Am J Cancer Res 2022;12(9):4120-4139
www.ajcr.us /ISSN:2156-6976/ajcr0144200

Original Article

Hepcidin as a prognostic biomarker in clear cell renal cell carcinoma

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Received May 16, 2022; Accepted August 22, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: Clear cell renal cell carcinoma (ccRCC) is a common malignancy of urologic neoplasms. Hepcidin is a pivotal modulator of iron metabolism involved in human cancers; however, the biological significance of hepcidin in ccRCC remains to be fully understood. Therefore, in this study, we evaluated the expression profiles of hepcidin in ccRCC from several public databases and found that hepcidin expression was upregulated in ccRCC, which was further validated in ccRCC cell lines, clinical samples, and tissue microarray (TMA) quantitative real-time PCR and immunohistochemistry. In addition, we found that the expression level of hepcidin was correlated with the age, T stage and pathologic stage of patients. Furthermore, hepcidin promoter methylation was significantly associated with the worse poor clinical parameters of ccRCC patients, and hepcidin was an independent prognostic factor. Mechanistically, enrichment analysis revealed that hepcidin participated in the immune-related and metabolism-related pathways. Hepcidin was positively correlated with not only immune infiltration and immune checkpoints but also tumor mutation burden and cytotoxic T lymphocyte. Finally, we validated the positive correlation of hepcidin with the marker of macrophage (CD68) in the TMA. Our findings provide insights into understanding the function and its underlying mechanism of hepcidin in ccRCC and suggest that hepcidin might serve as a potential predictive biomarker of response to immunotherapy and the prognosis of patients with ccRCC.

Keywords: Hepcidin, clear cell renal cell carcinoma, prognosis, immune infiltration, biomarker

Introduction

Renal cell carcinoma (RCC) has the highest mortality rate among genitourinary cancers [1, 2]. As the most common type of RCC, clear cell renal cell carcinoma (ccRCC) often presents as advanced stage and metastasis at the time of diagnosis due to the lack of clinical symptoms and biomarkers for early detection [3]. The conventional radiotherapy or chemotherapy generally fail to achieve satisfactory therapeutic results on the advanced or metastatic ccRCC [4]. Currently, the standard non-surgical treatment options are tyrosine kinase inhibitors-based targeted therapy, and immunotherapy that was recently developed and has shifted the paradigm of treatment on drug-resistant or inoperable ccRCC patients [5, 6]. Immune checkpoint inhibitors (ICIs) have shown remarkable efficacy as monotherapy or in combination with radio-chemotherapy, targeted drugs or surgery [7-12]. Unfortunately, a substantial proportion of ccRCC patients do not benefit from immunotherapy due to the inconsistent or insensitive responses to ICIs [13-16]. Therefore, it is crucial to identify novel prognostic predictors or therapeutic targets to improve the sensitivity to immunotherapy for ccRCC patients.

Numerous studies have reported that iron metabolism may govern tumorigenesis and progression due to its dual activity to trigger tumor growth and facilitate cell death [17, 18]. As highly metabolically active cells, tumor cells synthesize or secrete ferritin and influence the iron homeostasis [19]. Hepcidin is a polypeptide hormone encoded by the hepcidin antimicrobial peptide gene (HAMP), is a central regulator of iron metabolism and plays an important role in negatively maintaining the systemic iron balance of the body [20]. Hepcidin has

been found to strongly influence the iron metabolism in tumor cells [21, 22]. For example, iron metabolism disorder, induced by aberrant hepcidin, might be the major cause of certain malignant tumors, including prostate cancer [23], breast cancer [19], lung cancer [24], liver cancer [25], cholangiocarcinoma [26] and colon cancer [27]. Moreover, hepcidin is found to be overexpressed in metastatic patients compared to non-metastatic patients, and hepcidin predicts the poor overall survival (OS) in RCC [28, 29]. Nevertheless, so far, there are only few studies on the connection between hepcidin and ccRCC.

Metabolism and tumor microenvironment (TME) may interact to influence the iron requirement of cancer cells and immune cells, thereby modulating the immune response [30]. However, the composite effect of hepcidin in cooperation with immune infiltration in ccRCC remains unclear.

To determine the functional importance of hepcidin in ccRCC, we evaluated the expression profile of hepcidin in ccRCC by using bioinformatics analysis, which was further validated by experimental data. We found that hepcidin was upregulated in ccRCC and correlated with the poor OS of ccRCC patients. Furthermore, hepcidin might affect the OS of ccRCC patients through modulating immune infiltration and immune cells, especially microphages. Our findings suggest that hepcidin might serve as a promising biomarker for predicting the response to immunotherapy and the prognosis of patients with ccRCC.

Materials and methods

Patient samples

A total of 80 matched ccRCC tumor and adjacent normal specimens were obtained by radical nephrectomy in the First Affiliated Hospital of Nanjing Medical University during 2019. All diagnosis was confirmed by histopathological examination. Written informed consent was obtained from all patients.

Cell culture and quantitative real-time PCR

The normal human renal tubular epithelial cell line HK2 and ccRCC cell lines including 786-O, 769-P, Caki-1, and Caki-2 were cultured in their respective culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Quantitative real-time PCR (qRT-PCR) was performed to detect the hepcidin mRNA expression in three independent experiments. The primer sequence and reagents used in this study were listed in Table S1.

Tissue microarray (TMA) and immunohistochemistry (IHC)

TMA was constructed with samples from 80 ccRCC tissues and their paired adjacent nontumorous tissues. The clinicopathologic data and the follow-up information of patients were available. IHC was conducted to detect the expression of hepcidin and CD68 in the TMA of ccRCC. Histoscore (H-SCORE) was used to determine the protein expression level of hepcidin.

Datasets and data collection

Gene expression data and the clinicopathological information of 539 ccRCC samples and 72 para-carcinoma tissues were collected from TCGA (https://portal.gdc.cancer.gov/). The gene profiles of 28 normal kidney tissues were downloaded from the GTEx datasets (https://www.gtexportal.org). The expression of hepcidin in TCGA and GTEx datasets was analyzed using the same sequencing platform and library preparation to reduce potential batch effects. UALCAN (http://ualcan.path.uab.edu/) and Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) databases were used to access hepcidin mRNA expression in ccRCC.

Correlation of hepcidin gene methylation with clinicopathological parameters

The correlations between the promoter methylation of hepcidin and the clinicopathological parameters was examined in UALCAN. MethSurv (https://biit.cs.ut.ee/methsurv/) was utilized to provide the prognostic value of DNA methylation of hepcidin at CpG sites.

Survival analysis and cox regression analyses

Kaplan-Meier curves were analyzed with TCGA-KIRC dataset. Accordingly, the OS of ccRCC patients was assessed again by Kaplan-Meier Plotter database (KM Plotter, http://kmplot.

com). Receiver operating characteristic (ROC) curve was generated with "pROC" and "ggplot2" R package to analyze the predictive power of hepcidin.

Hepcidin-interacting genes and protein-protein interaction (PPI) network

GeneMANIA database (http://www.genemania.org/), STRING database (https://string-db.org/) and Cytoscape software (version 3.9) were utilized to build a PPI network of hepcidin, respectively. Ultimately, the "pheatmap" R package was used to visualize the cluster of iron-associated genes that were correlated with hepcidin.

Functional enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted to explore the potential function and signaling pathways related to hepcidin in ccRCC. In addition, the single-gene Gene Set Enrichment Analysis (GSEA) analysis of hepcidin was applied to explore the enrichment pathways. Subsequently, LinkedOmics (http://www.linkedomics.org/) was applied to validate the results from GO and KEGG analyses.

Tumor immune single cell analysis

Tumor Immune Single-Cell Hub (TISCH, http://tisch.comp-genomics.org) provided comprehensive cell type annotations and enabled interactive single-cell transcriptome visualization. The TME of ccRCC was explored via diverse datasets at the single-cell or cluster level.

Immune infiltration analysis

Based on the hepcidin expression data, the "ESTIMATE" R package was used to explore the immune infiltration of estimated stromal and immune cells (StromalScore, ImmuneScore and ESTIMATEScore). CIBERSORT algorithm was applied to calculate the immune scores to characterize 24 human immune cell subpopulations. Additionally, Spearman correlations between hepcidin and immune checkpoints (PDCD1, CD274 and CTLA4) were determined in GEPIA.

Immunotherapy response prediction

The response to immunotherapy was predicted by immune-checkpoints, tumor mutation burden (TMB) and cytotoxic T lymphocyte (CTL). Eight immune-checkpoint-relevant genes (SIGLEC15, TIGIT, CD274, HAVCR2, PDCD1, CTLA4, LAG3, and PDCD1LG2) were selected and their expression values were extracted. The correlation between hepcidin and CTL was analyzed using Tumor Immune Dysfunction and Exclusion database (TIDE, http://tide.dfci.harvard.edu/). Also, we elevated hepcidin expression in the patients who received Nivolumab therapy based on the gene expression date from clinical trials CheckMate 009 (NCT01358721), CheckMate 010 (NCT01354431) and CheckMate 025 (NCT01668784) [31, 32]. Brief details on study treatment, schedule, and patient number for each dataset included in the analyses are provided in Table S2.

Statistical analysis

R software (version 4.0.1), SPSS (version 26.0) and GraphPad Prism (version 8.0) were utilized to perform the statistical analysis. The rational statistical test was employed to compare two independent test series (Student's t-test) or more test series (ANOVA test). A two-sided $P<0.05$ indicated statistical significance.

Results

Hepcidin expression was upregulated in ccRCC

To investigate the functional importance of Hepcidin in ccRCC, we first assessed the expression of hepcidin in pan-cancers. We found that the expression of hepcidin was upregulated in most cancer types in TCGA database (Figure S1A). Similar elevated hepcidin expression was also observed in the paired pan-cancer samples (**Figure 1A**). Importantly, in ccRCC, the cancer type of our study interest, hepcidin mRNA expression was increased in the tumor samples from both TCGA (**Figure 1B-D**) and GSE53757 datasets (**Figure 1E**). The upregulation of hepcidin in ccRCC was further confirmed in other datasets such as GEPIA (Figure S1B, S1C) and UALCAN (Figure S1D).

Experimental validation of the hepcidin expression in ccRCC

To validate our findings from the bioinformatics analyses, we used several experimental approaches to examine the expression level of hepcidin in ccRCC. First, our qRT-PCR results

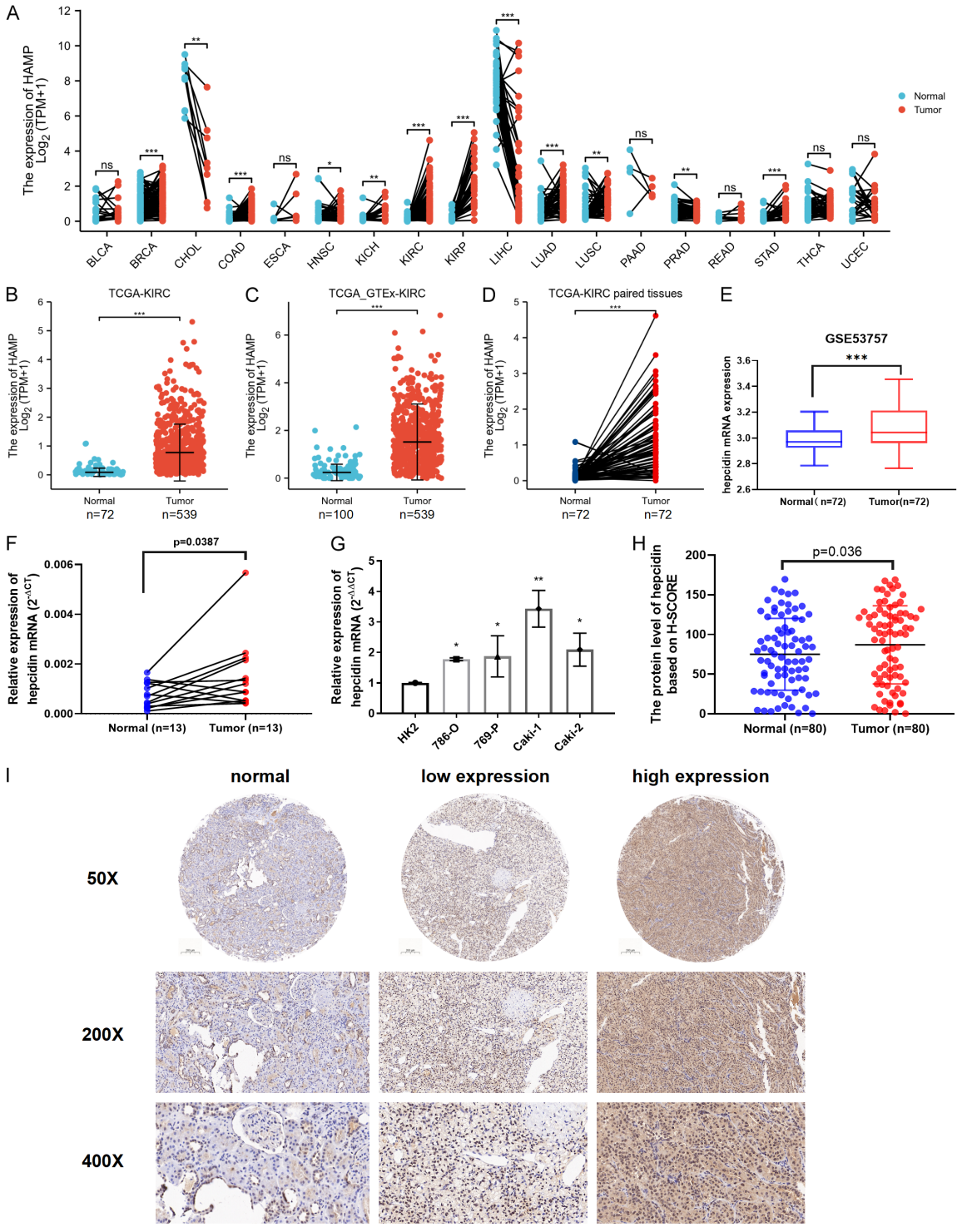

Figure 1. Overexpression of hepcidin in ccRCC. (A) Hepcidin expression in the paired samples in TCGA. (B, C) Hepcidin mRNA expression in ccRCC tumor and paracancerous tissues with TCGA (B) and GTEx-TCGA (C). (D) Hepcidin mRNA expression in 72 paired ccRCC tumor and adjacent normal tissues with TCGA. (E) Hepcidin mRNA expression in GSE53757 with GEO. (F) Hepcidin mRNA expression in 13 paired ccRCC tumor and adjacent normal tissues in our center. (G) Hepcidin mRNA expression in ccRCC cell lines. (H, I) Hepcidin protein expression in the TMA of ccRCC with IHC. Scale bars indicated 200 μm.

demonstrated that hepcidin mRNA level was upregulated in ccRCC tissues (**Figure 1F**). Similarly, hepcidin expression was elevated in ccRCC cell lines, including 786-O, 769-P, Caki-

Table 1. Correlation between hepcidin expression and clinicopathological characteristics in TMA of ccRCC (n=80)

Characteristics	Case	Hepcidin expression		X^2	*P* value
		Low	High		
All cases	80	37	43		
Age (years)				3.948	0.047*
<60	38	22	16		
≥60	42	15	27		
Gender				0.002	0.961
Male	56	26	30		
Female	24	11	13		
Laterality				0.453	0.501
Left	40	20	20		
Right	40	17	23		
T stage				6.772	0.009**
T1 & T2	53	30	23		
T3 & T4	27	7	20		
N stage				1.478	0.224
N0	75	36	39		
N1	5	1	4		
M stage				0.765	0.382
M0	76	36	40		
M1	4	1	3		
Pathologic stage				6.374	0.012*
I & II	51	29	22		
III & IV	29	8	21		
WHO grade				0.135	0.713
G1 & G2	45	20	25		
G3 & G4	35	17	18		

*P<0.05, **P<0.01.

1, and Caki-2, when compared to normal HK2 cells (**Figure 1G**). Next, IHC staining exhibited that hepcidin protein expression was higher in ccRCC tumor tissues than in normal tissues, as shown in **Figure 1H** and **1I**. Moreover, the IHC results indicated that hepcidin expression was significantly correlated with age, T stage, and pathologic stage in ccRCC (P<0.05) (**Table 1**).

Hepcidin expression correlated with the clinical features and the prognosis of patients with ccRCC

By employing logistic regression analysis, we found that hepcidin expression was associated with gender, T, N, M stage, tumor stage and grade of patients with ccRCC (**Table 2**), which was consistent with the previous tissue microarray (TMA) results. These data suggested that hepcidin expression might attribute to tumor progression and metastasis.

To evaluate the prognostic value of hepcidin, we performed univariate Cox analysis and found that hepcidin expression predicted poor overall survival (OS) in ccRCC patients (HR=1.533, P<0.001) (**Figure 2A**). Consistently, multivariate Cox analysis demonstrated that hepcidin was an independent prognostic factor to predict poor OS for patients with ccRCC (HR=1.261, P=0.048) (**Figure 2B**). We also observed that higher level of hepcidin was related to a poor OS in the TCGA cohort (P< 0.001) (**Figure 2C**), consistent with the survival result from K-M plotter (**Figure 2D**). We further examined the distribution of the risk score and survival status according to hepcidin expression in ccRCC patients (**Figure 2E**), and, at the end, a nomogram integrating four independent prognostic parameters (hepcidin, age, T and M stage) was built to predict the 1-, 3-, and 5-year survival of ccRCC patients (C-index =0.761) (**Figure 2F**).

To determine the diagnostic value of hepcidin, ROC analysis was performed, and the results illustrated that hepcidin could accurately distinguish ccRCC tissues from normal tissues (AUC=0.911) (**Figure 2G**). Furthermore, the ROC curve of OS in ccRCC was also conducted, and the AUC values for 1-, 3-, and 5-year ROC of hepcidin were 0.686, 0.631, and 0.627, respectively (**Figure 2H**). Together, these results suggested the potential of hepcidin as a new biomarker for the diagnosis and prognosis of patients with ccRCC.

Methylation of hepcidin in ccRCC

Since the methylation status in the promoter affects gene expression, we examined the methylation level of hepcidin gene in KIRC and found a lower methylation level in tumor tissues than in normal control tissues (**Figure 2I**). Furthermore, we determined that the degree of hepcidin gene methylation was decreased in ccRCC patients of different ages, genders, races, tumor stages, grades, and nodal metastasis (Figure S2A-F). These data suggested that hepcidin promotor methylation was involved in

Table 2. Correlation between hepcidin expression and clinicopathological features in TCGA-KIRC patients (n=539)

Characteristics	Odds Ratio (OR)	*P* value
Age (>60 vs. ≤60)	0.831 (0.592-1.164)	0.282
Gender (Male vs. Female)	1.597 (1.117-2.289)	0.010*
Laterality (Right vs. Left)	0.914 (0.651-1.283)	0.604
T stage (T3 & T4 vs. T1 & T2)	2.053 (1.434-2.954)	<0.001***
N stage (N1 vs. N0)	4.089 (1.279-18.161)	0.031*
M stage (M1 vs. M0)	2.306 (1.396-3.901)	0.001**
Pathologic stage (Stage III & Stage IV vs. Stage I & Stage II)	2.268 (1.591-3.250)	<0.001***
WHO grade (G3 & G4 vs. G1 & G2)	2.300 (1.626-3.266)	<0.001***

*P<0.05, **P<0.01, ***P<0.001.

hepcidin downregulation and that its methylation level was negatively correlated with the clinical features of ccRCC patients.

Network of hepcidin interacting genes and proteins

We utilized GeneMANIA and STRING databases to generate a gene-gene interaction network and PPI network, and the top 20 hepcidin-interacting genes included SLC40A1, HFE, TFRC, and CYBRD1 (**Figure 3A**, **3B**). In addition, we used Cytoscape to identify a batch of hub genes, including HFE, TFRC, EPO, and TFR2 (**Figure 3C**). Furthermore, we investigated the association of hepcidin with these hub genes in TCGA. As shown in **Figure 3D**, hepcidin was positively correlated with HFE, EPO, TFR2, FAM132B and TMPRSS6, while negatively correlated with SLC11A2 and TFRC in KIRC.

Enrichment analysis of hepcidin and its correlated genes

We further identified genes that were co-expressed with hepcidin in TCGA-KIRC database. The top 30 genes co-expressed with hepcidin were shown (**Figure 4A**, **4B**). A total of 509 positively associated genes and 106 negatively associated genes were stratified with a |Spearman's r| >0.4 and P<0.05. Then, GO and KEGG analyses uncovered that the genes positively associated with hepcidin were enriched in immune-related response, including T cell activation, regulation of lymphocyte activation, cytokine binding, and cytokine receptor activity (**Figure 4C**). Interestingly, hepcidin was inversely associated with genes enriched in metabolism-related processes, such as lipid modification, fatty acid catabolic process, propanoate metabolism (**Figure 4D**).

Moreover, we explored the hepcidin-related signaling pathways in ccRCC by using a gene set enrichment assay (GSEA). As our KEGG analysis indicated that the top 10 signaling pathways positively correlated with hepcidin were mainly related to various immune functions, including cytokine-cytokine receptor interaction, chemokine signaling pathway, intestinal immune network for IgG production, and antigen processing and presentation (**Figure 4E**), GSEA analysis revealed that hepcidin negatively regulated metabolism-related pathways, including the pathways of glycolysis, gluconeogenesis, proximal tubule bicarbonate reclamation, oxidative phosphorylation, and pyruvate metabolism (**Figure 4F**). In consistent with these findings, enriched GO annotations and KEGG pathways in KIRC were also identified by LinkedOmics, suggesting that hepcidin expression was involved in T cell activation and Toll receptor activity (Figure S3).

Correlation between hepcidin and TME

Two KIRC data sets (KIRC_GSE111360 and KIRC_GSE139555) in TISCH were used to decipher the relationship between hepcidin expression and tumor microenvironment (TME). We found that hepcidin expression in KIRC was enriched in proliferative T cells, followed by monocytes/macrophages (**Figure 5A**). The distribution and the 12 types of immune-related cells in TME were shown in **Figure 5B**, **5C**. Notably, monocytes and macrophages were the most enriched immune cells, and hepcidin expression was correlated with the degree of monocytes/macrophages infiltration (**Figure**

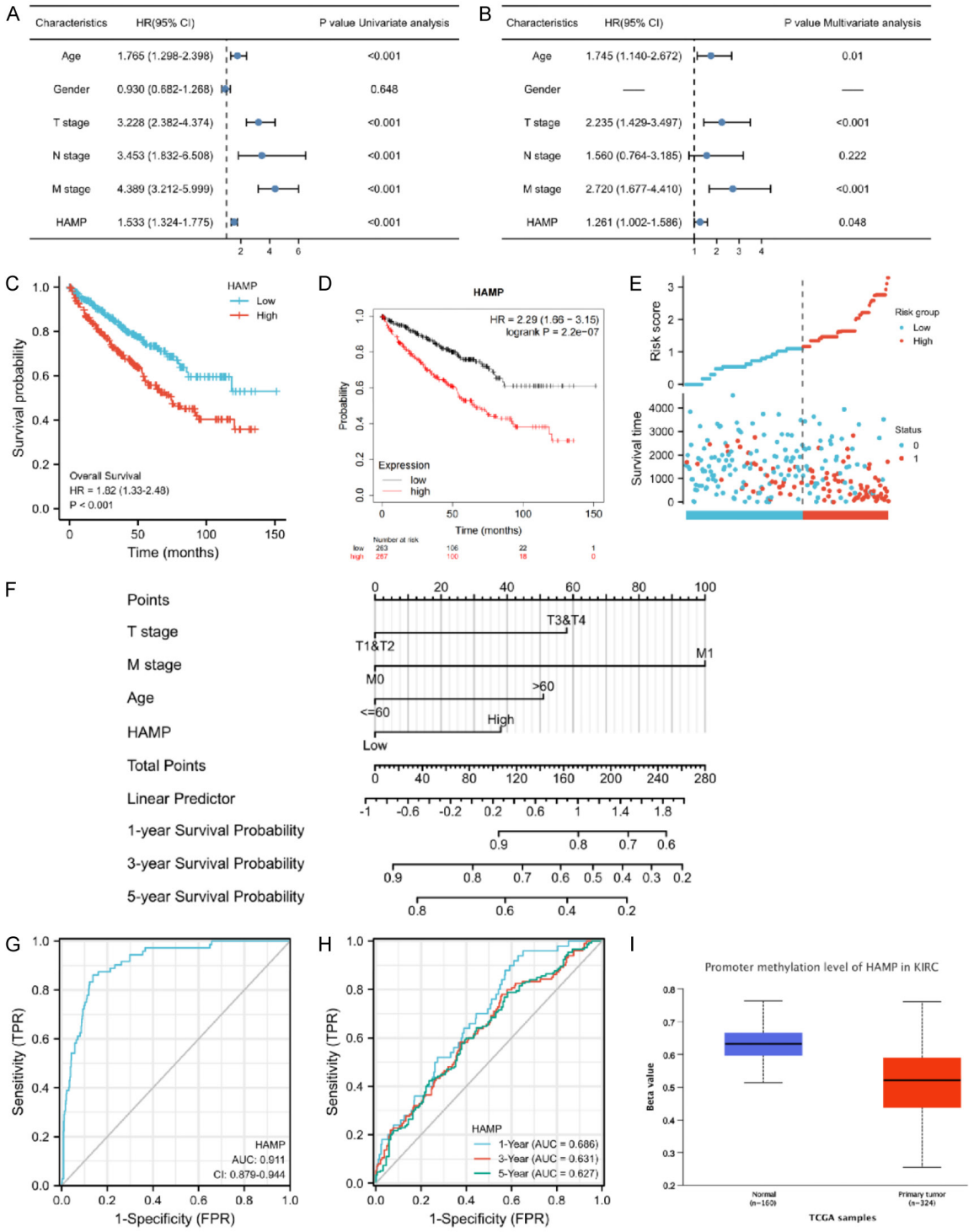

Figure 2. Hepcidin Expression Correlates with Clinical Features and Prognosis of ccRCC Patients. (A, B) Univariate (A) and multivariate (B) Cox regression analysis of hepcidin with clinical characteristics on OS. (C, D) Survival analysis in TCGA (C) and K-M plotter (D) for OS in ccRCC, respectively. (E) Distribution of the risk score and survival time based on hepcidin expression in ccRCC. (F) Prognostic nomogram for ccRCC patients. (G) ROC analysis of hepcidin between tumor and adjacent normal tissues in KIRC. (H) ROC analysis of hepcidin for 1-year, 3-year and 5-year OS in KIRC. (I) DNA methylation level of hepcidin in KIRC on UALCAN platform.

5D-G). Thus, our findings suggested that hepcidin might modulate TME and that monocytes/macrophages might play an important role in KIRC.

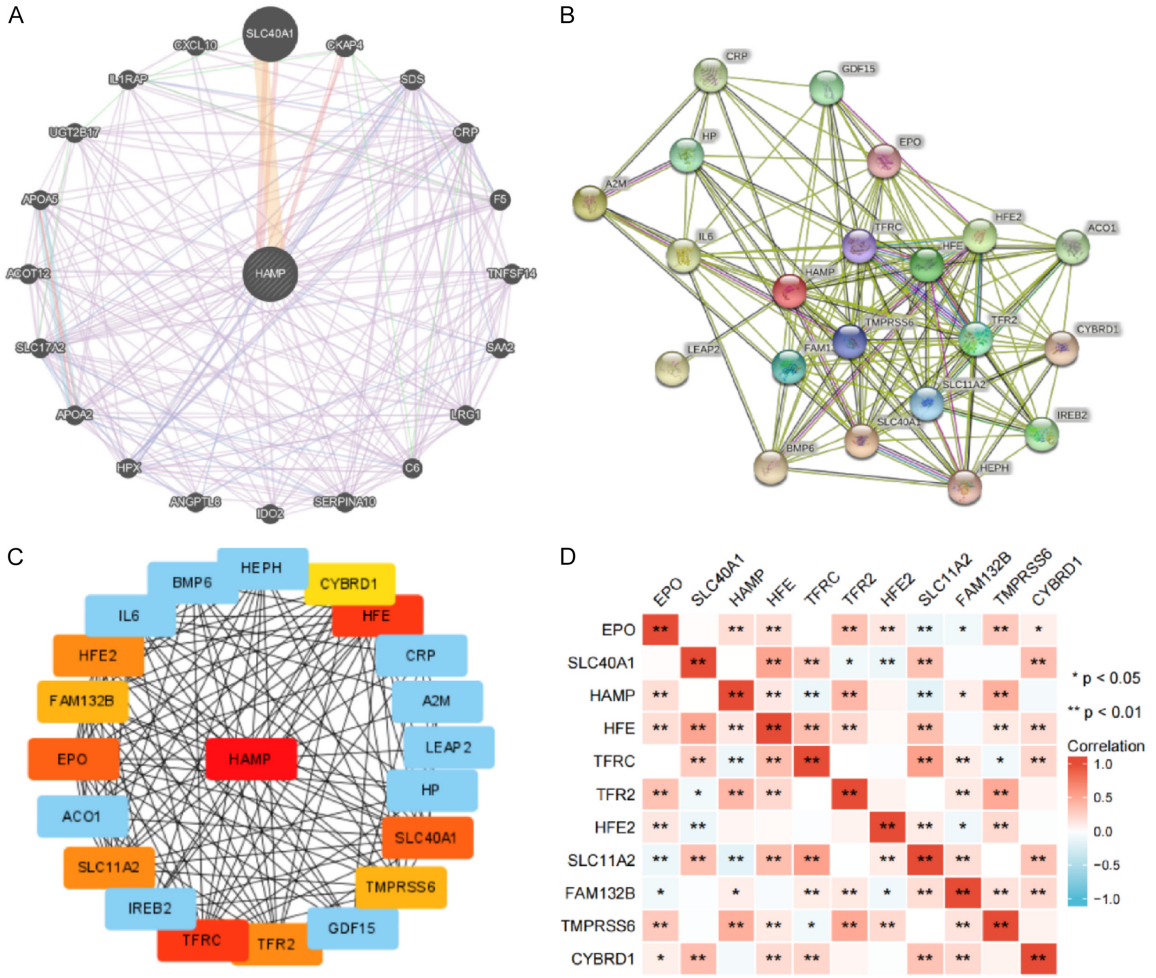

Figure 3. The interaction network of hepcidin. (A-C) The interaction genes of hepcidin in GeneMANIA (A), STRING (B) and Cytoscape (C). (D) Correlations of hepcidin with iron regulated-related genes in KIRC.

Hepcidin expression correlates with immune infiltration in ccRCC

The correlation landscape indicated that T cells had a positive association with most of the infiltrating immune cells, especially cytotoxic cells (**Figure 6A**). Meanwhile, patients with high hepcidin expression presented a higher StromalScore, ImmuneScore and ESTIMATEScore than those with low hepcidin expression (P<0.001) (**Figure 6B**). Notably, the presence of 24 immune cell subtypes was different between the high and low hepcidin expression subgroups, as most of the immune cells were abundant in the high hepcidin expression group (**Figure 6C**).

The results in TIMER further demonstrated that hepcidin had a significantly positive correlation with the level of macrophages, B cells, neutrophils, and dendritic cells in KIRC (**Figure 7A**). Moreover, the somatic copy number alterations (SCNA) analysis suggested that the arm-level deletion of hepcidin was significantly correlated with the infiltrating immune cells in KIRC (**Figure 7B**). Finally, we estimated the associations between hepcidin and the diverse signatures of immune cells in KIRC (**Table 3**).

Importantly, we explored the relationship between hepcidin and the common immune checkpoints in KIRC with data from cBioPortal. The alteration rate of hepcidin, PD-1, PD-L1 and CTLA-4 in ccRCC was 0.7%, 1.7%, 0.7% and 2.4%, respectively (**Figure 7C**). And hepcidin was positive correlated with the mRNA level of PD-1 in KIRC by GEPIA analysis (**Figure 7D**), which was further confirmed in TISIDB database (**Figure 8A**). Interestingly, hepcidin expression was positively correlated with several immune inhibitors, including SIGLEC15, TIGIT, HAVCR2, PDCD1, CTLA4, LAG3, and PDCD1LG2, in various tumors including ccRCC (**Figure 8B**, **8C**) and likewise was positively associated with CTL and TMB (**Figure 8D**, **8E**).

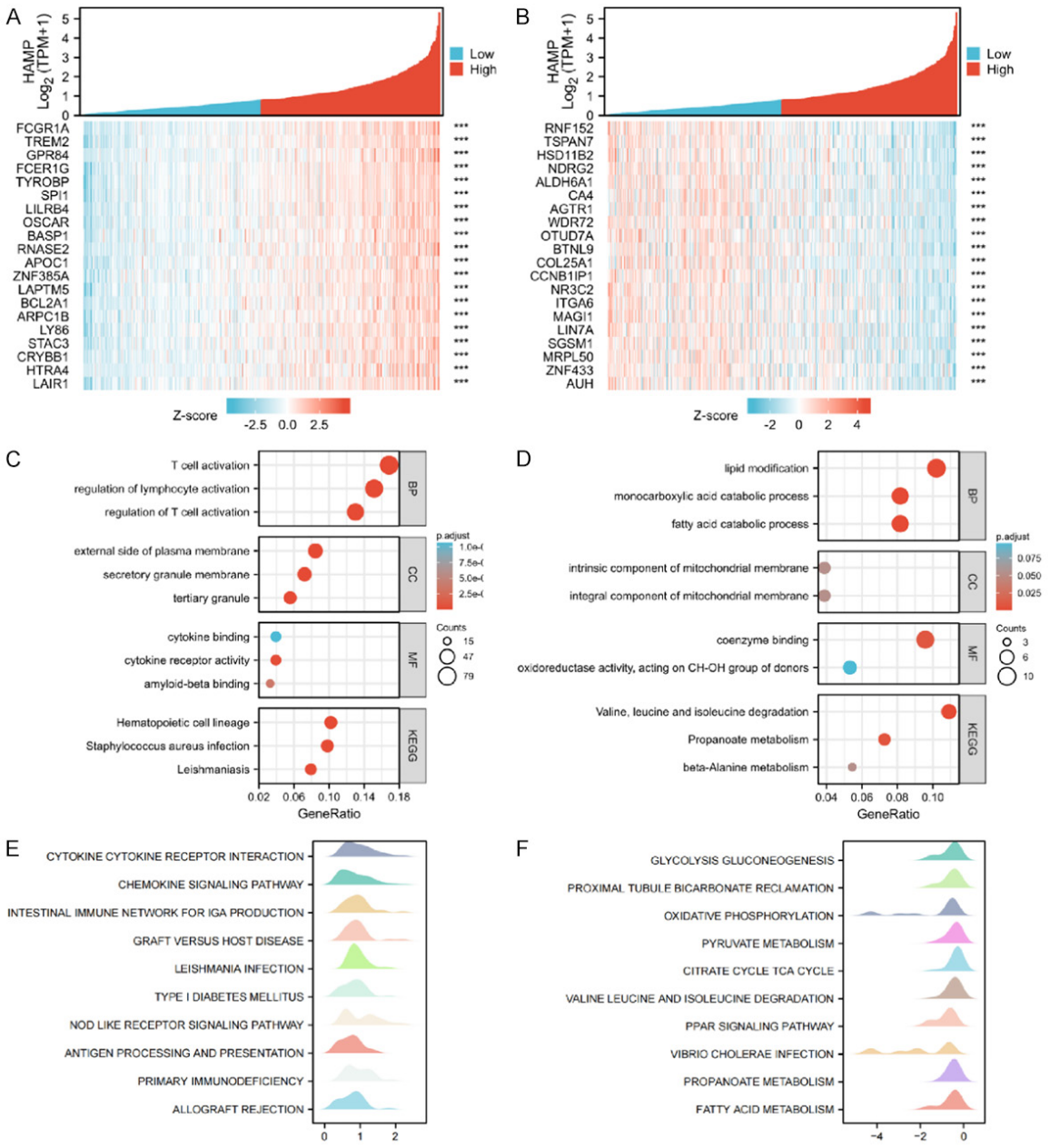

Figure 4. GO/KEGG enrichment analysis and GSEA analysis for hepcidin in TCGA-KIRC patients. (A, B) The top 20 genes with a positive (A) and negative (B) correlation of hepcidin in KIRC. (C, D) Functional enrichment terms positively (C) and negatively (D) correlated with hepcidin in GO/KEGG categories. (E, F) Top 10 enrichment plots positively (E) and negatively (F) correlated with hepcidin from GSEA.

These results suggested that hepcidin might modulate the immune activation in the TME of ccRCC, which provided the basis of potentially targeting hepcidin in immunotherapy for ccRCC. However, it should be noted that based on the analysis of data from three clinical cohorts (CheckMate 009, CheckMate 010, and CheckMate 025), hepcidin expression was no difference between Nivolumab response and non-response group of patients (Figure S4), although further studies are needed to validate this result.

Prognostic value of hepcidin with immune cells in KIRC

By performing multivariate Cox regression analysis, we determined that hepcidin (HR=1.379,

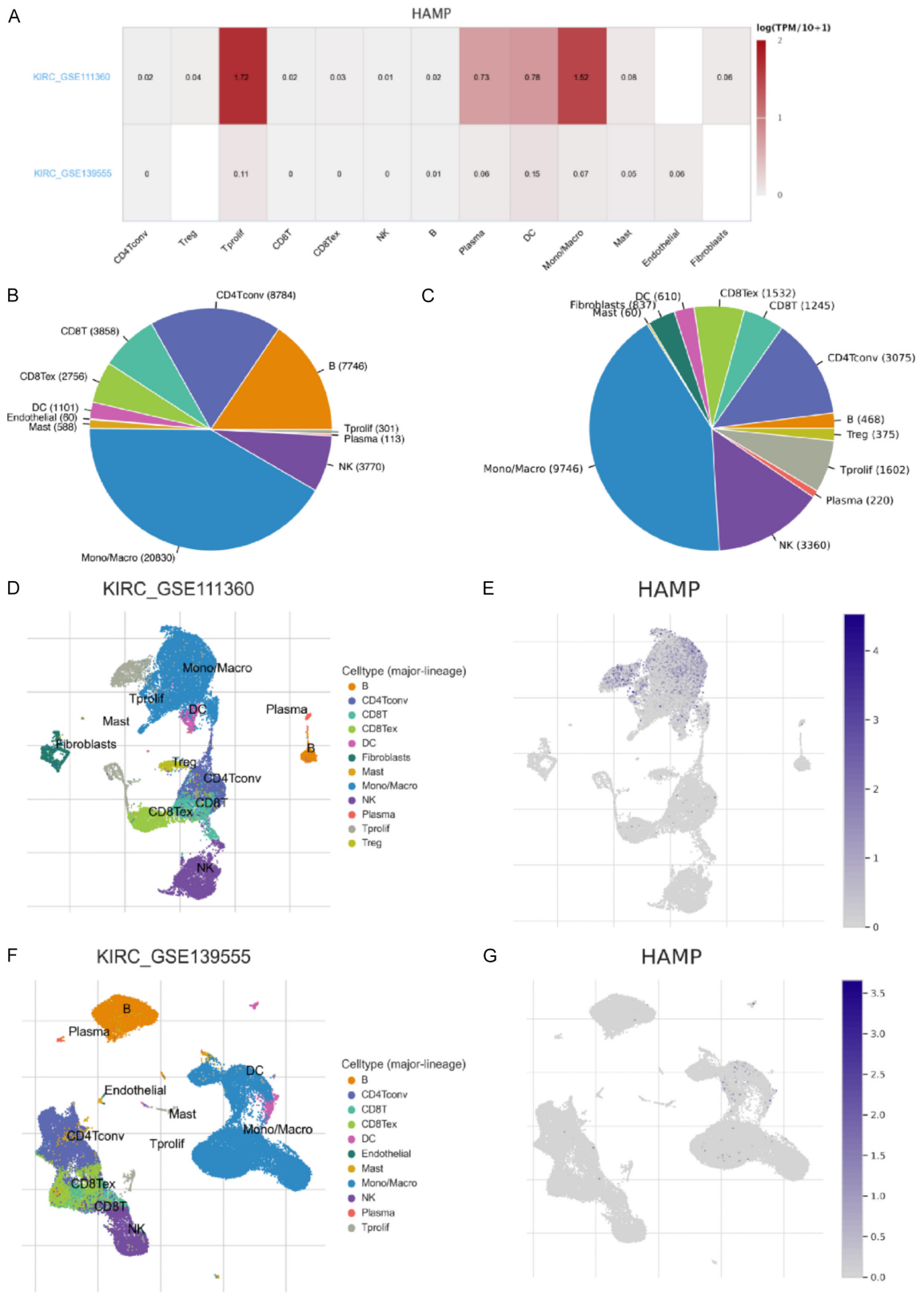

Figure 5. Correlation between hepcidin and TME of ccRCC with TISCH database. (A) Correlation of hepcidin with TME in KIRC. (B, C) The annotation and distribution of the immune cell types in KIRC_GSE111360 and KIRC_GSE139555 dataset. (D-G) The proportion of hepcidin in different immune cell types in KIRC_GSE111360 dataset (D, E) and in KIRC_GSE139555 dataset (F, G).

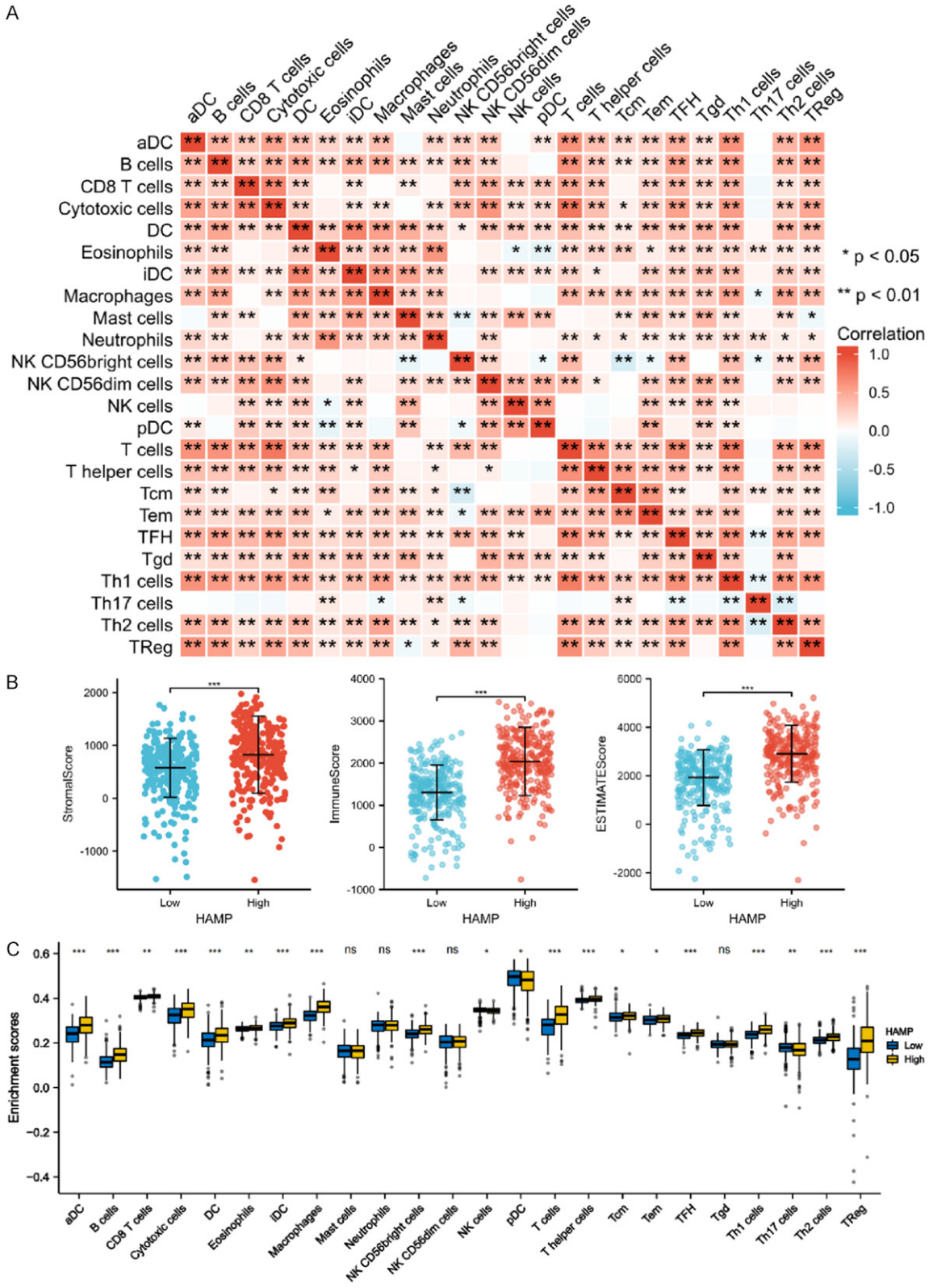

Figure 6. Hepcidin influences immune infiltration in ccRCC. A. Correlation matrix of the proportion of immune cells. B. StromalScore, ImmuneScore and ESTIMATEScore in low and high hepcidin expression groups. C. Composition of immune cell components difference between low and high expression hepcidin subgroups.

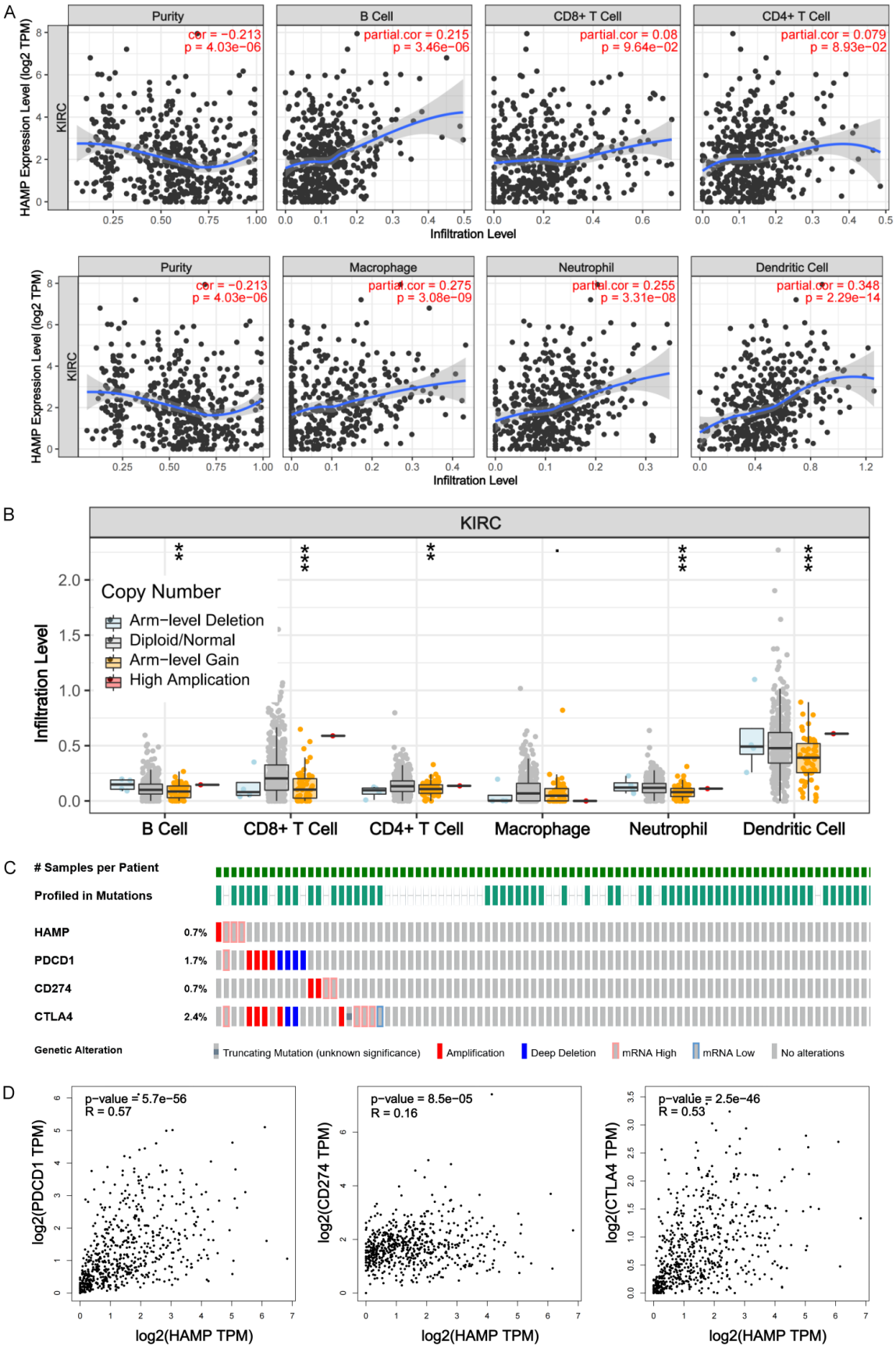
A
Purity
B Cell
CD8+ T Cell
CD4+ T Cell
cor = −0.213
p = 4.03e−06
partial.cor = 0.215
p = 3.46e−06
partial.cor = 0.08
p = 9.64e−02
partial.cor = 0.079
p = 8.93e−02
HAMP Expression Level (log2 TPM)
KIRC
Infiltration Level
Purity
Macrophage
Neutrophil
Dendritic Cell
cor = −0.213
p = 4.03e−06
partial.cor = 0.275
p = 3.08e−09
partial.cor = 0.255
p = 3.31e−08
partial.cor = 0.348
p = 2.29e−14
B
KIRC
Copy Number
Arm−level Deletion
Diploid/Normal
Arm−level Gain
High Amplication
Infiltration Level
B Cell
CD8+ T Cell
CD4+ T Cell
Macrophage
Neutrophil
Dendritic Cell
C
Samples per Patient
Profiled in Mutations
HAMP
PDCD1
CD274
CTLA4
0.7%
1.7%
0.7%
2.4%
Genetic Alteration
Truncating Mutation (unknown significance)
Amplification
Deep Deletion
mRNA High
mRNA Low
No alterations
D
p−value = 5.7e−56
R = 0.57
p−value = 8.5e−05
R = 0.16
p−value = 2.5e−46
R = 0.53
log2(PDCD1 TPM)
log2(CD274 TPM)
log2(CTLA4 TPM)
log2(HAMP TPM)

Figure 7. Correlation between hepcidin and immune infiltration in KIRC. A. Hepcidin has a positive correlation with tumor infiltrating of KIRC in TIMER. B. Correlations of SCNA in hepcidin with immune infiltration of KIRC in TIMER. C. OncoPrint of alterations in hepcidin, PD-1, PD-L1 and CTLA-4 from cBioPortal. D. Correlations of hepcidin with PD-1, PD-L1 and CTLA-4 in KIRC via GEPIA.

Table 3. Correlation analysis between hepcidin and markers of immune cells in TIMER

Description	Gene markers	KIRC			
		None		Purity	
		Cor	*P*	Cor	*P*
B cell	CD19	0.376	***	-0.213	***
	CD79A	0.425	***	0.375	***
T cell (general)	CD3D	0.440	***	0.435	***
	CD3E	0.422	***	0.452	***
	CD2	0.429	***	0.437	***
CD8+ T cell	CD8A	0.382	***	0.443	***
	CD8B	0.370	***	0.394	***
Monocyte	CD86	0.546	***	0.379	***
	CD14	0.598	***	0.565	***
	CSF1R	0.443	***	0.598	***
TAM	CD11b	0.434	***	-0.114	*
	CCL2	0.016	0.718	0.455	***
	CD68	0.456	***	-0.013	0.777
	IL10	0.417	***	0.491	***
M1 Macrophage	NOS2	-0.273	***	0.413	***
	IRF5	0.274	***	-0.287	***
	PTGS2	0.062	0.151	0.277	***
	CD40	0.002	0.958	0.051	0.274
M2 Macrophage	MRC1	-0.064	0.137	-0.008	0.866
	CD163	0.343	***	-0.068	0.145
	VSIG4	0.483	***	0.371	***
	CD200R1	0.360	***	0.510	***
	MS4A4A	0.412	***	0.358	***
Neutrophils	CEACAM8	-0.107	*	0.421	***
	CCR7	0.366	***	0.456	***
Natural killer cell	KIR3DL1	-0.135	**	0.383	***
	KIR3DL2	0.010	0.825	-0.104	*
	KIR3DL3	0.026	0.550	0.024	0.614
	KIR2DS4	-0.096	*	0.022	0.631
Dendritic cell	HLA-DPB1	0.434	***	-0.077	0.100
	HLA-DRA	0.420	***	0.457	***
	HLA-DPA1	0.367	***	0.451	***
	NRP1	-0.242	***	0.049	0.290
	ITGAX	0.461	***	-0.261	***

*P<0.05, **P<0.01, ***P<0.001.

P<0.001) and macrophage (HR=0.043, P=0.007) were two independent prognostic factors in TIMER (**Table 4**), which further indicated that hepcidin might impact on the prognosis of ccRCC patients through regulating immune infiltration in TME.

Validation of the correlation between hepcidin and immune infiltration

Lastly, we validated the correlation between hepcidin and immune infiltration by IHC staining of tumor samples. Given that macrophages were significantly correlated with hepcidin in TIMER and that both influenced the prognosis of ccRCC patients, we chose macrophage marker CD68 to validate the results obtained from bioinformatics analyses. **Figure 9A**, **9B** showed the IHC results of hepcidin and CD68 staining in ccRCC tumor TMA. H-SCORE calculated based on the intensity and degree of IHC staining revealed that hepcidin protein expression was positively correlated with CD68 (r=0.370, P<0.001) in ccRCC (**Figure 9C**), consistent with the immune infiltration results in TIMER.

Discussion

Over the past decades, a significant advancement on understanding the oncogenesis of ccRCC and identifying the prognostic biomarkers for ccRCC has been achieved [33-35]; however, there are still gaps in the clinical diagnosis and treatment of ccRCC. In our present study, we found that the expression of hepcidin was higher in ccRCC tumor tissues than in normal tissues via a series of bioinformatics analyses. More importantly, we validated this result in ccRCC cell lines and tumor tissues by qRT-PCR and IHC. These findings were consistent with two previous publications in which the authors determined that the serum hepcidin levels was correlated with poor prognosis of patients with ccRCC [28, 29].

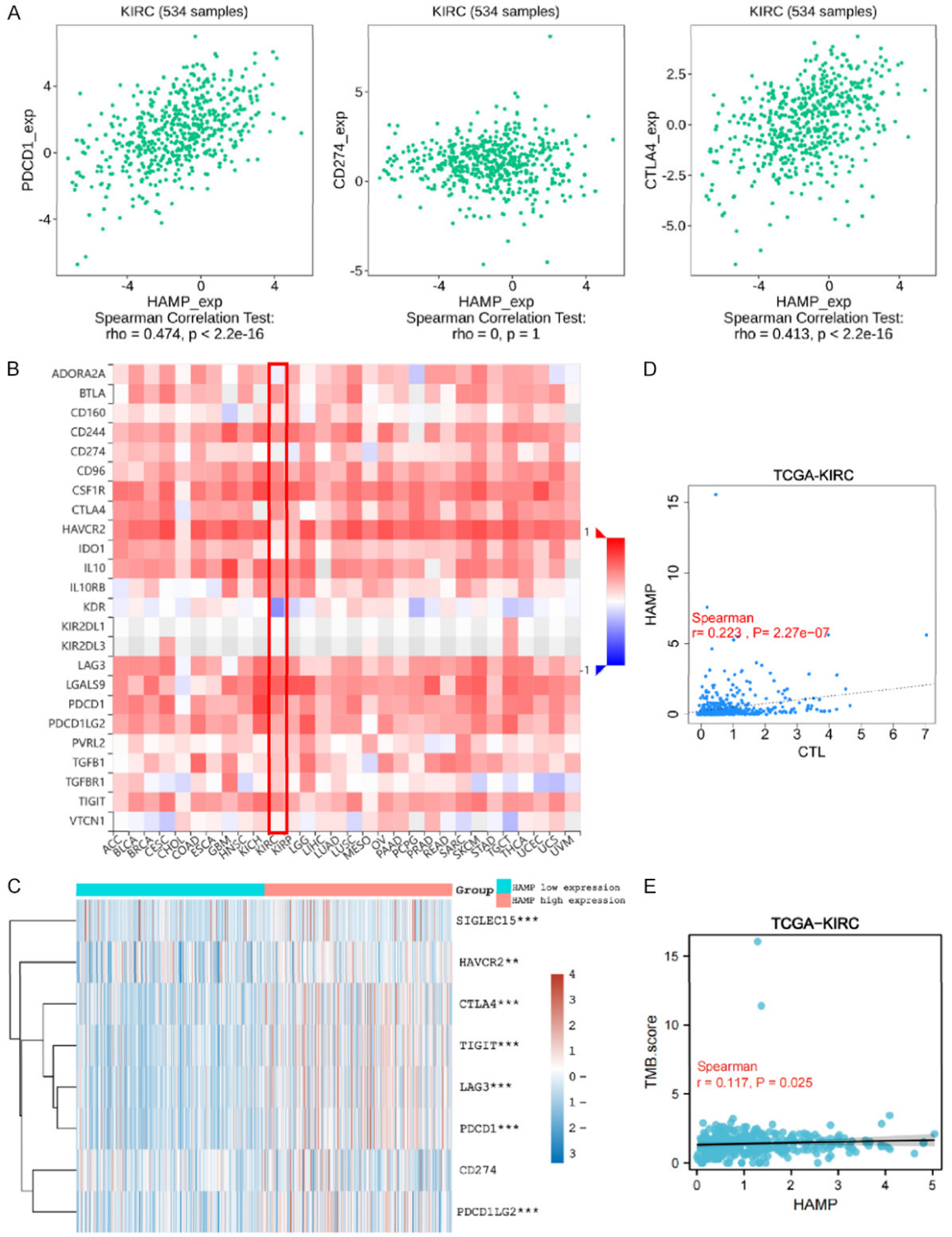

Figure 8. Correlation between hepcidin and immune inhibitors. (A) Hepcidin expression is positively correlated with PD-1, PD-L1 and CTLA4 in KIRC. (B, C) Heatmap analysis of the correlation between immune inhibitors and hepcidin expression in tumors (B) and in KIRC (C). (D, E) Hepcidin expression is positively associated with CTL (D) and TMB (E).

Human hepcidin is primarily synthesized in liver and other organs or tissues such as kidney [36]. Many studies have reported that the extrahepatic hepcidin not only modulates iron

Table 4. Multivariable Cox regression of hepcidin and immune cells of KIRC in TIMER

Characteristics	Hazard Ratio (HR)	*P* value
B Cell	0.314 (0.018-5.647)	0.432
CD8+ T Cell	0.434 (0.093-2.012)	0.286
CD4+ T Cell	1.044 (0.082-13.244)	0.973
Macrophage	0.043 (0.004-0.425)	0.007**
Neutrophil Cell	14.708 (0.278-779.138)	0.184
Dendritic Cell	1.009 (0.185-5.490)	0.992
Hepcidin	1.379 (1.234-1.540)	<0.001***

***P*<0.01, ****P*<0.001.

homeostasis but also has a significant impact on tumor development via regulating iron metabolism [37, 38]. For instance, Toshiyama *et al* reported that elevated expression of hepcidin predicted the shorter OS in pancreatic cancer patients (*P*=0.0049) [39]. Consistent to this finding, we found that high expression of hepcidin was significantly correlated with patients' gender, pathologic stage, and tumor grade in TCGA-KIRC. Similarly, our IHC analyses confirmed that hepcidin expression was markedly correlated with the patients' age, T stage, and pathologic stage in the TMA of ccRCC. Furthermore, Cox regression analyses demonstrated that hepcidin was an independent prognostic factor for poor OS in patients with ccRCC. Together, these results suggested that hepcidin might be used as a biomarker to predict the prognosis of patients with ccRCC.

Currently, the mechanism for hepcidin upregulation in ccRCC remains unclear. Therefore, in our study, we analyzed the methylation of HAMP promoter and found a significant decrease in HAMP promoter methylation in ccRCC. Gene promoter methylation is a chemical modification that can regulate the expression of the gene and lead to gene silencing during tumorigenesis [40]. Studies have shown that HAMP was transcriptionally repressed in liver cancer tissues concurrent with its promoter hypermethylation [41]. In our study, we also observed a reduced hepcidin expression possibly due to its promoter hypermethylation, and the methylation of HAMP was correlated with the clinical features of ccRCC patients, further indicating the role of epigenetic DNA methylation in hepcidin function in ccRCC. Nevertheless, further studies are needed to elucidate the mechanisms underlying epigenetic modifications of hepcidin and their associations with hepcidin expression in ccRCC.

By performing PPI analysis, we revealed that the hepcidin-interacting hub genes, including HFE, TRFC and EPO, were mostly enriched in iron metabolism. Iron metabolism and active oxidative damage may be the central link to ferroptosis [42]. Altered iron level promotes reactive oxygen species, thus triggering ferroptosis or malignant transformation [43]. Since alterations in hepcidin and its interacting genes could contribute to iron overload, it is conceivable that hepcidin could regulate ferroptosis as well. Therefore, it is significant to explore the correlation between ccRCC and ferroptosis and the involvement of hepcidin in it. Moreover, we found several enriched genes, such as IL-6, CRP and C6, were functionally related to macrophage expansion, recruitment, proliferation, differentiation, or polarization. For example, IL-6 was reported to be involved in the macrophage differentiation and could enhance macrophage activation and antigen presentation [44]. Liao *et al* found that IL-6 plays a critical role in aldosterone-induced macrophage recruitment and infiltration in the myocardium [45]. Regarding CRP, Devaraj *et al* stated that CRP could induce M-CSF release and macrophage proliferation [46]. With regard to C6, it was reported that macrophage recruitment and activation was inhibited in C6-deficient rats [47].

To further explore the functional mechanisms of hepcidin in ccRCC, we conducted GO/KEGG and GSEA analysis. Our findings suggested that hepcidin-related genes were enriched in immune response- and metabolism-related processes. These processes have been demonstrated as the critical regulators of ccRCC [48, 49], and more importantly, emerging studies have shown that the crosstalk between immune and metabolism microenvironments influences cancer development [30]. For example, Wang *et al* reported that CD8+ T cells could facilitate cancer cell's ferroptosis with reduced expression of SLC3A2 and SLC7A11 during immunotherapy, thus enhancing the anti-tumor effect [50]. In our study, we determined that macrophage was an independent factor for better prognosis of ccRCC (HR=

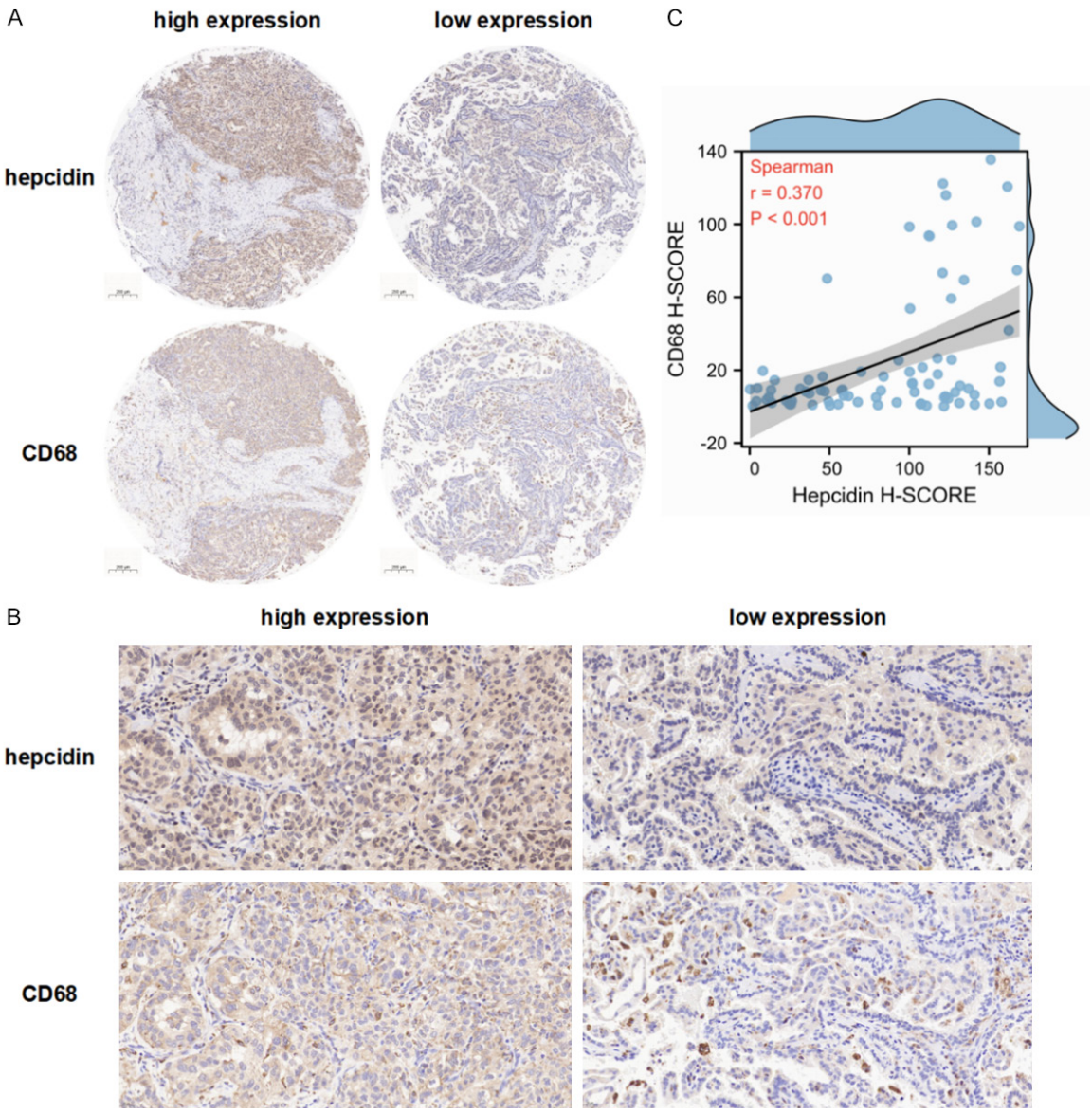

Figure 9. Validation of the correlation between hepcidin protein expression and immune infiltration via IHC analysis of TMA. (A, B) IHC analysis of hepcidin and CD68 in TMA of ccRCC at 50× (A) and 400× (B) magnification. Scale bars indicated 200 μm. (C) The correlation of hepcidin protein expression with CD68.

0.043, *P*=0.007). TISCH study also found that hepcidin was expressed to a certain extent in immune cells and had a strong correlation with proliferative T cells and monocytes/macrophages. Additionally, Katharina *et al* recently proposed that the cancer cells of ferroptosis were efficiently phagocytosed via macrophages clearance in human cancer cells [51]. Likewise, in our analysis, macrophages in anti-tumor immunity suggested that hepcidin was involved in the immune regulatory network via facilitating immune activation in ccRCC. Although we did not explore the mechanism by which macrophage was expanded along with the high hepcidin expression in ccRCC in our study, the relationship between macrophage and hepcidin has previously been reported in other diseases. First, macrophages are also able to produce hepcidin although to a low extent [52]. Second, hepcidin expression is increased when iron is overload; hence increased hepcidin expression occurs when macrophages secretes more iron [53]. Lastly, macrophages secrete IL-6 and IL-6, which can also trigger hepcidin induction via the IL-6R/STAT3 pathway [54]. Through all these different mechanisms, macrophage cells

are expanded along with high hepcidin expression. In the future, we will apply co-culture ccRCC cells with macrophages cells (THP-1) and other approaches, e.g., flow cytometry, real-time imaging, or cytokine release assays to further explore the mechanism involved in hepcidin and microphage activity.

As the major components in TME, infiltrating immune cells control tumor suppression and immune escape [9]. Elevated StromalScore and ImmuneScore have been reported to significantly correlate with the advanced clinicopathological features and poor prognosis of ccRCC patients [55, 56]. Consistently, we showed that StromalScore and ImmuneScore were higher in hepcidin high expression group, supporting the predictive role of hepcidin for poor prognosis in ccRCC. Nonetheless, the association of hepcidin with immune infiltration in ccRCC has not been fully understood. Therefore, in the present study, we estimated the immune composition of ccRCC and discovered that hepcidin was correlated with immune cell infiltration and, together with immune cells, affected the prognosis in ccRCC. Via TIMER, we found a significant association between hepcidin and most immune cell marker sets, which was further validated by IHC of TMA, as the expression of microphage marker CD68 was indeed correlated with hepcidin expression in ccRCC. The specific regulatory mechanism of how hepcidin was related to the immune infiltration needs further investigation.

Lastly, we found that hepcidin was positively associated with immune checkpoints, TMB, and CTL. Previous studies have shown that several biomarkers, including TMB, PD-1/PD-L1 expression, and CTL, can be used to predict the benefits of ICIs therapy. High TMB indicates that more tumor neoantigens are exposed, which provide the targets for ICI blockade therapy [57]. With regard to CTL, CTL cells express CD8 coreceptor and are the primary immune cells to eliminate cancer cells; hence the level of CTL infiltration is also a predictor for the outcomes of immunotherapy [58]. Our findings in the function of hepcidin may provide the rationale of using hepcidin inhibitor in combination therapy with immunotherapy in ccRCC.

In summary, our study elucidated the functional connection between iron metabolism with hepcidin and immunotherapy in ccRCC. In addition, we used the single-cell RNA sequencing data to explore the correlation between hepcidin and TME, while most published studies only examined the immune infiltration via TIMER. Furthermore, we found hepcidin was correlated with the predictors of immunotherapy. Finally, we experimentally verified the bioinformatics analysis results by using clinical samples, cancer cells, and TMA of ccRCC, and the correlation between hepcidin and immune infiltration was validated by IHC. Our findings suggested hepcidin as a potential biomarker to predict the prognosis and the immune response as well as the iron metabolism in ccRCC patients. Our findings also provided new insight into understanding the role of hepcidin and its clinical application in ccRCC.

However, there are several limitations in this study. First, our conclusions were mostly from the bioinformatics analysis of genomic data. The definitive role of hepcidin in ccRCC needs to be further tested *in vivo* and *in vitro*. Second, the precise underlying mechanisms of how hepcidin and ferroptosis function in ccRCC need to be elucidated. Lastly, the role of hepcidin in response to immunotherapy should be validated in ccRCC patients.

Collectively, we preliminarily explored the prognostic value of hepcidin in ccRCC with bioinformatics prediction and experimental validation. Further, we found that hepcidin might be a potential prognostic biomarker and a reference to predict immune response and iron metabolism in ccRCC patients. These findings may aid understanding of the role of hepcidin and its clinical application in ccRCC.

Acknowledgements

This work was supported by Scientific Research Foundation of Huashan Hospital Northern Branch (No. HSBY2019003), National Natural Science Foundation of China (No. 81970603 and No. 82100807).

Disclosure of conflict of interest

None.

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Table S1. Main reagents and consumable used in this article

Reagents	Vendor	Catalogue Number	Information
Antibody-HAMP	Affinity Biosciences	DF6492	IHC (1:100)
Antibody-CD68	ProteinTech	66231-2-Ig	IHC (1:800)
Primer-HAMP	Tsingke Biotechnology	/	Forward: 5'-CTGACCAGTGGCTCTGTTTTCC-3'
			Reverse: 5'-AAGTGGGTGTCTCGCCTCCTTC-3'
Primer-Actin	Tsingke Biotechnology	/	Forward: 5'-ATGACTTAGTTGCGTTACACC-3'
			Reverse: 5'-GACTTCCTGTAACAACGCATC-3

Table S2. Summary of clinical studies for RCC patients who received Nivolumab treatment

Study	Treatment	Dose and schedule
CheckMate 009 (NCT01358721), phase I dose escalation	Nivolumab	0.3, 2, and 10.0 mg/kg, Q3W
CheckMate 025 (NCT01668784), phase III	Nivolumab	3.0 mg/kg, Q2W
CheckMate 010 (NCT01354431), phase II dose ranging	Nivolumab	0.3, 2, and 10.0 mg/kg, Q3W

Q2W: every 2 weeks, Q3W: every 3 weeks.

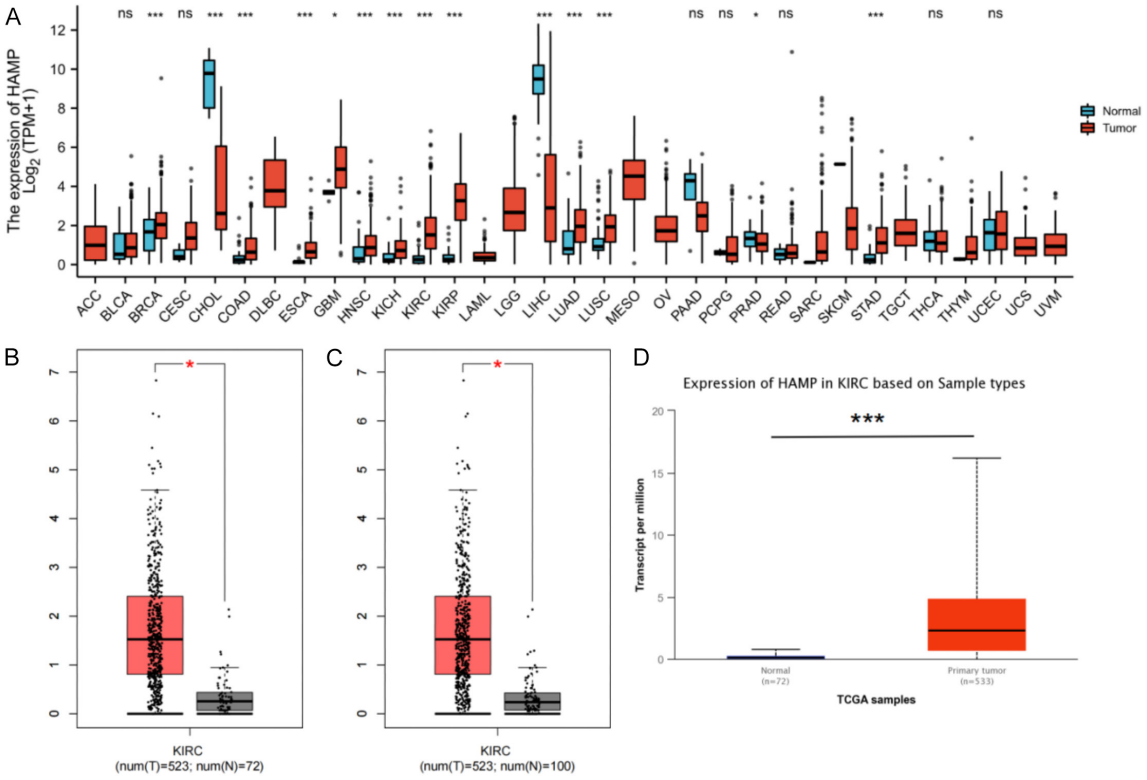

Figure S1. Expression of hepcidin in human cancers and ccRCC. (A) Hepcidin expression in human cancers with TCGA samples. (B, C) Overexpression of hepcidin in KIRC tumor tissues in GEPIA with (B) or without GTEx (C) samples. (D) Hepcidin expression in KIRC upregulated in UALCAN.

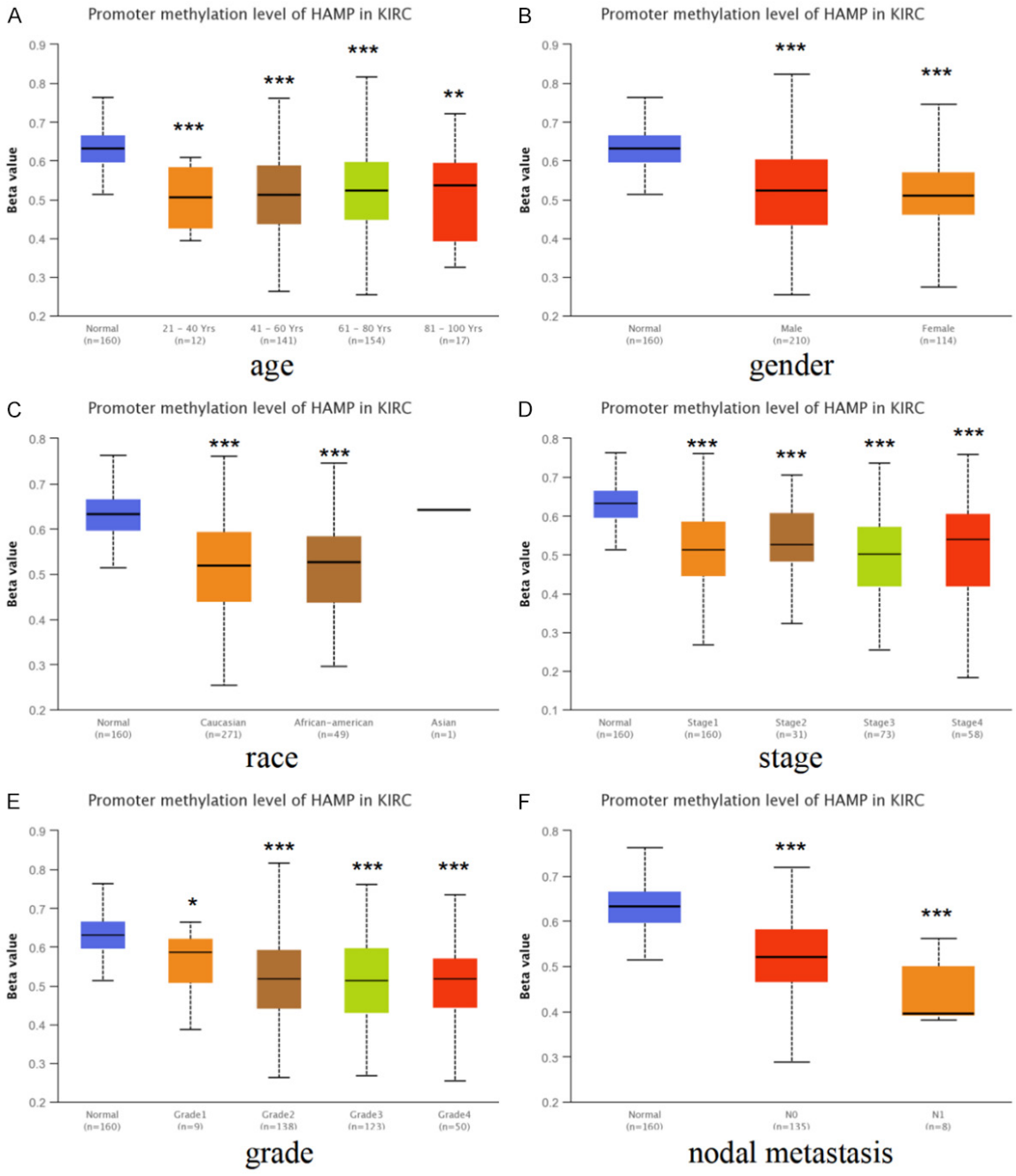

Figure S2. Promoter methylation level of hepcidin in subgroups of ccRCC patients based on clinicopathologic features via UALCAN, including age (A), gender (B), race (C), tumor stages (D), grade (E) and lymph node metastasis (F). (*P<0.05, **P<0.01, ***P<0.001).

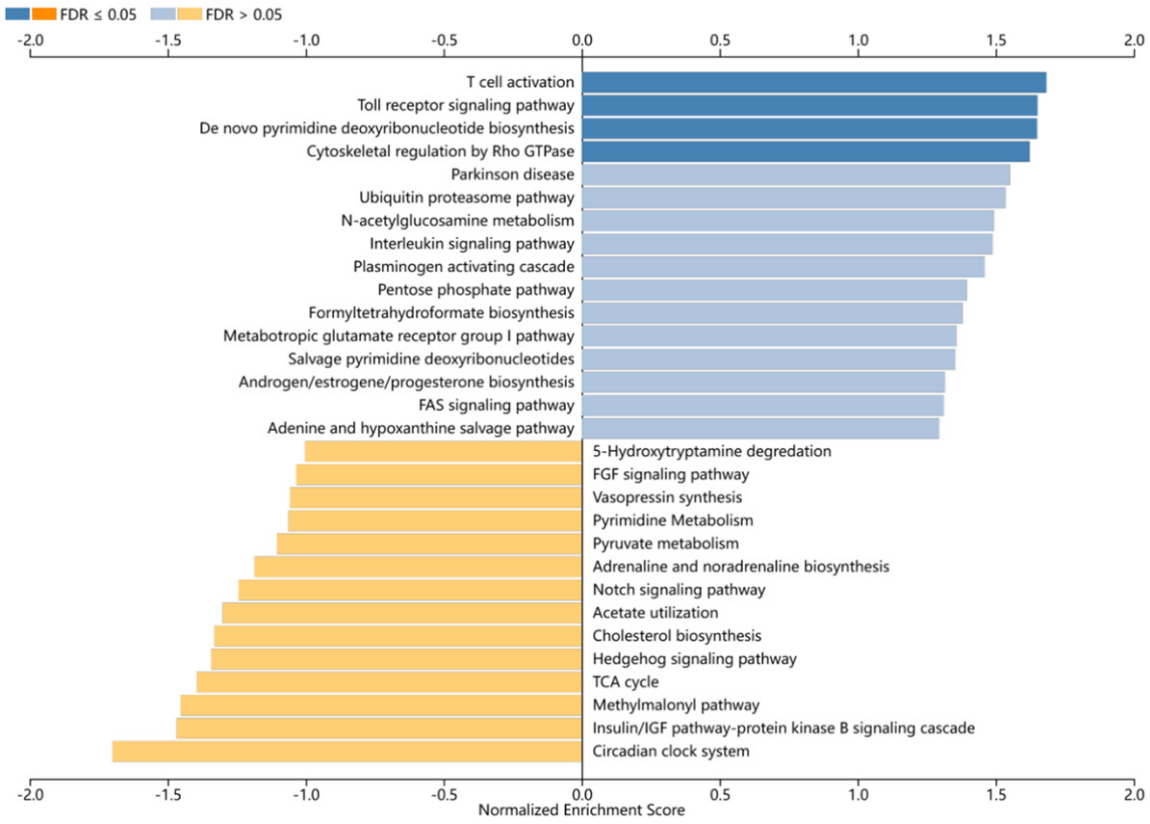

Figure S3. Significantly enriched GO annotations and KEGG pathways of hepcidin in KIRC with LinkedOmics.

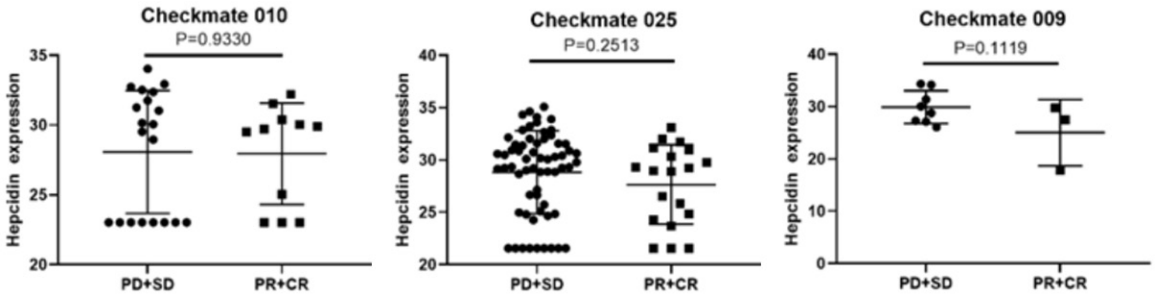

Figure S4. Hepcidin expression in the patients who have response and non-response to Nivolumab (CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease).