differential *PROZ* expression between tumor and adjacent normal tissues in clinical HCC samples. We observed a significant decrease in *PROZ* expression in HCC. These results indicated that different signaling pathways might regulate *PROZ* expression, and that *PROZ* plays distinct roles in various tumors.

A previous study examining *PROZ* expression in the blood or tissue of patients with various cancer types (including acute leukemia, multiple myeloma, lung cancer, gastric cancer, breast cancer, and ovarian cancer) showed a negative correlation between plasma *PROZ* levels with advanced stages of multiple myeloma [21]. By contrast, patients with advanced lung cancer showed higher *PROZ* levels [10]. Although these studies suggested that *PROZ* might be a prognostic biomarker, the effect of *PROZ* on survival was not directly assessed. Based on public databases, we first demonstrate a positive correlation of *PROZ* expression with OS, PFS, DSS, and RFS, indicating that

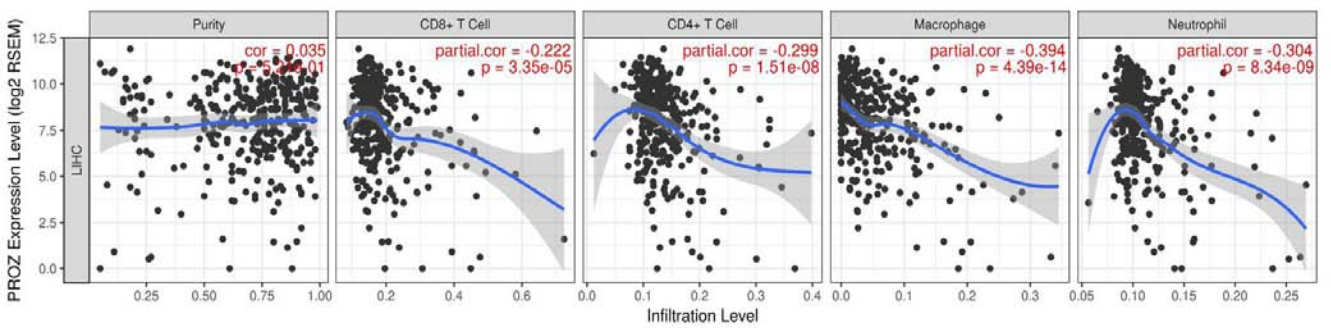

Figure 4: Correlation of *PROZ* expression with tumor-infiltrating cells in HCC based on purity-corrected analysis.

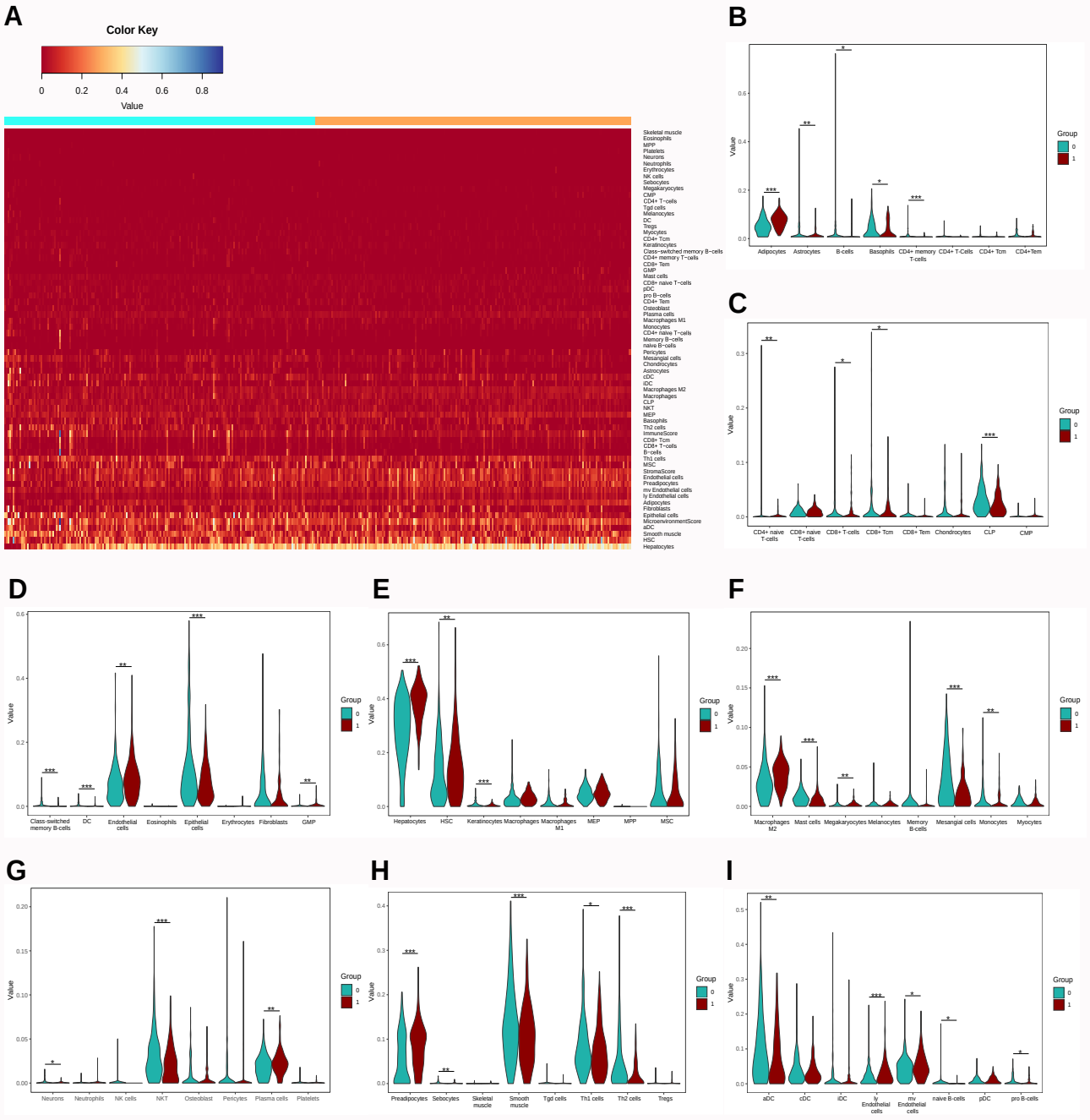

Figure 5: Immune cell infiltration patterns between *PROZ* high and low expression group. (A) Heatmap of immune infiltration level. (B-I) Violin plot of immune infiltration level between *PROZ* low (blue) and high (red) expression group.

PROZ acts as a tumor-suppressor gene. It was further validated by using clinical HCC patients information's that over-expression of *PROZ* showed positive correlation with HCC patient's survival. In particular, we found a significant role of *PROZ* in predicting the progression of early HCC. Neumann et al. [22] reported that *PROZ* matched their tumor-suppressor gene, but was found to increase the viability and clonogenicity of HCC cells. The complexity of the human body and the fact that tumor progression is affected by multiple factors, including several factors in the tumor microenvironment, can potentially explain this discrepancy between results from *in vivo* and *in vitro* analyses.

To further understand the impact and underlying mechanism of *PROZ* in HCC, we examined its association with the tumor microenvironment. We found broad negative correlations between *PROZ* expression and tumor-infiltrating cells and their marker genes. However, a positive correlation was observed between the expression of *PROZ* and INOS, a marker of M1 macrophages. Tregs and T cell exhaustion suppress or limit anti-tumor immunity [23-26]. TAMs include M1 macrophages that suppress tumor progression and M2 macrophages that promote tumor development [27]. Therefore, our results suggest that higher *PROZ* expression may increase the anti-tumor effect of infiltrating immune cells in the local microenvironment of HCC. The relation of *PROZ* with monocytes and neutrophils further suggests a potential influence on inflammation. Since the precise mechanism remains unclear, the roles of *PROZ* in tumor-infiltrating cells should be determined in further experiments.

Thromboembolism is a leading cause of death among cancer patients [28]. High Interleukin-6 (IL-6) expression is a well-known risk factor for HCC development, which tends to be much higher in patients with stage III cancer than in those with stage I and II HCC [29]. The finding that *PROZ* expression was increased in patients with stage I HCC suggests a potential association with decreased liver function. Plasma *PROZ* levels were found to be inversely correlated with plasma IL-6 levels in patients with acute leukemia and non-Hodgkin's lymphoma [30]. However, the association of *PROZ* and IL-6 in HCC is unknown. Moreover, further study is needed to determine whether *PROZ* suppresses HCC progression by influencing thromboembolism and IL-6.

In summary, *PROZ* was identified as a tumor-suppressor gene, which appears to have a particularly important role in early HCC progression that may be associated with its impact on tumor-infiltrating cells.

Author Contributions

Conception and design of studies: CLY, XDJ. Analysis and interpretation: WLL, ZRL. Drafting article and tables: CLY, WLL Critical review and discussion: WC. The authors read and approved the final manuscript.

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Ethics Approval and Consent Statement

The study was approved by the ethics review committee of Chinese PLA General Hospital.

Data Availability

All datasets presented in this study are availability in https://www.jianguoyun.com/p/DSji1WoQ1r32CRjo6JQE. And upon request by contact with the corresponding author.

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