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Copper-binding proteins genes set predicting the overall survival and immune infiltration in hepatocellular carcinoma by bioinformatic analysis

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ABSTRACT

Abnormal Copper (Cu) accumulation shared a close association with hepatocellular carcinoma (HCC), but the regulatory role of Copper-binding proteins in HCC remains largely unknown. The aim of study was to identify the potential regulatory role of Cu-binding proteins, including copper homeostasis maintainer and the downstream effectors of Cu, in the progression of HCC. We conducted a comprehensive bioinformatic analysis of Cu-binding proteins in HCC using data from TCGA and ICGC database. Univariate cox regression analysis was conducted, and four prognostic Cu-binding proteins was identified to be differentially expressed between the normal liver tissues and HCC tissues. In addition, the Cu-binding proteins-based predictive signature (CuPscore) model was generated using the least absolute shrinkage and selection operator (LASSO) cox regression model. Here, we identified the crucial prognostic value of CuPscore in HCC. The pathological stage and CuPscore were independent risk factors for the prognosis of HCC patients. Pathological stage and CuPscore-based nomogram model exhibited great performance in predicting the prognosis of HCC patients. We also observed that the CuPscore shared a close association with several immunomodulatory molecules and the proportion of several tumor infiltrating immune cells, suggesting a potential value of CuPscore in predicting the response to immunotherapy in HCC. Our results demonstrated the prognostic value of Cu-binding proteins and its correlation with immune microenvironment in HCC, providing a therapeutic basis for the precision medicine strategy through targeting Cu-binding proteins in HCC.

1. Introduction

Hepatocellular carcinoma, one of the most lethal digestive cancers, accounts for the third most frequent cancer-related death in the world, with the 1-year and 3-year survival rates being 20% and 5%, respectively [1,2]. Surgery is the most effective treatment method for HCC, but most HCC patients are diagnosed at advanced stage, losing the opportunity to receive radical resection. Despite the development of targeted therapy and immunotherapy-based comprehensive treatment strategy, the overall 5-year survival of HCC patients still remains to be poor [3]. These necessitate the discovery of novel therapeutic targets and effective combinational treatment strategies for HCC.

Copper, an indispensable metal ion for living organisms, plays a crucial role in various kinds of physiological activity through acting as a cofactor of key enzymes involved in biological functions [4]. The unique electronic structure confers Cu the ability to transfer between oxidized and reduced state, regulating the activity of Cu-binding enzymes required for the normal growth and development. However, the redox property is also one of the main risks for the toxicity of Cu [5]. Cu toxicity is more tightly correlated with mismetalation of other metalloproteins [6], disruption of iron sulfur clusters [7], and cuproptosis [8]. Organisms have evolved a Cu homeostasis-maintaining system for Cu uptake, transport and storage, minimizing the Cu-caused toxic effect. After being incorporated into cells through copper transporter CTR1, Cu was shuttled to the Cu-transporting ATPases ATP7A and ATP7B in the *trans*-Golgi network in a Cu chaperone ATOX1-dependent manner. The ATPases use the energy derived from the hydrolysis of ATP to

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List of abbreviation

ATP7B	ATPase Copper Transporting Beta
AUC	area under the curve
CCS	Copper chaperone for superoxide dismutase
CIBERSORT	Cell-Type Identification Using Expression-Based Regression and Regularization
COMMD1	copper metabolism domain containing 1
COX1	cytochrome c oxidase I
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CTR1	Copper Transporter 1
CTR2	Copper Transporter 2
Cu	Copper
Cu1+	cuprous
Cu2+	cupric
CuPscore	Cu-binding proteins-based predictive signature
DBH	dopamine beta-hydroxylase
DEGs	differentially expressed genes
F5	factor V
GPC1	Recombinant Glypican 1
GSVA	Gene set variation analysis
HCC	hepatocellular carcinoma
HIF1-α	hypoxia-inducible factor 1-alpha
ICB	immune checkpoints blockade
LASSO	least absolute shrinkage and selection operator
LIHC	liver hepatocellular carcinoma
LOX	lysyl oxidase
MAP2K1	Mitogen-activated protein kinase kinase 1
MEMO1	mediator of cell motility 1
MICA	MHC Class I Polypeptide-Related Sequence A
MICB	MHC Class I Polypeptide-Related Sequence B
OS	overall survival PD-1: Programmed Death 1
PFS	progression-free survival
ssGSEA	single sample gene set enrichment analysis
S100A12	S100 calcium binding protein A12
TCGA	the Cancer Genome Atlas
TGF-β1	Transforming Growth Factor Beta 1
TIM-3	T-Cell Immunoglobulin and Mucin Domain-Containing Protein 3
VEGF-A:	vascular endothelial growth factor A
VTCN1	Transmembrane Domain Containing 1

incorporate Cu into the Cu-dependent enzymes in the secretory pathway, ensuring that the intracellular concentration of Cu remains within the physiological range [9–11]. The increased serum level of Cu in cancer patients, as well as the increased Cu level in tumor tissues, supported the possibility of increased demand of cancer cells for Cu [12]. Mounting evidence revealed the important role of Cu in the malignant phenotype of cancer. Cu was reported to regulate the migration ability of cancer cells through activating the metabolic and proliferative enzymes [12]. In addition, Cu was also reported to modulate the angiogenesis progress, a critical hallmark of cancer, through increasing the expression of angiogenic factors [13]. Considering the great potential of Cu to serve as a novel therapeutic target for cancer treatment, great efforts have been made to develop Cu-chelating compounds-based anti-cancer therapies [12,14]. Pre-clinical studies have revealed the anti-cancer efficiency of several Cu chelating agents [5]. For example, tetrathiomolybdate, a copper chelating agent applicated in the treatment of Wilson disease, a genetic copper overload disorder, significantly inhibited angiogenesis and metastasis through targeting NF-κB signaling in breast cancer [15]. The close association between Cu accumulation and HCC was proposed by the observation of increased incidence of HCC in patients with Wilson disease [16], as well as the increased serum level of copper in HCC patients [17,18]. Tetrathiomolybdate, a Cu-specific chelator, significantly inhibited the proliferation of HCC cells, suggesting a critical role of Cu in the malignant phenotype of HCC [19]. Takashi Himoto et al. reported that Cu accumulation could activate hypoxia-inducible factor 1-alpha (HIF1-α) to promote the hepatocarcinogenesis [20].

A recent study established the Cu proteome (partial list), which means proteins could bind with Cu [21]. These proteins include two main kinds of proteins: One is the Cu-transporting proteins that participate in regulating Cu homeostasis, such as Copper Transporter 1 (CTR1) responsible for transporting Cu to cytoplasmic [22], and COX11, COX17, SCO1, SCO2 that are responsible for providing Cu to the mitochondria; The other one is Cu-dependent enzymes that serve as the downstream biological effector of Cu, which account for nearly half of the proteins identified to be able to bind with Cu. Furthermore, several other proteins that are allosterically modulated by Cu such as PDE3B and ULK1 [23,24]. The Cu proteome also includes some proteins with unknown functions. S. Blockhuys et al. reported that the mRNA expression level of several Cu-binding proteins was dysregulated in various kinds of tumor [21]. Several Cu-binding proteins have been revealed to participate in regulating the malignant phenotype of cancer cells. For example, the Cu deprivation-caused dysfunction of cytochrome *c* oxidase I (COX1), a Cu-binding protein, significantly reduced oxidative phosphorylation, thereby inhibiting cancer cells proliferation [25]. Lysyl Oxidase (LOX), a Cu-depending enzyme, could be secreted by cancer cells to generate a pre-metastatic niches via stimulating collagen cross-linking and fibronectin synthesis [26,27]. Mitogen-activated protein kinase kinase 1 (MAP2K1), a Cu-dependent enzyme involved in the MAPK signaling pathway, plays a significant regulatory role in the malignant phenotype of cancer cells [28]. Several other Cu-dependent enzymes, such as mediator of cell motility 1 (MEMO1), SPARC, and copper metabolism domain containing 1 (COMMD1), are reported to participate in regulating the malignant phenotype of cancer cells [29–31]. In HCC, the dysregulation of Cu-binding protein was observed [21]. However, little is known about the regulatory role of Cu-binding protein in HCC progression.

Although previous studies revealed the association between Cu accumulation and hepatocellular carcinoma, little is known about the underlying mechanisms of Cu-mediated tumor progression. Cu-binding proteins are important regulators of Cu homeostasis and downstream effectors of Cu, and it is reasonable to speculate that Cu-binding proteins might play a significant role in the Cu-mediated tumor progression. Here, we conducted a systematic analysis of the role of Cu-binding proteins in HCC, exploring its expression pattern, prognostic value, putative immune microenvironment regulatory effect in HCC. Our bioinformatic analysis generated a novel Cu-binding proteins-based nomogram model for predicting the prognosis of HCC patients and facilitated our understanding of the therapeutic application of Cu-binding proteins in HCC treatment.

2. Materials and methods

2.1. Patient cohort and data collection

A total of 50 adjacent normal tissues and 373 HCC samples and clinicopathological information from the Cancer Genome Atlas (TCGA) and 240 HCC samples from ICGC cohorts were included in the present study. The TCGA cohort was used as the training cohort and the ICGC cohort with 240 patients were used for validation. RNA expression profile and clinical parameters of patients in the ICGC cohort were downloaded from the ICGC database (https://dcc.icgc.org/). Data in the

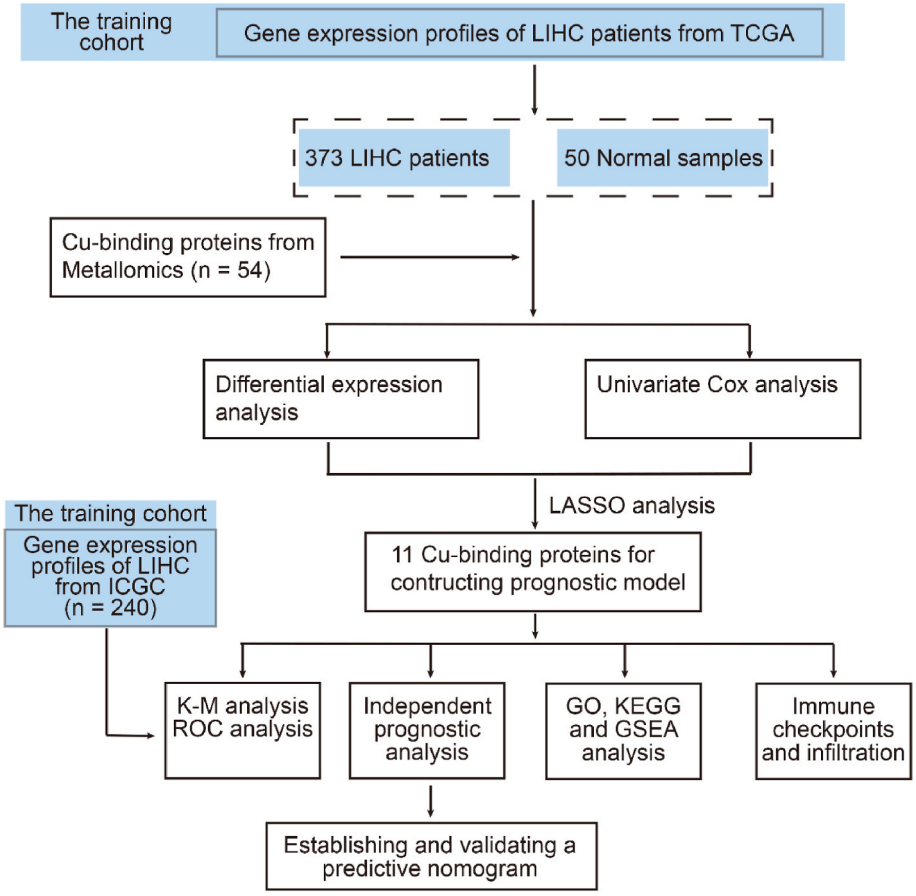

Fig. 1. The workflow of this study.

TCGA cohort were downloaded from the UCSC website (https://xenabrowser.net/datapages/). The Ethical Committee of our hospital approved this study.

2.2. Pathway correlation analysis

To analyze the effects of Cu-binding proteins on glioma development and progression, we used the R software Gene set variation analysis (GSVA) package to evaluate the gene set of Cu-binding proteins in normal and HCC tissues. The parameter of method was set to 'single sample gene set enrichment analysis (ssGSEA)'. In ssGSEA, the expression values of the genes in the gene set are first ranked, and then the cumulative sum of the gene expression values is calculated for each gene in the set. This cumulative sum is then plotted against the rank of the genes, and the resulting plot is used to determine the enrichment score for the gene set in the sample.

2.3. Establishment of the CuP-related model

In the training set, the differentially expressed genes (DEGs) of Cu-binding gene set between the normal and HCC tissues were analyzed using the *t*-test method. After that, we performed univariate Cox regression analysis to screen prognostic genes of the DEGs and four crucial genes (GPC1, F5, LOX and S100A12) were finally confirmed as prognostic factors for HCC. Then, LASSO Cox regression model was used to screen out the most robust markers related to survival and construct the prognostic CuP model through the R 'glmnet' package. The four CuP genes were integrated using the LASSO algorithm to construct a predictive model for the CuPscore:

$$\text{CuPscore} = \Sigma\ (LASSO\ coefficient\ of\ RNAi \times RNAi\ expression)$$

The optimal cut-off value was determined using the X-tile software [32]. According to the calculated CuPscores, these patients were classified into CuP_low and CuP_high groups. Subsequently, the overall survival (OS) and progression-free survival (PFS) between CuP_low and CuP_high groups were compared to validate the model's prognostic value using the R 'survival' package.

2.4. Survival analysis

The OS of HCC patients were downloaded from the TCGA and ICGC database. The optimal cut-off value was calculated by X-tile software and used to divide samples into CuP_low and CuP_high groups. Samples with low expression levels of these proteins were assigned to the CuP_low group, while samples with high expression levels were assigned to the CuP_high group. This cut-off value was based on the expression levels of the Cu-binding proteins in each sample. The Kaplan–Meier method was used to plot the survival curves for the CuP_low and CuP_high groups. The log-rank test was used to evaluate the differences in survival time between the CuP_low and CuP_high groups. The COX regression analysis was used to evaluate the mortality risks associated with the expression levels of the Cu-binding proteins. the predictor variable was the expression level of the Cu-binding proteins, and the outcome variable was death.

2.5. Immune profile analysis

After dividing the samples into CuP_low and CuP_high groups based on their expression levels of Cu-binding proteins, we analyzed the association of CuP group and immune checkpoints using the Mann-Whitney *U* test. To assess the relationship between the expression levels of the immune checkpoint proteins and the immune

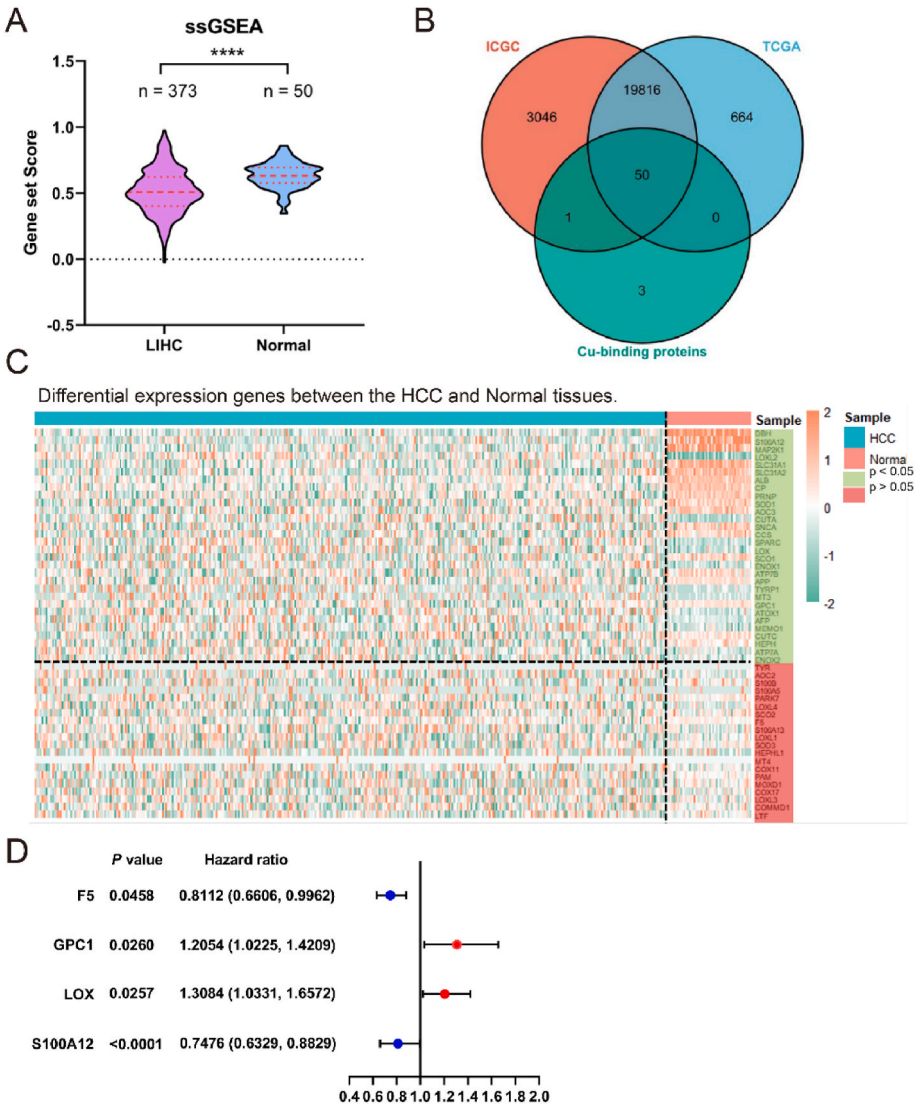

Fig. 2. Identification of prognostic Cu-binding proteins. (A) ssGSEA analysis of the human copper proteome in normal and liver hepatocellular carcinoma (LIHC) tissues. (B) The venn plot of the human copper proteome, mRNAs in TCGA and ICGC cohorts. (C) Differentially expressed Cu-binding Proteins between hepatocellular carcinoma (HCC) and normal tissues from TCGA. (D) The univariate Cox analysis found 4 Cu-binding Proteins.

microenvironment of the tumors, the proportion of 22-type infiltrating immune cells in each tumor were calculated using the cibersortx software (https://cibersortx.stanford.edu) [33].

2.6. Statistical analysis

Statistical analyses and figure plots were performed using the R software (version 4.1.0, http://www.r-project.org), GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA, USA) software and SPSS Statistics 25 (IBM Corp., Armonk, NY, USA). RNA expression data were standardized using a Z-score method. The *t*-test method was used to identify DEGs. The relationship between CuP model and other categorical variables was analyzed using the Chi-square test. The univariate and multivariate Cox analyses were performed using the SPSS software. All statistical tests were two-sided, and statistical significance was set at $^*P < 0.05$.

3. Results

3.1. Acquisition of Cu-binding proteins-related DEGs

Blockhuys et al. previously identified fifty-four Cu-binding proteins and analyzed the dysregulation of these proteins in breast cancer [21]. To explore the expression pattern and the regulatory role of these Cu-binding proteins in HCC, we conducted a comprehensive bioinformatic analysis of these proteins in HCC using data from TCGA cohort and ICGC cohort. The process of data collection and analysis was summarized in Fig. 1. A total number of 373 cancer tissues and 50 normal tissues from the TCGA cohort and 240 HCC tissues from the ICGC cohort was collected. Using the R 'GSVA' package, we performed the ssGSEA analysis to measure the expression levels of the gene set of Cu-binding proteins. The results of ssGSEA showed that the scores of the Cu-binding proteins gene set in liver cancer tissues were significantly different from that in normal liver tissues (Fig. 2A). Among the fifty-four Cu-binding proteins, fifty proteins' mRNA expression information was obtained in both ICGC and TGCA database (Fig. 2B). We analyzed the expression of all these Cu-binding proteins in HCC, and found that thirty Cu-binding proteins, such as dopamine beta-hydroxylase (DBH), S100

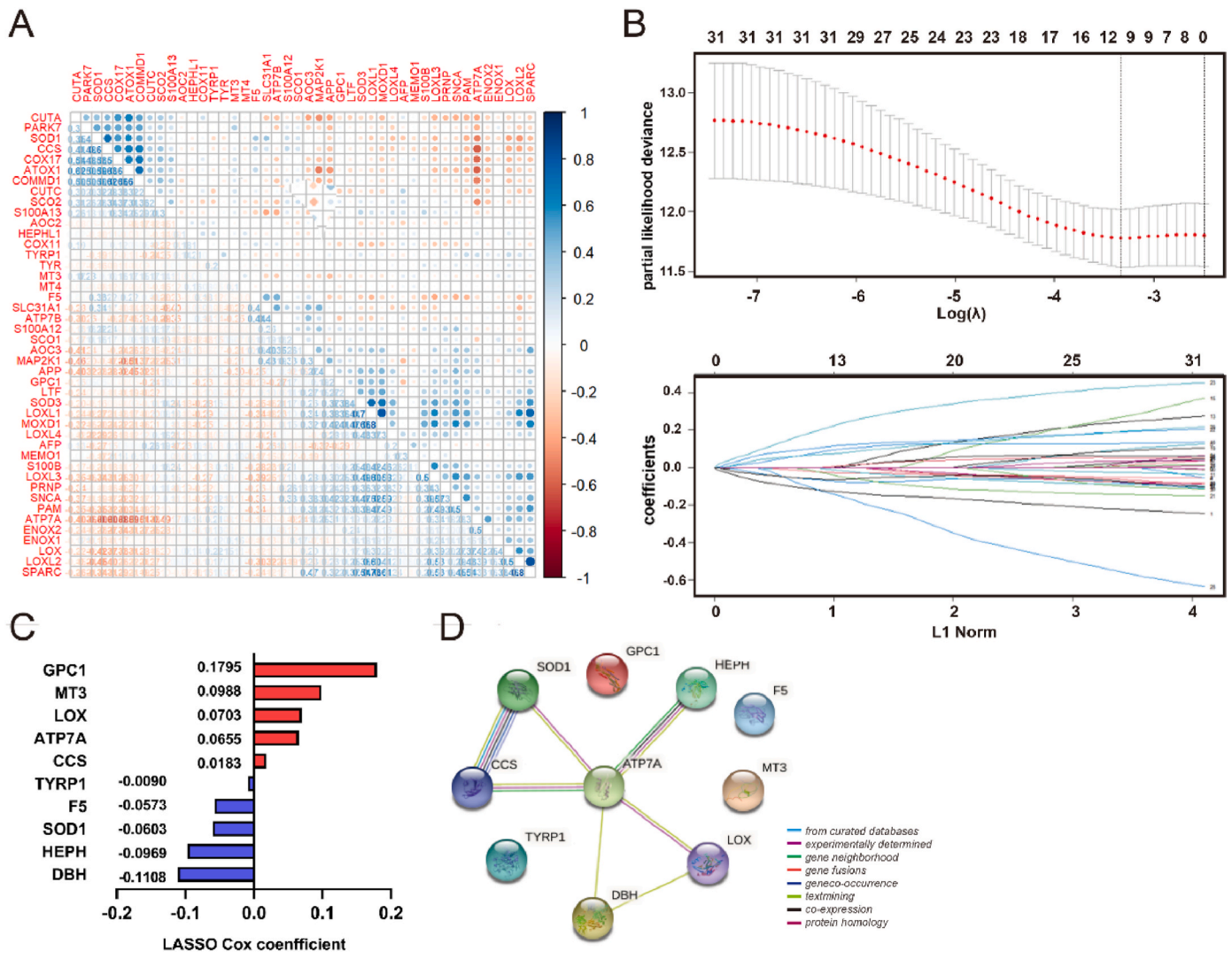

Fig. 3. Construction of the Cu-binding proteins (CuP) signature. (A) The linear correlation analysis among 31 Cu-binding proteins in hepatocellular carcinoma (HCC). (B) The most robust predictive genes were identified using the least absolute shrinkage and selection operator Cox regression algorithm. (C) An ensemble of 10 genes remained with nonzero coefficients. (D) The protein-protein interactions among the 10 genes.

calcium binding protein A12 (S100A12), Mitogen-Activated MAP2K1, LOXL2, ATPase Copper Transporting Beta (ATP7B), were differentially expressed between the liver cancer tissues and normal liver tissues (Fig. 2C). Through analyzing the prognostic value of these proteins, we found that four Cu-binding proteins coagulation factor V (F5), Recombinant Glypican 1 (GPC1), lysyl oxidase (LOX), S100A12, and shared a close association with the prognosis of patients with HCC ($p < 0.05$) (Fig. 2D).

3.2. Construction of Cu-binding proteins-Related prognostic signature

DEGs was analyzed and the results showed that the expression level of these genes can be influenced (Fig. 3A). To further identify the most robust prognostic biomarkers for the survival of HCC patients, we conducted the LASSO cox regression model. Ten-fold cross-validation was conducted and the min-λ value was selected for overcoming the overfitting in the LASSO cox regression model (Fig. 3B). The results of regression model revealed that an ensemble of ten genes (GPC1, MT3, LOX, ATP7A, CCS, TYRP1, F5, SOD1, HEPH and DBH) had individual nonzero coefficients (Fig. 3C), and these ten proteins were further integrated into establishing the CuPscore. The correlation network among the 10 Cu-binding genes was analyzed and the results revealed a tight connection among these molecules (Fig. 3D).

To further clarify the prognostic value of the CuPscore, we analyzed the risk scores distribution and the survival status of each patient, and the results suggested that the high score of CuPscore model got associated with poor prognosis of patients with HCC (Fig. 4A and B). The Kaplan–Meier curve analysis revealed that patients with higher CuPscore had a shorter OS and PFS (Fig. 4C and D). To further clarify the prognostic effect of CuPscore model in HCC, we conducted the time-dependent ROC analysis to assess the value of the CuPscore model in predicting the survival time of patients with HCC. We performed ROC analyses and the results revealed that the area under the curve (AUC) at 1-, 3- and 5-year OS were 0.69, 0.70 and 0.73 respectively (Fig. 4E). Furthermore, PCA analysis was performed to descript the expression features of CuPscore model-included genes. The results showed that patients in CuPscore-high and CuPscore-low groups had different expression patterns of genes enrolled in CuPscore model (Fig. 4F).

3.3. Verification of the Cu-binding proteins-Related prognostic signature

To further evaluate the prognostic value of the CuPscore model, we assessed the efficiency of CuPscore in predicting the prognosis of HCC patients using data from a new independent cohort downloaded from

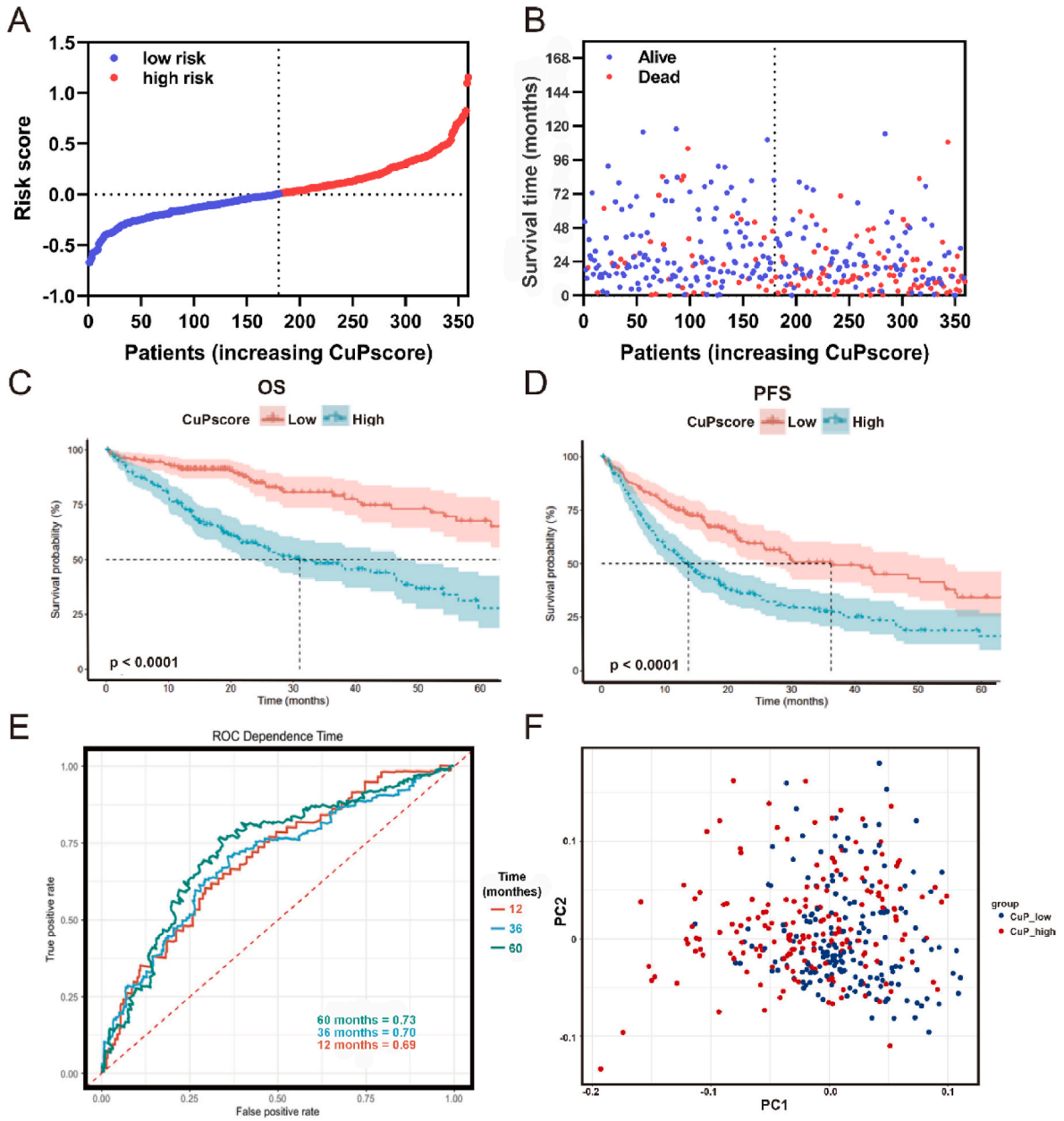

Fig. 4. Prognostic analysis of the CuP signature in the TCGA set. (A, B) The distribution and median value of the CuP signature. (C, D) Kaplan-Meier curves for the OS and PFS of hepatocellular carcinoma (HCC) patients in the CuP_low group and CuP_high group. (E) AUC of time-dependent ROC curves at 12-, 36- and 60-months. (F) PCA analysis showed different distribution patterns.

ICGC database (https://dcc.icgc.org/). X-tile software was previously reported to be a bio-informatic tool for the optimization of outcome-based cut-point [32]. Here, we divided all the HCC patients into CuPscore-low group and CuPscore-high group according to the cut-off value, which was determined by the X-tile software. Consistent with the previous results observed in the TCGA cohort, data from the ICGA cohort also exhibited that HCC patient with high CuPscores had a poor prognosis (Fig. 5A and B). The Kaplan–Meier analysis also exhibited that the HCC patients with higher CuPscore had a shorter OS compared with patients with lower CuPscore (Fig. 5C). The correlation between CuP-score and PFS of HCC patients from ICGC cohort was not analyzed as a result of the information of PFS in the ICGC cohort was not available. In addition, we use CuPscore to conduct a predictive model for the OS of HCC patients from ICGA cohort, and the AUC of predicting 1- and 3-year OS were 0.63 and 0.55 respectively (Fig. 5D). PCA analysis was performed to descript the different distributions of Cu-binding proteins between CuP_low and CuP_high groups. Consistently, the results of PCA showed the different distribution pattern between HCC patients with high CuPscore or low CuPscore (Fig. 5E).

3.4. Correlation analysis between clinical information and CuPscore

Correlation analyses were performed to investigate the relationship between the Cu-binding proteins signature and the clinicopathological characteristics of HCC patients. As shown in the heatmap and scatter diagrams, CuPscore were closely associated with the tumor grade ($p < 0.001$), T stage ($p < 0.0001$), pathological stage ($p < 0.0001$) and survival status ($p < 0.0001$) (Fig. 6A–E). To further clarify the independent risk factor for the OS of HCC patients, we conducted the univariate and multivariate Cox regression analyses. The results showed that the CuP-score represents an independent risk factor ($p < 0.001$, HR: 4.894, 95% CI: 2.584–9.269) for the OS of HCC patients (Fig. 6F). These results

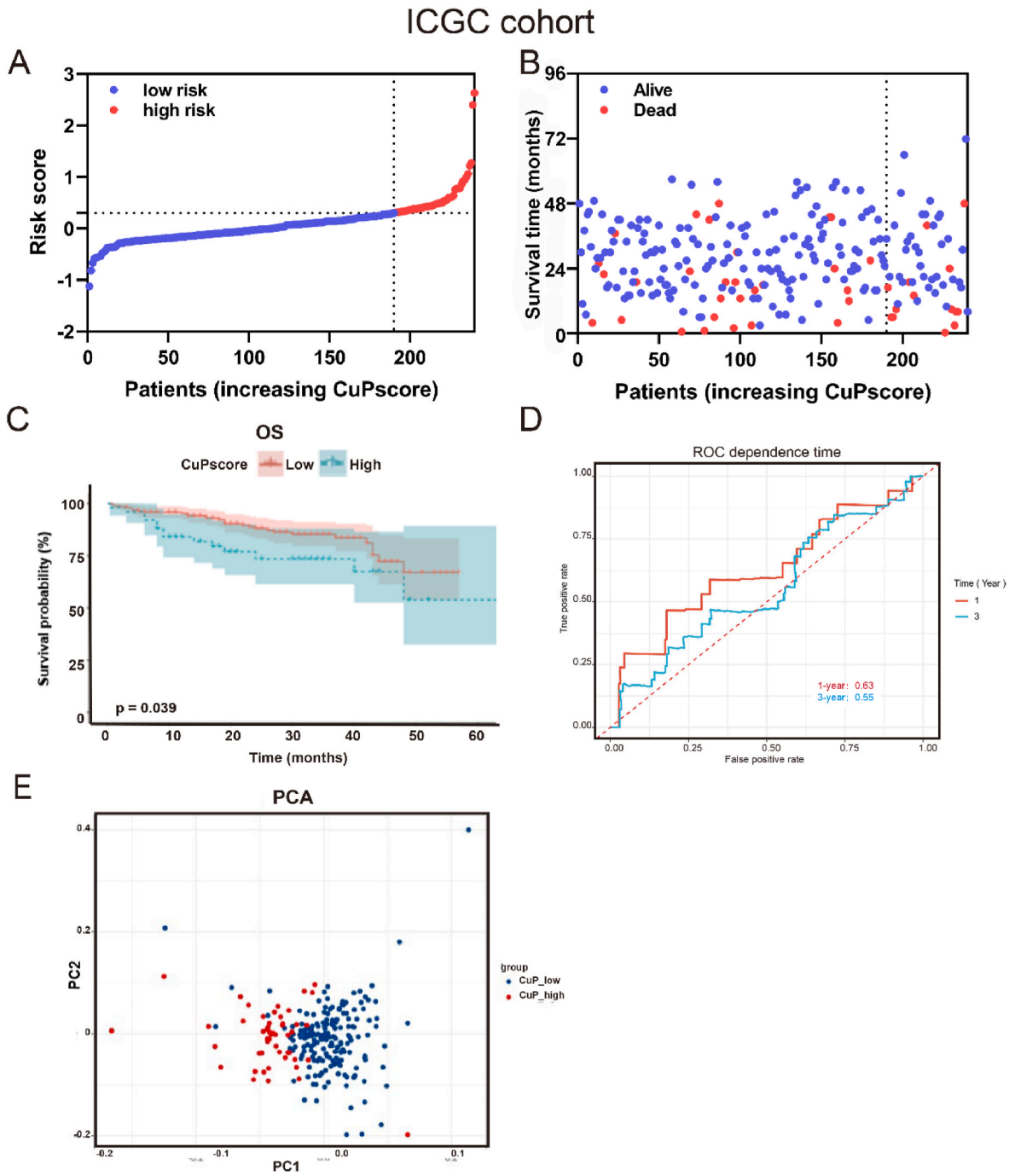

Fig. 5. Validation of the CuP signature in the ICGC cohort. (A, B) The distribution and median value of the CuP signature in the ICGC cohort. (C) Kaplan–Meier curves for the OS of hepatocellular carcinoma (HCC) patients in the CBP_low group and CBP_high group from the ICGC cohort. (D) AUC of time-dependent ROC curves at 1-, and 3-year. (E) PCA analysis showed different distribution patterns.

indicated the significant prognostic value of Cu-binding protein signature in HCC.

3.5. Establishment and validation of a nomogram model

Considering that the pathological stage and CuPscore were independent prognostic indicators for HCC patients, we established a nomogram using pathological stage and CuPscore to predict the 1-, 3- and 5-year OS rate of patients with HCC (Fig. 7A). The result of calibration chart revealed that pathological stage and CuPscore-based nomogram had a well predictive efficiency for the OS of HCC patients, especially for the prediction of 3-year OS rate (Fig. 7B). Furthermore, the AUC of nomogram-based predication efficiency of the 1-, 3- and 5-year OS were 0.70, 0.76 and 0.80, respectively, which is better in predicting the OS of HCC patients than only using pathological stage or CuPscore (Fig. 7C). The Kaplan–Meier curve analysis also revealed that the OS of HCC patients is significantly shorter in patients with higher nomogram scores (Fig. 7D).

3.6. Correlation between CuPscores and the expression of immune checkpoints

Recently, immune checkpoints-based immunotherapy has emerged as a significant anticancer strategy and markedly improved the

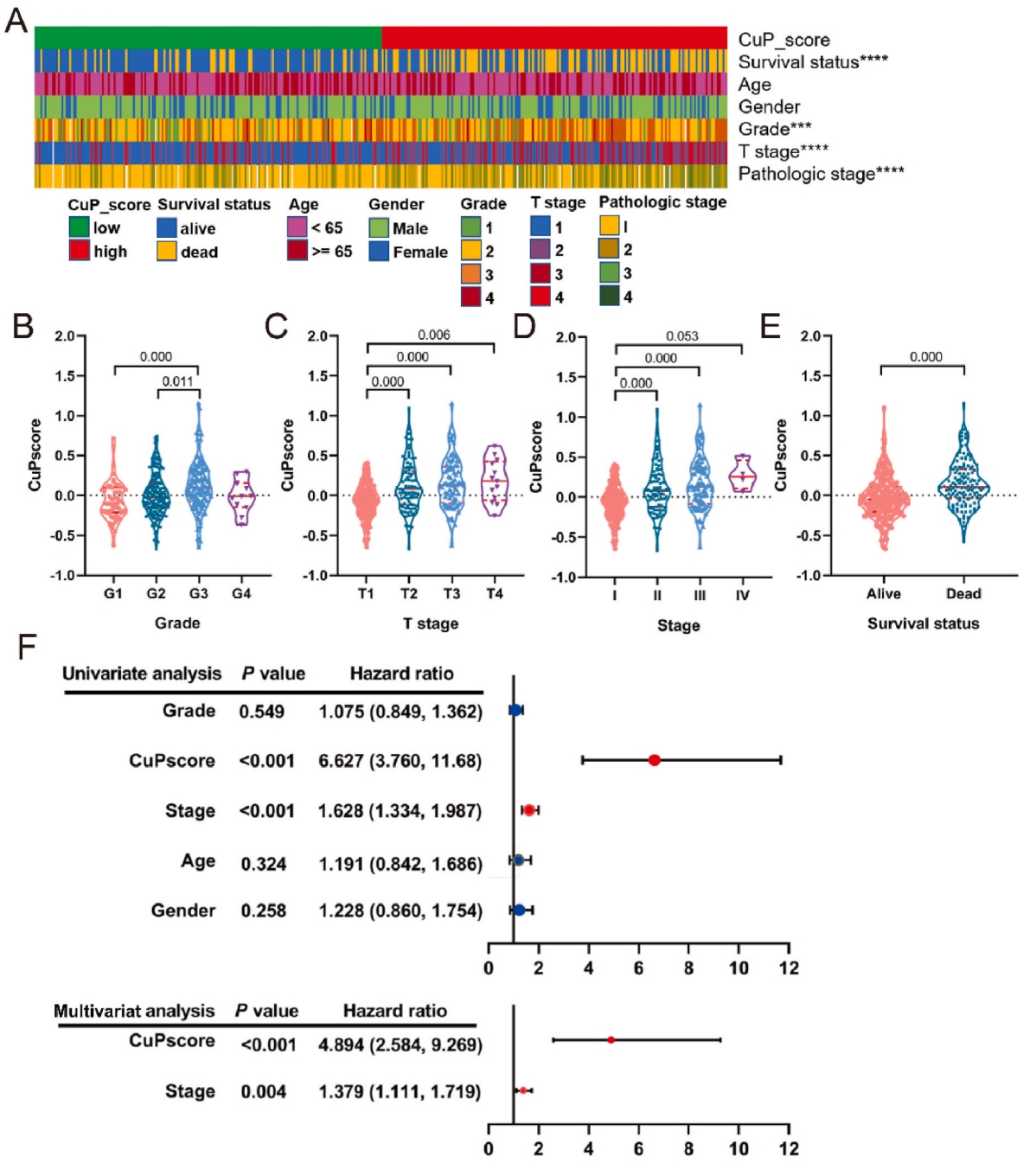

Fig. 6. Relationships between the CuP signature and clinicopathological characteristics. (A) Heatmap of the clinicopathological characteristics and the scores of CuP signature. (B–E) The scores of CuP signature in different groups classified by clinical characteristics. (F) Univariate and multivariate Cox regression analyses regarding OS in the TCGA set. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

prognosis of HCC patients. Several immunomodulatory molecules, such as PD-1, CTLA4, CD276, and TIM-3, was reported to be associated with the response to immune checkpoints blockade (ICB) therapy [34]. In order to analyze the potential association between CuPscore and the response to ICB therapy, we investigated the relationship between CuPscore and the expression level of immunomodulatory molecules. The results showed that HCC patients with a higher CuPscore had a significantly higher expression level of numerous immunomodulatory molecules, such as PD-1, CTLA4, CD276, TIM-3, VEGFA, VEGFB, IL-10, TGF-β1, VTCN1, MICA and MICB (Fig. 8A and B). These results indicated that the high CuPscore might got closely associated with an immunosuppressive microenvironment, as well as a poor response to ICB therapy.

3.7. Correlation between CuPscores and the infiltration of immune cell in HCC

Our previous analysis indicated a potential role of CuPscore in predicting the response to ICB therapy in HCC for the observation that CuPscore shared a close association with the expression of numerous immunomodulatory molecules. To further clarify the relation between CuPscore and the response to ICB therapy, we analyzed the correlation between CuPscore and the tumor-infiltrating immune cells, which was regarded as an important regulator of the immunotherapy and tumor progression [35]. Cell-Type Identification Using Expression-Based Regression and Regularization (CIBERSORT) algorithm was previously reported to be used for analyzing the proportions of immune infiltrated

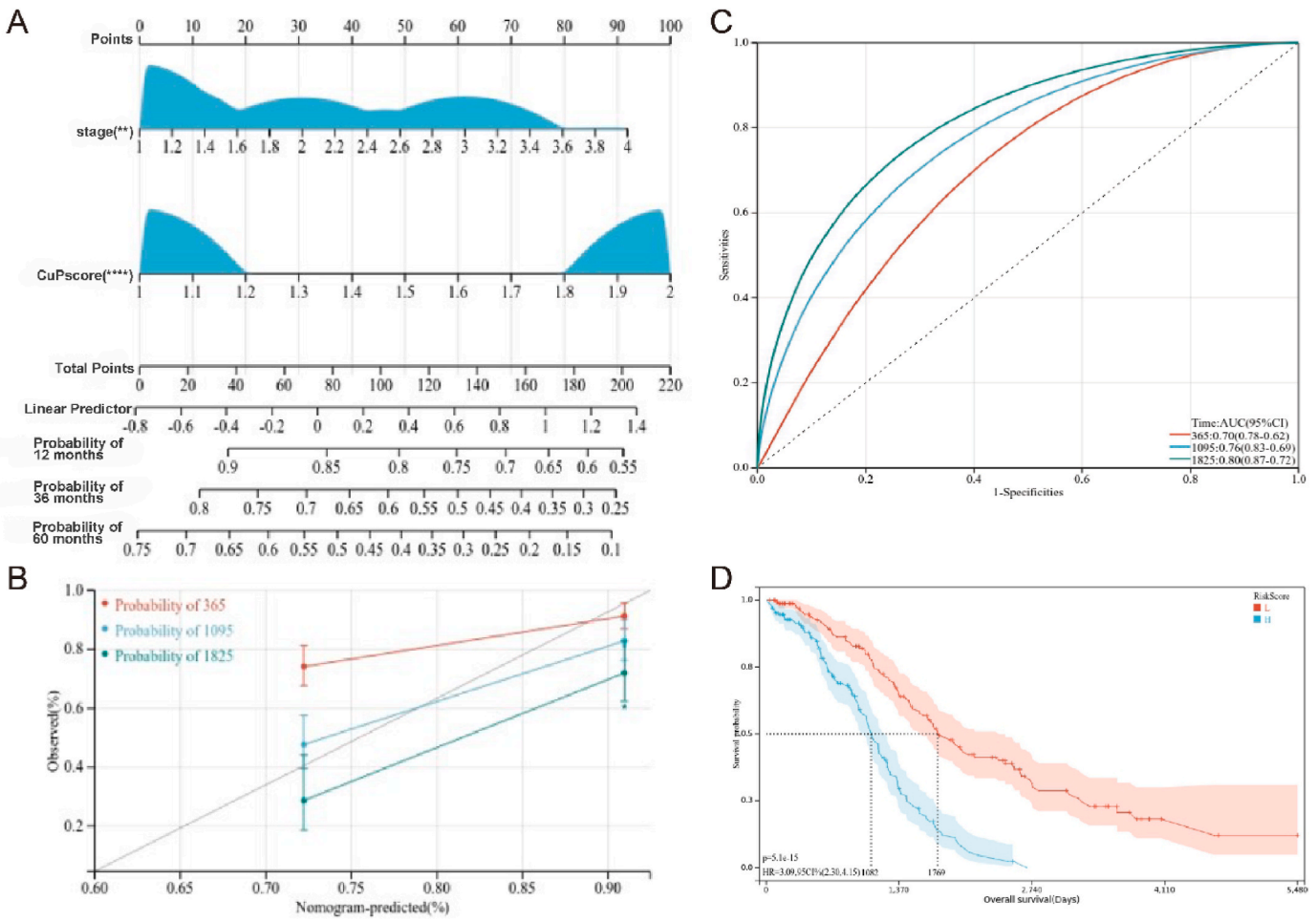

Fig. 7. Nomogram to predict OS in hepatocellular carcinoma (HCC) patients. (A) Nomograms using prognostic factors identified by multivariate Cox analysis in the TCGA set. (B) The calibration curve for determining the reliability of the nomogram to predict the 1- (365 day), 3- (1095 day), and 5- (1825 day) OS. (C) AUC of time-dependent ROC curves at 1- (365 day), 3- (1095 day), and 5- (1825 day) in the TCGA cohort. (D) Kaplan–Meier curves for the OS of HCC patients in the low nomogram score group and high nomogram score group in the TCGA cohort.

cells [36]. Here, we conducted the CIBERSORT algorithm to analyze the relationship between CuPscore and the proportion of tumor-infiltrating immune cells in HCC. The results revealed that patients with high CuPscore had a markedly decreased proportion of monocyte, $CD8^+$ T cells, γ^+T cells and mast cells; whereas patients with low CuPscore had a decreased proportion of M0 macrophages (Fig. 9A and B). These results indicated that the high CuPscore predicted a potential poor response to ICB therapy.

4. Discussion

Although great efforts have been made in developing the comprehensive therapeutic strategies, the prognosis of HCC patients still remains to be poor, with the 5-year survival rate being 18%. Exploration of the underlying mechanism and prognostic biomarkers may facilitate the precision medicine of cancer patients. Further discovery of the underlying mechanism of tumor progression can develop the novel therapeutic strategies for HCC.

As a trace element, Cu got involved in various biological activities and played an essential role in the living matter. The reversible process of oxidation between cuprous (Cu^{1+}) and cupric (Cu^{2+}) conferred Cu the ability to act as a cofactor for various kinds of Cu-dependent enzymes, facilitating the biological cellular process [37]. Previous studies have already reported that Cu homeostasis played a critical role in the physiological activities including mitochondrial respiration, elastin cross-linking, free radical scavenging, redox chemistry, and iron absorption [38]. However, the disruption of Cu homeostasis could also lead to the accumulation of reactive oxygen species and proteasome inhibition, causing cellular toxicity [39]. Abnormal accumulation of Cu was previously reported to be associated with the development of several disease, such as wilson's disease and neuroinflammation [40,41]. Recently, increasing number of studies have revealed that Cu metabolic reprogramming and Cu-dependent cellular process are crucial for cancer development, and it is important to uncover the underlying regulatory mechanisms of Cu in cancer development. The close association between Cu accumulation and HCC was proposed by the observation of increased incidence of HCC in patients with Wilson disease [16], as well as the increased serum level of copper in HCC patients [17,18]. Takashi Himoto et al. reported that Cu accumulation could activate HIF1-α to promote hepatocarcinogenesis [20]. It was reported that COMMD10 downregulation-mediated Cu accumulation lead to the radio-resistance of HCC through inhibiting the ubiquitin degradation of HIFα, which can initiate the transcription of ceruloplasmin and SLC7A11 to inhibit the ferroptosis [42]. Recent studies identified cuproptosis as an important regulator in cancer progression [8], and the signature of cuproptosis shared a close association with the prognosis in several kinds of cancer patients [43,44]. As the important transporters and the downstream effectors of Cu, Cu-binding proteins were also reported to be critical regulators of several kinds of tumors and got closely associated with the prognosis of cancer patients [45]. However, little is known about the role of Cu-binding proteins in HCC.

Here, we conducted a comprehensive bioinformatic analysis of Cu-

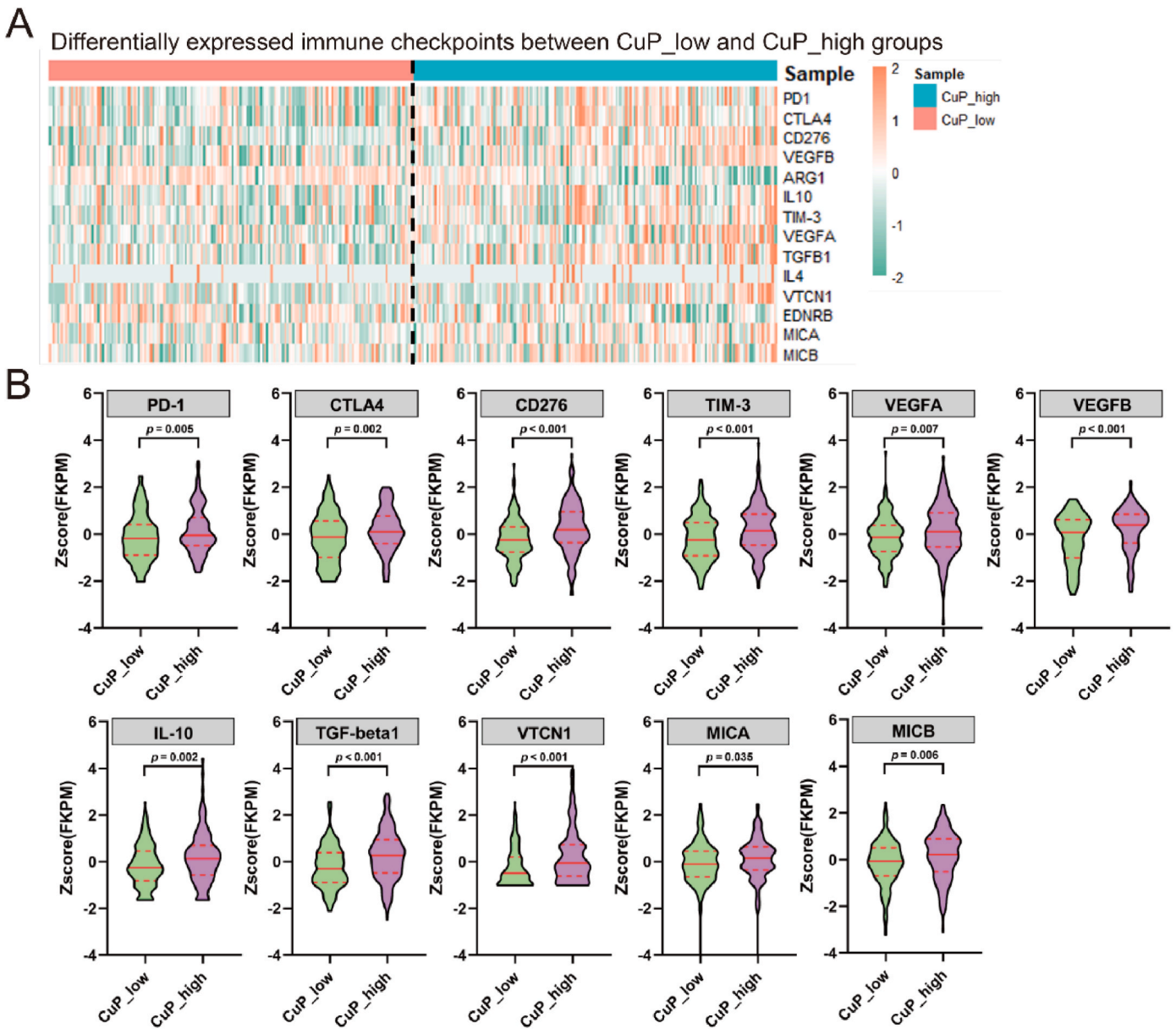

Fig. 8. Relationships between the CuP signature and immune suppressive checkpoints. (A) Heatmap of immune suppressive checkpoints between the CuP_low and CuP_high group. (B) Differentially expressed immune checkpoints between the CuP_low and CuP_high group.

binding proteins in HCC using data from TCGA and ICGC cohort. We observed the different expression pattern of fifty-four Cu-binding proteins in HCC and normal liver tissues, and found that the expression of four Cu-binding proteins got closely associated with the prognosis of HCC patients. Further prognosis analysis revealed that four Cu-binding proteins F5, GPC1, LOX and S100A12, shared a close association with the prognosis of patients with HCC ($p < 0.05$). GPC1 is a member of the phosphatidylinositol glycan family of acetyl heparan sulfate glycoproteins, and the serum level of GPC1 expression level was reported to be a prognostic marker for HCC [46]. LOX is an extracellular enzyme that plays a critical role in covalent cross-link formation in collagen fibrils [47]. Sun et al. reported the positive correlation between LOX family and the infiltration of various immune cells, and LOX family may be a potential diagnostic and prognostic biomarker for HCC [48]. S100A12 is a member of the calgranulin S100 protein subfamily, a group of inflammatory molecules primarily composed of calcium-binding proteins [49]. Cai et al. reported that high expression of S100A12 correlated with poor prognosis of HCC patients receiving surgical resection [50]. These findings further supported the results of our study.

To identify the most robust prognostic biomarkers from the Cu-binding proteins, we conducted the LASSO cox regression model and generated a ten genes-composed CuPscore, which shared a close association with the prognosis of HCC patients. Time-dependent ROC analysis revealed the excellent efficiency of the CuPscore model in predicting the survival time of HCC patients. The role of CuPscore in predicting the prognosis of HCC patients was also confirmed from another HCC cohort form ICGC database. Analyzing the correlation between CuPscore and the clinical information, we observed that CuPscore were significantly associated with the tumor grade ($p < 0.001$), T stage ($p < 0.0001$), pathological stage ($p < 0.0001$) and survival status ($p < 0.0001$). Further univariate and multivariate Cox regression analyses indicated that CuPscore was an independent risk factor ($p < 0.001$, HR: 4.894, 95%CI: 2.584–9.269) for the OS of HCC patients. Nomogram model was commonly conducted to predict the prognosis of cancer patients. Here, we found that CuPscore and HCC pathological stage-based nomogram model had a great performance in predicting the prognosis of HCC

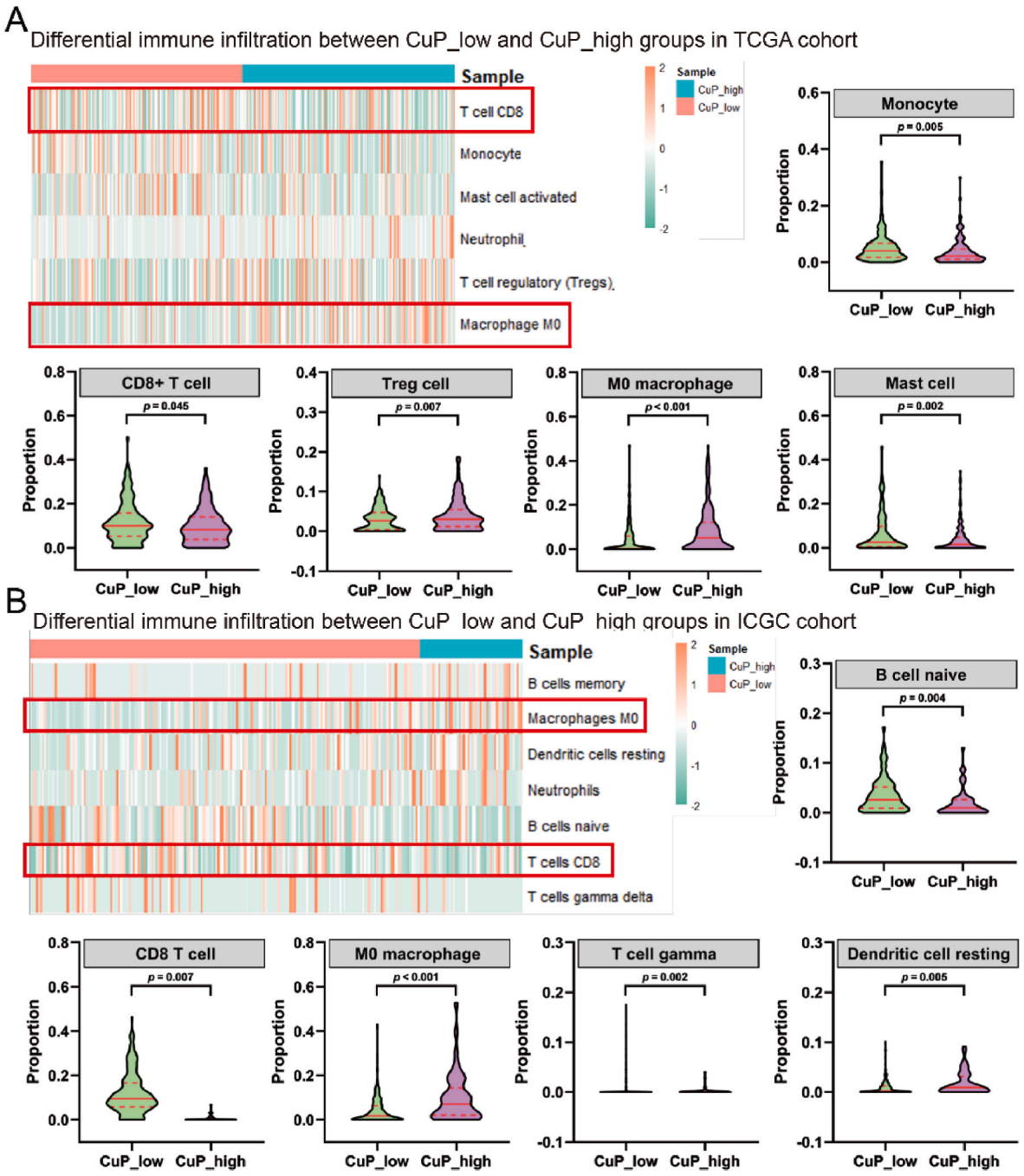

Fig. 9. Immune infiltration analyses. (A) Differential immune infiltrations, including $CD8^+$ T cells and M0-type macrophage, between the CuP_low and CuP_high group in the TCGA cohort. (B) Differential immune infiltrations, including $CD8^+$ T cells and M0-type macrophage, between the CuP_low and CuP_high group in the ICGC cohort.

patients. These results revealed that CuPscore exhibited great potential in acting as a prognostic biomarker in HCC.

Immunotherapy, including immune checkpoint inhibitor or CAR-based adoptive cell immunotherapy, has evolved into a significant treatment strategy for anti-cancer therapy and markedly improved the prognosis of cancer patients. Identifying patients resistant or sensitive to immunotherapy, which will facilitate the precise treatment, still remains to be a challenge. Several immunomodulatory molecules, such as Programmed Death 1 (PD-1), Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), CD276, and T-Cell Immunoglobulin and Mucin Domain-Containing Protein 3 (TIM-3), was reported to be associated with the response to immunotherapy [34]. To explore the potential association between CuPscore and immunotherapy in HCC, we analyzed the relationship between CuPscore and the expression level of immunomodulatory molecules, and the result indicated that CuPscore closely associated with the expression level of several immunomodulatory molecules, such as PD-1, CTLA4, CD276, TIM-3, vascular endothelial growth factor A (VEGF-A), VEGFB, IL-10, Transforming Growth Factor Beta 1 (TGF-β1), V-Set and Transmembrane Domain Containing 1 (VTCN1), MHC Class I Polypeptide-Related Sequence A (MICA) and MHC Class I Polypeptide-Related Sequence B (MICB), hinting a negative regulatory role of Cu-binding proteins in HCC immune microenvironment. Tumor-infiltrating immune cells also got closely associated with the response to immunotherapy. Our analysis of the correlation between

CuPscore and immune cell infiltration revealed that HCC patients with high CuPscore had a markedly decreased proportion of monocyte, $CD8^+$ T cells, γ^+T cells and mast cells; whereas patients with low CuPscore had a decreased proportion of M0 macrophages, indicating that the high CuPscore predicted a potential poor response to ICB therapy. These results suggested a potential regulatory role of Cu-binding protein in immune microenvironment and the response to immunotherapy in HCC. Considering that the expression level of Cu-binding proteins in our study was determined by the RNA-seq data and Cu-binding proteins could be regulated both post-transcriptionally and post-translationally [51–53], whether the protein expression level of Cu-binding proteins shared a close association with the immune microenvironment and the prognosis of HCC remains to be explored, and further *in vitro* and *in vivo* experiment is needed to further clarify these results.

Of course, there were several limitations in our study. First, we only analyzed the mRNA expression level of Cu-binding proteins using data from TCAG and ICGC database, and the prognostic value of the protein expression level of Cu-binding proteins in HCC remains to be further explored considering that Cu-binding proteins could be regulated both post-transcriptionally and post-translationally [54–56]. Second, although we identified the prognostic value of Cupscore composed of ten Cu-binding proteins, it is difficult to explain the regulatory role of these Cu-binding proteins in HCC, further *in vitro* assay is needed to analyze the functional role of these proteins in HCC. Finally, the results derived from bioinformatic analysis needs to be further confirmed in real-word application.

5. Conclusion

Overall, our study conducted a comprehensive bioinformatic analysis of Cu-binding protein, exploring its expression pattern and clinical value in HCC. The results indicated the abnormal expression of thirty Cu-binding proteins in HCC and the prognostic value of CuPscore and Cu-binding proteins-based nomogram model in predicting the prognosis of HCC patients. Furthermore, we also observed a close association between CuPscore and the expression level of immunomodulatory molecules, as well as the percentage of tumor-infiltrating immune cells, and CuPscore might serve as a predictor for response to immunotherapy in HCC.

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Author contributions

LM and LC contributed to conception and design of the study. DJ performed the statistical analysis. LM wrote the first draft of the manuscript; HC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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