

# CENPO is Associated with Immune Cell Infiltration and is a Potential Diagnostic and Prognostic Marker for Hepatocellular Carcinoma

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**Purpose:** To examine the expression, clinical significance, and potential regulatory mechanism of centromere protein O (CENPO) in hepatocellular carcinoma (HCC).

**Methods:** CENPO expression in pan-cancer was studied using the TCGA-GTEX database, in HCC and normal liver tissues using the GEO and TCGA databases, and in clinical HCC samples by RT-qPCR. The diagnostic value of CENPO was assessed using receiver operating characteristic curves. Univariate and multivariate regression analyses of factors associated with HCC prognosis were performed. CENPO function and its mechanism in HCC were explored using GO, KEGG, and GSEA analyses of differentially expressed genes (DEGs). Association of CENPO expression with immune cell infiltration and immune checkpoint-associated molecules was conducted using TCGA data and the TIMER2.0 database. Relationships between CENPO expression and DNA methylation were analyzed using the UALCAN and cBioPortal databases. CENPO expression in HCC cell lines was detected using RT-qPCR.

**Results:** CENPO is upregulated in most cancers, including HCC and cell lines, and is a potential biomarker for HCC diagnosis (AUC = 0.936, 95% CI: 0.911–0.960). Higher CENPO expression was associated with poorer outcomes in patients with HCC (OS,  $p = 0.004$ ; DSS,  $p = 0.002$ ; PFI,  $p < 0.001$ ), and CENPO was an independent predictor of factors influencing overall survival in HCC. DEGs between samples with high and low CENPO levels were enriched in various biological processes, including activation of the G2M checkpoint and other signaling pathways, while CENPO expression correlated with HCC immune cell infiltration and immune checkpoint-associated molecules, as well as CENPO promoter methylation ( $p < 0.001$ ).

**Conclusion:** In HCC and cell lines, CENPO is overexpressed, a potential diagnostic marker and an indicator of poor prognosis. CENPO may regulate HCC development by influencing nuclear division and tumor immune infiltration and is regulated by methylation, making it a potential target for HCC immunotherapy.

**Keywords:** HCC, diagnosis, prognosis, biomarker, immune checkpoints, methylation

## Introduction

Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer, accounting for approximately 75–85% of all liver carcinomas, and is associated with poor patient prognosis. In 2020, there were approximately 900,000 new cases of HCC and 830,000 deaths from the disease.<sup>1</sup> HCC is treated using various methods, including surgery, radiotherapy, interventional therapy, targeted therapy, and traditional Chinese medicine, with surgical resection being the most effective therapy option for patients with HCC.<sup>2</sup> Patients are frequently diagnosed when they have advanced illness, because clinical symptoms of HCC are non-specific and screening procedures are largely ineffective, and patients often experience disease recurrence or subsequent metastases following surgery.<sup>3</sup> Consequently, the short- and long-term outlook for patients diagnosed with liver cancer is dismal. Identification of biomarkers that can be used for early diagnosis of liver cancer and to predict its prognosis is therefore of considerable importance.

Centromere protein O (CENPO) belongs to the centromere protein (CENP) family. The *CENPO* gene is located on human chromosome 2p23.3, contains eight exons, and encodes a 34-kDa CENPO protein, also known as interphase centromere complex protein 36, which consists of 300 amino acids. *CENPO* is essential for spindle formation, chromosome segregation, and cell cycle checkpoint signaling during mitosis, while its increased expression is associated with poor prognosis in rectal cancer,<sup>4</sup> gastric cancer,<sup>5</sup> bladder cancer,<sup>6</sup> and chronic granulocytic leukemia.<sup>7</sup> Recent studies have also shown an association between *CENPO* and breast cancer chemoresistance.<sup>8</sup> Nevertheless, the activity and mechanism underlying the role of CENPO in HCC remain unknown. In this study, we investigated the role of *CENPO* in HCC using bioinformatics and molecular biology approaches.

Transcriptome information was retrieved from The Cancer Genome Atlas (TCGA), the Gene Expression Omnibus (GEO), and the Genotype-Tissue Expression (GTEx) databases, with the aim of investigating the differential expression of *CENPO* in liver cancer and normal liver tissues. Next, we analyzed associations between *CENPO* expression and the clinicopathological characteristics of HCC. Further, we assessed the value of *CENPO* as a prognostic factor in HCC and we used gene enrichment analysis to assess the potential functions of *CENPO* in HCC. *CENPO* expression and immune cell infiltration were also examined to determine their potential contribution to promotion of HCC. Finally, we investigated the relationship between *CENPO* expression and methylation, with the aim of determining the mechanisms underlying *CENPO* upregulation in HCC.

## Materials and Methods

### Collection and Analysis of RNA-Seq Data

*CENPO* mRNA expression data in pan-cancer was downloaded using the UCSC XENA browser.<sup>9</sup> Using data from TCGA database, the expression of CENPO in 374 HCC tissues and 50 normal liver tissues was investigated.<sup>10</sup> RNA-seq data in FPKM format were translated to TPM format, and then log<sub>2</sub> transformed for analysis. The GSE4184 dataset (platform: GPL570), including 20 cases of HCC and 20 para-cancer tissue expression profiles,<sup>11</sup> was downloaded from the GEO database and used to analyze *CENPO* expression levels.

### Tissue Specimens

The study was conducted in accordance with the principles of the Declaration of Helsinki, and the protocol was approved by the North Sichuan Medical College Hospital Ethics Committee (IRB Review Approval Notice, File Number: 2022ER243-1). Fifteen pairs of primary HCC and normal liver specimens were acquired from the Department of Hepatobiliary Surgery, Affiliated Hospital of North Sichuan Medical College (Nanchong, China). All patients provided informed consent. At least two pathologists each made a diagnosis for each case. No patients underwent radiotherapy, chemotherapy, or immunotherapy prior to surgery. After surgical resection, liver cancer and liver tissue samples were collected and immediately frozen in liquid nitrogen, before transfer to -80°C freezers for storage.

### Cell Culture

The human Huh7, MHCC97L, Hep3B, HepG2 HCC cell lines, and normal liver THLE-2 cells were purchased from the American Type Culture Repository and stored in liquid nitrogen at the Institute of Hepatobiliary and Pancreatic Diseases, North Sichuan Medical College. All cells were cultured in DMEM containing 10% fetal bovine serum (VivaCell; Shanghai, China), 100 units/mL penicillin (VivaCell; Shanghai, China), and 100 mg/mL streptomycin (VivaCell; Shanghai, China). Cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator. The culture medium was changed every 48 hours and cells in log phase were used for RNA extraction.

### RNA Extraction and qRT-PCR Assays

TRIzol reagent (Invitrogen) was used to extract RNA from tissues or cells, and the concentration and purity of the extracted RNA was determined using a NanoDrop 2000 (Thermo Scientific). Reverse transcription of extracted RNA into cDNA was conducted using a Superscript II Reverse Transcription Kit (Vazyme Biotech, Nanjing, China). Real-time PCR assays were conducted on a LightCycler® 96 RT-qPCR system (Roche, Switzerland) using SYBR Green PCR Kits (Vazyme Biotech, Nanjing, China).

*GAPDH* served as the endogenous control. The primers used in this investigation were synthesized by Sangon Biotechnology (Shanghai, China), as follows: *CENPO*, Forward, 5'-TTACAGACCAACATCCAGCACTTCC-3', Reverse, 5'-GCAAGG GCTTCGTACAGAACAGAG-3'; and *GAPDH*, Forward, 5'-AAGGTCATCCCTGAGCTGAA-3', Reverse, 5'-TGACAAA GTGGTCGTTGAGG-3'. PCR amplification conditions: 95°C, 15 sec; 95°C, 10 sec; 60°C, 30 sec, 40 cycles. Results were analyzed by calculating the relative expression of *CENPO* mRNA using the  $2^{-\Delta\Delta C_t}$  method.

## Survival and Prognosis Association Analysis

After exclusion of patients with missing information, clinical features and RNA-seq data of patients with HCC were retrieved from TCGA; a total of 374 patient profiles were acquired for analysis in this study. To investigate the relationships between *CENPO* expression and patient clinicopathological characteristics, patients were divided into a high expression group (n = 187) and a low expression group (n = 187) according to median expression of *CENPO*. Associations of *CENPO* expression with overall survival (OS), disease-specific survival (DSS), and progression-free interval survival (PFI) of patients with HCC were analyzed using the R package “survival” (version 3.6.3). The R package “pROC” (version 1.17) was used to estimate the sensitivity of *CENPO* mRNA as a diagnostic indicator for HCC and the results were visualized with the R package “ggplot2” (version 3.3.3).

## Analysis of Differentially Expressed Genes and Biological Function Analysis

Patients were separated into two groups according to median expression of *CENPO*, and analysis of differential gene expression between the two groups conducted using the R program “DESeq2” (version 3.6.3). Genes with adjusted p values < 0.05 and |fold change| ≥ 1 were screened and defined as Differentially Expressed Genes (DEGs). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs between the high and low *CENPO* expression groups were conducted using the R package “clusterProfiler” (version 3.14.3).<sup>12</sup>

## Gene Set Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis (GSEA) was used to determine whether an a priori defined gene set was statistically significant and to determine the consistency of differences between two biological states.<sup>13</sup> Hence, GSEA was conducted using the R package “clusterProfiler” (version 3.14.3) using the cluster Profiler “h.all.v7.2.symbols.gmt [Hallmarks]” gene set available from the Molecular Signature Database (<http://software.broadinstitute.org/gsea/msigdb/>). The interchange of gene sets was 1000 per analysis. Biological pathways with a false discovery rate < 0.05 and p < 0.05 were considered to be significantly enriched. The R package “ggplot” (version 3.3.3) was used to visualize the top five signaling pathways.

## Analysis of Immune Cell Infiltration

To evaluate HCC tumor-immune infiltration, we applied single-sample GSEA using the R package “GSVA” (version 3.6)<sup>14</sup> to assess correlations between numbers of infiltrated HCC tumor-associated immune cells and *CENPO* mRNA expression. To calculate p values and assess correlations, the Wilcoxon rank sum and Spearman rank correlation tests were applied. Associations among patient prognosis, *CENPO* expression, and levels of immune cell infiltration were analyzed using the “Outcomes” module of the TIMER 2.0 database.<sup>15</sup> The “Corr” module was used to analyze correlations between *CENPO* expression and immune checkpoint molecules.

## Analysis of Correlations Between *CENPO* Expression and Promoter Methylation

Analysis of differences in *CENPO* promoter methylation levels between HCC and normal liver tissue was performed using the data from the UALCAN database.<sup>16</sup> The “Plot” module of cBioPortal for Cancer Genomics was used to evaluate correlations between *CENPO* expression and methylation using Spearman and Pearson tests.<sup>17</sup>

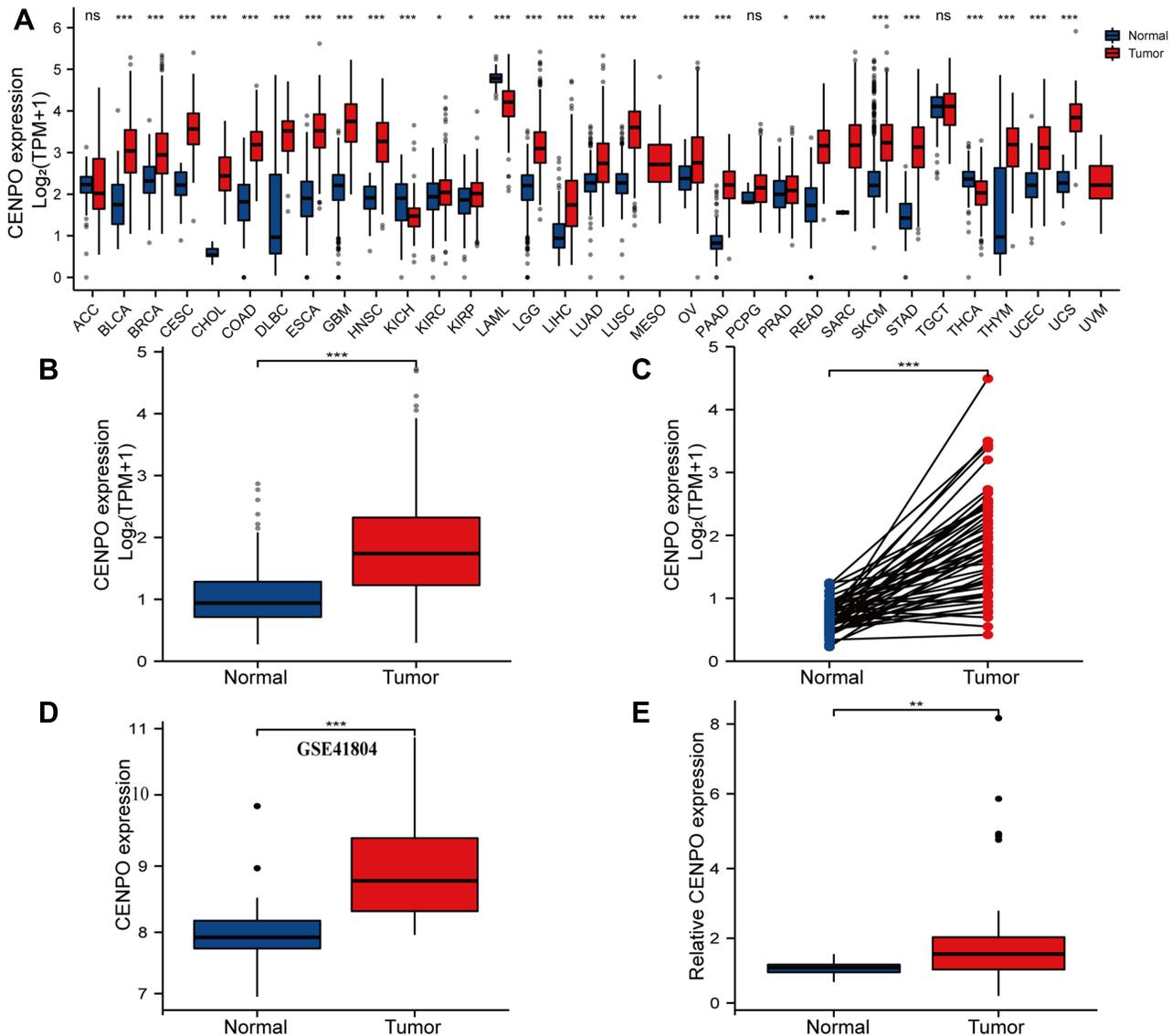
## Statistical Analysis

Statistical analyses and data visualization were conducted using R software (version 3.6.3) and GraphPad Prism software. P < 0.05 was set as the threshold for statistical significance.

## Results

### Pan-Cancer *CENPO* mRNA Expression Analysis

TCGA database contains RNA sequencing data from 33 cancers, including LIHC. Analysis of the RNA-seq profiles of all 33 cancers demonstrated that *CENPO* mRNA was highly expressed in 24 cancer types, including HCC, pancreatic adenocarcinoma, and cholangiocarcinoma, while its expression was down-regulated in kidney chromophobe, acute myeloid leukemia, and thyroid carcinoma (Figure 1A). *CENPO* mRNA expression levels were significantly higher in HCC than in normal liver tissues (Figure 1B), and in HCC tissues compared with adjacent non-cancerous tissues



**Figure 1** *CENPO* mRNA expression in pan cancer and LIHC (HCC). (A) Pan-cancer *CENPO* mRNA expression. (B) *CENPO* mRNA expression in 374 unpaired liver cancer tissues and 50 normal liver tissues from TCGA. (C) *CENPO* mRNA expression in 50 paired liver cancer and normal liver tissues from TCGA. (D) *CENPO* mRNA expression in 20 paired liver cancer and normal liver tissues in the GEO dataset (GSE 41804). (E) Analysis of *CENPO* mRNA expression in 15 paired clinical liver cancer and normal liver tissues by RT-qPCR. ns, not significant; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Abbreviations:** ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, lower grade glioma; LIHC, Liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma. PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

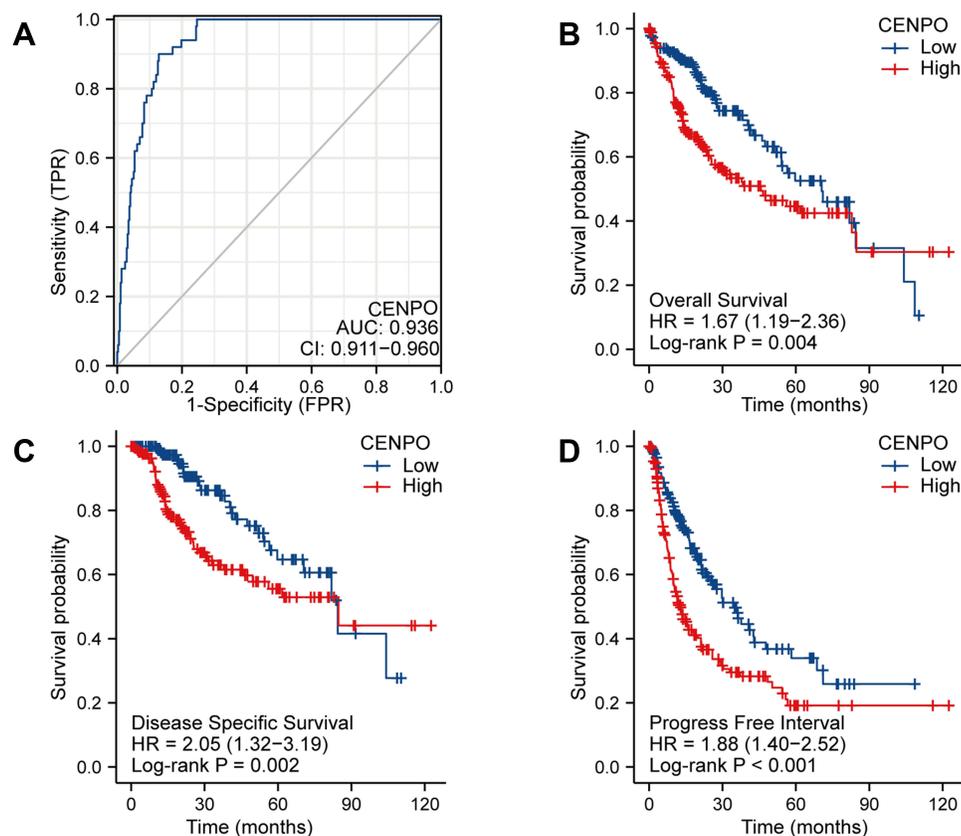
(Figure 1C) (paired samples *t*-test). Further, we validated the overexpression of *CENPO* in HCC using data from the GEO database (GSE41804 dataset; platform, GPL570) (Figure 1D). Next, *CENPO* expression was measured in paired HCC and corresponding normal liver tissues ( $n = 15$ ) by RT-qPCR and the results demonstrated that *CENPO* mRNA was over-expressed in HCC tissue (Figure 1E).

## Analysis of the Diagnostic Value of *CENPO* and Association of *CENPO* Expression with Clinical Characteristics of Patients with HCC

To explore the clinical diagnostic value of *CENPO* in HCC, we evaluated the feasibility of distinguishing HCC from normal tissue using receiver operating characteristics (ROC) curve analysis. The calculated area under the curve (AUC) value (AUC = 0.936, CI = 0.911–0.960) indicated that *CENPO* had high accuracy as a diagnostic indicator for HCC (Figure 2A). Patients were divided into high and low *CENPO* expression groups ( $n = 187$  each) according to median *CENPO* expression and correlations between *CENPO* expression levels and patient clinicopathological characteristics were investigated. High expression of *CENPO* in HCC was significantly and positively correlated with pathological stage ( $p = 0.002$ ), T stage ( $p < 0.001$ ), histological grade ( $p < 0.001$ ), and vascular invasion ( $p = 0.035$ ) (Chi-square or Fisher's exact test). *CENPO* expression was also positively correlated to alpha fetoprotein (AFP;  $p < 0.001$ ) levels and was negatively related to age ( $p = 0.012$ ) (Wilcoxon rank-sum test) (Table 1).

## High *CENPO* Expression is Associated with Worse Survival Prognosis in Patients with HCC

To explore the prognostic value of *CENPO* in HCC, we evaluated the relationships between *CENPO* mRNA levels and OS, DSS, and PFI in patients with HCC. Kaplan–Meier analysis showed that, compared with the low expression group, patients with high *CENPO* mRNA expression had significantly worse OS (hazard ratio (HR) = 1.67, 95% CI: 1.19–2.36,



**Figure 2** Relationship between *CENPO* mRNA expression and HCC diagnosis and prognosis. (A) Diagnostic value of *CENPO* expression in HCC. TPR, true positive rate; FPR, false positive rate. (B) Association between *CENPO* expression and overall survival. (C) Association between *CENPO* expression and disease-specific survival. (D) Association between *CENPO* expression and progression-free interval.

**Table 1** Analysis of the Association Between *CENPO* Expression and Clinicopathological Features of HCC in TCGA-LIHC Data

Characteristic	Low <i>CENPO</i> Expression (n = 187)	High <i>CENPO</i> Expression (n = 187)	P-value
Sex, n (%)			0.077
Female	52 (13.9%)	69 (18.4%)	
Male	135 (36.1%)	118 (31.6%)	
Ethnicity, n (%)			0.267
Asian	71 (19.6%)	89 (24.6%)	
Black or African American	10 (2.8%)	7 (1.9%)	
White	96 (26.5%)	89 (24.6%)	
Age (years), median (Q1, Q3)	64 (54.5, 69)	59.5 (50, 67.75)	<b>0.012</b>
BMI (kg/m <sup>2</sup> ), median (Q1, Q3)	24.93 (22.38, 28.77)	24.22 (21.11, 27.99)	0.180
AFP (ng/mL), median (Q1, Q3)	6 (3, 25)	113 (9, 2505)	<b>&lt; 0.001</b>
Albumin (g/dl), median (Q1, Q3)	4 (3.5, 4.3)	4 (3.5, 4.3)	0.923
Prothrombin time (s), median (Q1, Q3)	1.1 (1, 9)	1.1 (1, 9.3)	0.376
Child-Pugh grade, n (%)			1.000
A	123 (51%)	96 (39.8%)	
B	12 (5%)	9 (3.7%)	
C	1 (0.4%)	0 (0%)	
Fibrosis Ishak score, n (%)			0.677
0	42 (19.5%)	33 (15.3%)	
1–2	19 (8.8%)	12 (5.6%)	
3–4	13 (6%)	15 (7%)	
5–6	47 (21.9%)	34 (15.8%)	
T stage, n (%)			<b>&lt; 0.001</b>
T1	110 (29.6%)	73 (19.7%)	
T2	40 (10.8%)	55 (14.8%)	
T3	30 (8.1%)	50 (13.5%)	
T4	4 (1.1%)	9 (2.4%)	
N stage, n (%)			0.623
N0	124 (48.1%)	130 (50.4%)	
N1	1 (0.4%)	3 (1.2%)	
M stage, n (%)			1.000
M0	133 (48.9%)	135 (49.6%)	
M1	2 (0.7%)	2 (0.7%)	

(Continued)

**Table I** (Continued).

Characteristic	Low <i>CENPO</i> Expression (n = 187)	High <i>CENPO</i> Expression (n = 187)	P-value
Pathologic stage, n (%)			<b>0.002</b>
I	102 (29.1%)	71 (20.3%)	
II	39 (11.1%)	48 (13.7%)	
III	30 (8.6%)	55 (15.7%)	
IV	3 (0.9%)	2 (0.6%)	
Histologic grade, n (%)			<b>&lt; 0.001</b>
G1	38 (10.3%)	17 (4.6%)	
G2	104 (28.2%)	74 (20.1%)	
G3	40 (10.8%)	84 (22.8%)	
G4	3 (0.8%)	9 (2.4%)	
Vascular invasion, n (%)			<b>0.035</b>
No	118 (37.1%)	90 (28.3%)	
Yes	48 (15.1%)	62 (19.5%)	
Residual tumor, n (%)			<b>0.218</b>
R0	169 (49%)	158 (45.8%)	
R1	6 (1.7%)	11 (3.2%)	
R2	1 (0.3%)	0 (0%)	

**Notes:** Bold indicates a statistically significant difference with  $p < 0.05$ .

**Abbreviations:** *CENPO*, centromere protein O; T stage, Tumor stage; N stage, Lymph node stage; M stage, Metastasis stage; BMI, body mass index; AFP, alpha fetoprotein.

$p = 0.004$ ), DSS (HR = 2.05, 95% CI: 1.32–3.19,  $p = 0.002$ ), and PFI (HR = 1.88, 95% CI: 1.40–2.52,  $p < 0.001$ ) (Figure 2B–D). Next, univariate and multivariate Cox analyses were conducted to identify independent predictors of OS in patients with HCC. Univariate analysis showed that *CENPO* expression ( $p = 0.004$ ), T-stage ( $p < 0.001$ ), M-stage ( $p = 0.017$ ), and pathological stage ( $p < 0.001$ ) were associated with OS in patients with HCC (Table 2). Further, multivariate Cox analysis showed that *CENPO* expression ( $p = 0.014$ ) and T-stage ( $p = 0.013$ ) remained significant predictors of poorer OS (Table 2). These findings suggest that *CENPO* is an independent predictor of OS in patients with HCC and a promising potential prognostic marker for HCC.

## Functional Enrichment Analysis of DEGs Between HCC with High and Low *CENPO* Expression

Next, GO, KEGG, and GSEA were used to probe the functions and pathways involving DEGs between patients with high and low *CENPO* expression. The results suggest that the DEGs were involved in several GO-biological process terms, including protein activation cascade, nuclear division, complement activation, and humoral immune response (Figure 3A). Further, KEGG pathway analysis indicated that the DEGs were mainly enriched in signaling pathways, including bile secretion, retinol metabolism, metabolism of xenobiotics by cytochrome P450 and the drug metabolism cytochrome P450 signal pathway (Figure 3B). The top five GSEA results indicated that hallmark G2M\_checkpoint, E2F\_targets, spermatogenesis, mitotic spindle, and KRAS signaling were significantly enriched in the high *CENPO* expression group (Figure 3C).

**Table 2** Univariate and Multivariate Cox Analyses of Factors Influencing Overall Survival in HCC

Characteristic	Total (n)	Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P value	HR	95% CI	P value
CENPO	373						
Low	187	1					
High	186	1.678	1.181–2.384	<b>0.004</b>	1.742	1.117–2.716	<b>0.014</b>
Sex	373						
Female	121	1					
Male	252	0.793	0.557–1.130	0.200			
Ethnicity	361						
Asian	159	1					
Black or African American & White	202	1.341	0.926–1.942	0.121			
Age (years)	373						
≤ 60	177	1					
> 60	196	1.205	0.850–1.708	0.295			
BMI (kg/m <sup>2</sup> )	336						
≤ 25	177	1					
> 25	159	0.798	0.550–1.158	0.235			
AFP (ng/mL)	279						
≤ 400	215	1					
> 400	64	1.075	0.658–1.759	0.772			
Prothrombin time (s)	296						
≤ 4	207	1					
> 4	89	1.335	0.881–2.023	0.174			
Fibrosis Ishak score	214						
0	75	1					
1–6	139	0.772	0.465–1.281	0.316			
T stage	370						
T1 & T2	277	1					
T3 & T4	93	2.598	(1.826–3.697)	<b>&lt; 0.001</b>	2.003	1.160–3.459	<b>0.013</b>
N stage	258						
N0	254	1					
N1	4	2.029	0.497–8.281	0.324			
M stage	272						
M0	268	1					
M1	4	4.077	1.281–12.973	<b>0.017</b>	2.098	0.643–6.845	0.219

(Continued)

Table 2 (Continued).

Characteristic	Total (n)	Univariate Analysis			Multivariate Analysis		
		HR	95% CI	P value	HR	95% CI	P value
Pathologic stage	349						
I	173	I					
II, III, IV	176	2.090	1.429–3.055	<b>&lt; 0.001</b>	1.571	0.874–2.823	0.131
Histologic grade	368						
G1	55	I					
G2, G3, G4	313	1.188	0.721–1.958	0.499			
Residual tumor	344						
R0	326	I					
R1, R2	18	1.604	0.812–3.169	0.174			
Vascular invasion	317						
No	208	I					
Yes	109	1.344	0.887–2.035	0.163			

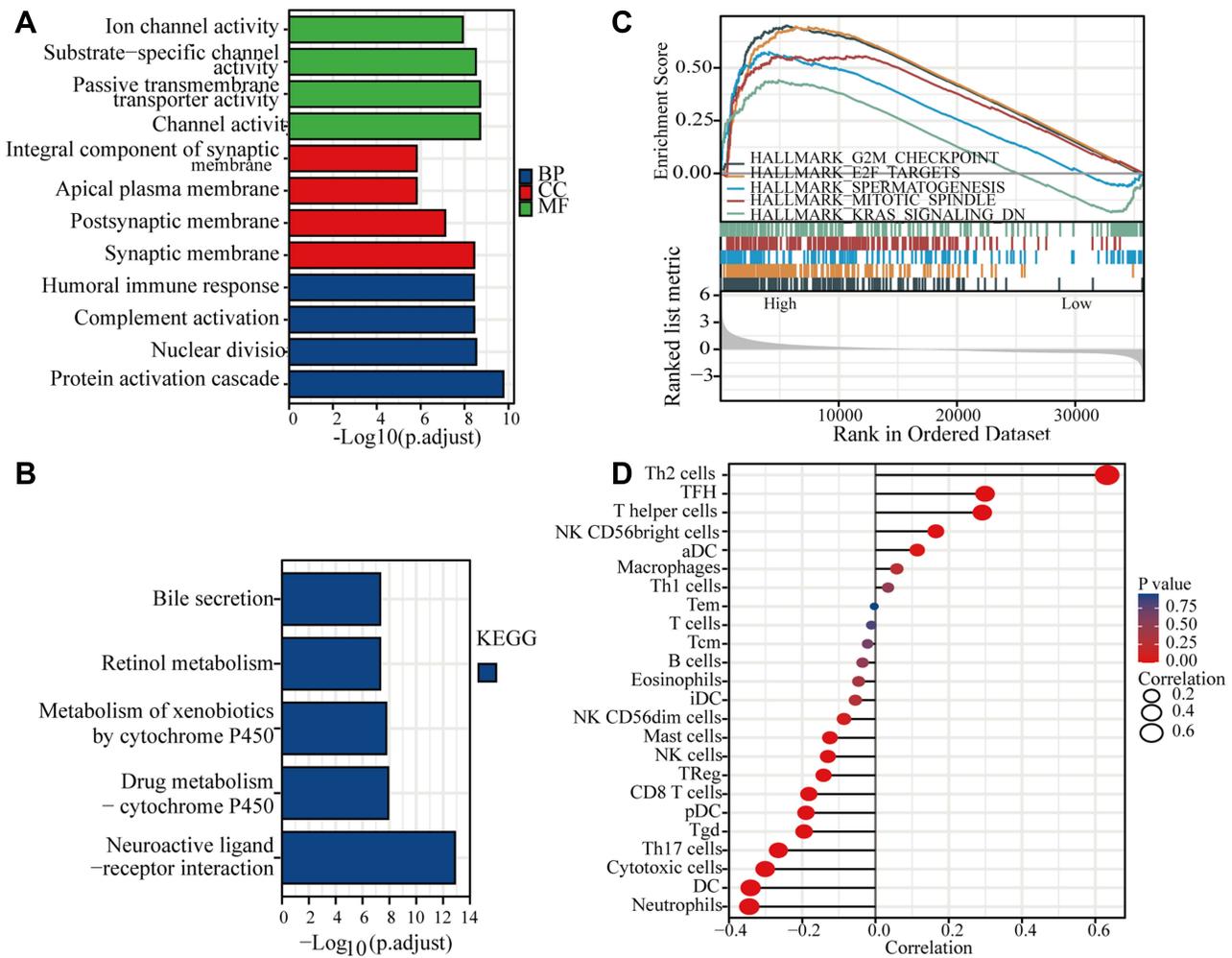
**Notes:** Bold indicates a statistically significant difference with  $p < 0.05$ .

**Abbreviations:** CENPO, centromere protein O; T stage, Tumor stage; N stage, Lymph node stage; M, Metastasis stage; BMI, body mass index; AFP, alpha fetoprotein; HR, hazard ratio; CI, confidence interval.

## Expression of *CENPO* is Associated with Immune Cell Infiltration

The results of our functional exploration of DEGs between high and low *CENPO* expression groups indicated that *CENPO* was associated with complement activation and humoral immune responses; therefore, we speculated that there may be an association between *CENPO* expression in HCC and immune cell tumor infiltration, and analyzed the correlation between *CENPO* expression and the abundance of infiltrating tumor immune cells. To explore possible correlations between *CENPO* mRNA levels and levels of 24 immune cell types, we used the “ssGSEA” package in R. The results are presented as Spearman’s  $r$  values and showed that *CENPO* expression was significantly correlated with Th2 cells, dendritic cells (DC), T helper cells, T follicular helper cells (TFH), neutrophils, cytotoxic cells, Th17 cells, plasmacytoid DC (pDC), CD8 T cells, gamma delta T (Tgd), natural killer (NK) CD56<sup>bright</sup> cells, regulatory T cells (Tregs), NK cells, mast cells, and activated DC (aDC) (Figure 3D). Further, *CENPO* expression was positively correlated with levels of Th2 cells (Figure 4A) ( $r = 0.632$ ,  $p < 0.001$ ), T helper cells (Figure 4B) ( $r = 0.292$ ,  $p < 0.001$ ), and TFH cells (Figure 4C) ( $r = 0.299$ ,  $p < 0.001$ ), NK CD56<sup>bright</sup> cells (Figure 4D) ( $r = 0.165$ ,  $p = 0.001$ ), and aDC infiltration (Figure 4E) ( $r = 0.114$ ,  $p = 0.028$ ). In addition, *CENPO* expression was negatively correlated with DCs (Figure 4F) ( $r = -0.341$ ,  $p < 0.001$ ), neutrophils (Figure 4G) ( $r = -0.345$ ,  $p < 0.001$ ), cytotoxic cells (Figure 4H) ( $r = -0.301$ ,  $p < 0.001$ ), Th17 cells (Figure 4I) ( $r = -0.265$ ,  $p < 0.001$ ), pDCs (Figure 4J) ( $r = -0.190$ ,  $p < 0.001$ ), CD8 T cells (Figure 4K) ( $r = -0.182$ ,  $p < 0.001$ ), Tgd (Figure 4L) ( $r = -0.195$ ,  $p < 0.001$ ), Tregs (Figure 4M) ( $r = -0.142$ ,  $p = 0.006$ ), NK cells (Figure 4N) ( $r = -0.130$ ,  $p = 0.012$ ), and mast cells (Figure 4O) ( $r = -0.124$ ,  $p = 0.016$ ).

The above results encouraged us to further explore the associations between *CENPO* expression and the abundance of tumor infiltrating immune cells. Interestingly, we observed significant differences in the degree of tumor immune cell infiltration between the high and low *CENPO* expression groups. In the *CENPO* high expression group, tumor-infiltrating immune cells Th2 cells (Figure 5A) ( $p < 0.001$ ), T helper cells (Figure 5B) ( $p < 0.001$ ), TFH cells (Figure 5C) ( $p < 0.001$ ), NK CD56<sup>bright</sup> cells (Figure 5D) ( $p = 0.021$ ), and aDC (Figure 5E) ( $p = 0.024$ ) were more abundant. However, in the *CENPO* low expression group, DCs (Figure 5F) ( $p < 0.001$ ), neutrophils (Figure 5G) ( $p < 0.001$ ), cytotoxic cells (Figure 5H) ( $p < 0.001$ ), Th17 cells (Figure 5I) ( $p < 0.001$ ), pDCs (Figure 5J) ( $p = 0.002$ ), CD8 T cells (Figure 5K) ( $p = 0.017$ ), Tgd (Figure 5L) ( $p = 0.033$ ), Tregs (Figure 5M) ( $p = 0.009$ ), NK cells (Figure 5N) ( $p = 0.013$ ), and mast cells (Figure 5O) ( $p = 0.014$ ) had a higher infiltration abundance. This is consistent with the results of the previous correlation analysis between *CENPO* expression and immune cells. Finally, we



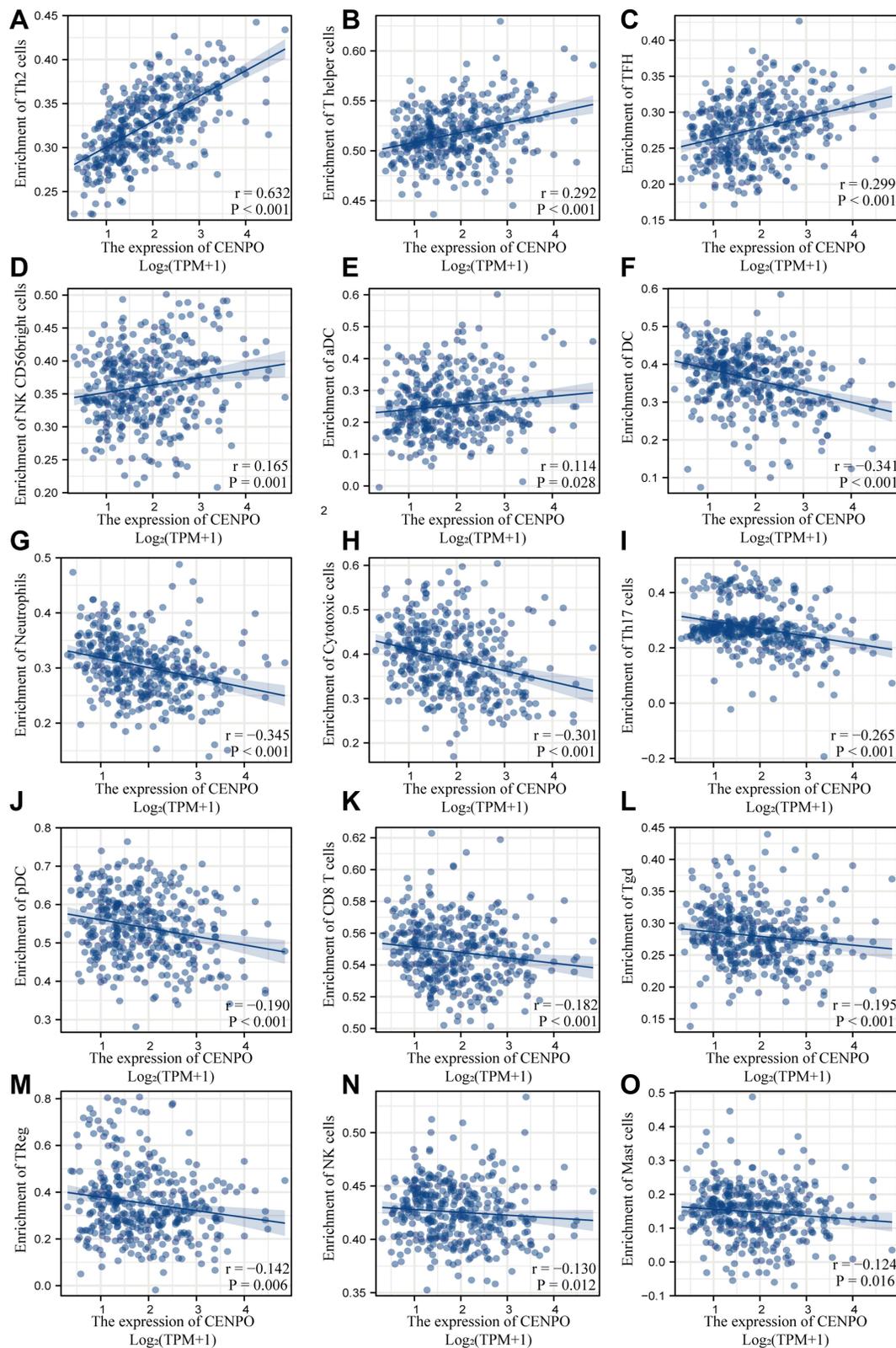
**Figure 3** GO, KEGG, and GSEA evaluation of DEGs between HCC with high and low *CENPO* expression. **(A)** Biological processes enriched for DEGs identified by GO analysis. **(B)** Pathways enriched for DEGs according to KEGG analysis. **(C)** Top five pathways with the highest GSEA enrichment analysis scores. **(D)** Correlation between *CENPO* expression and the abundance of 24 immune cell infiltrates in TCGA-LIHC.

**Abbreviations:** TFH, T follicular helper cells; aDC, activated dendritic cell; iDC, interdigitating dendritic cell; Treg, regulatory T cells; pDC, plasmacytoid dendritic cell; Tgd, gamma delta T cells; DC, dendritic cell.

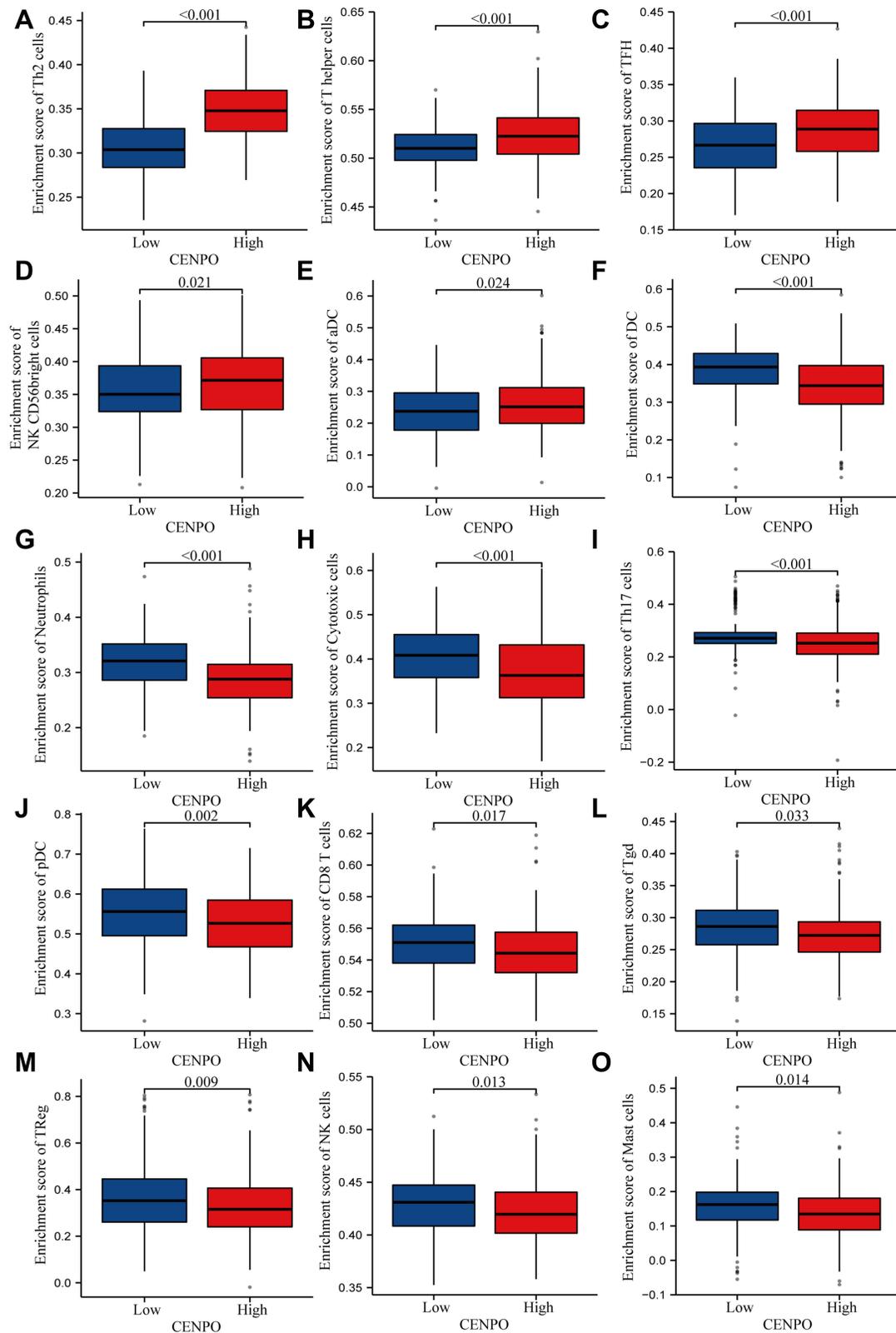
evaluated the associations between *CENPO* and immune cell infiltration with clinical survival outcomes in patients with liver cancer, according to the TIMER 2.0 online database. Low levels of CD4<sup>+</sup> memory resting T cells and endothelial cells were associated with poor prognosis in patients with HCC and high *CENPO* expression ( $p < 0.05$ ) (Figure 6A and B). Further levels of neutrophil and macrophage M2 infiltration were significantly associated with poor prognosis in patients in the low *CENPO* expression group ( $p < 0.05$ ) (Figure 6C and D). These findings suggest that *CENPO* has an important role in the immune infiltration of HCC.

## *CENPO* Function in HCC Immune Evasion

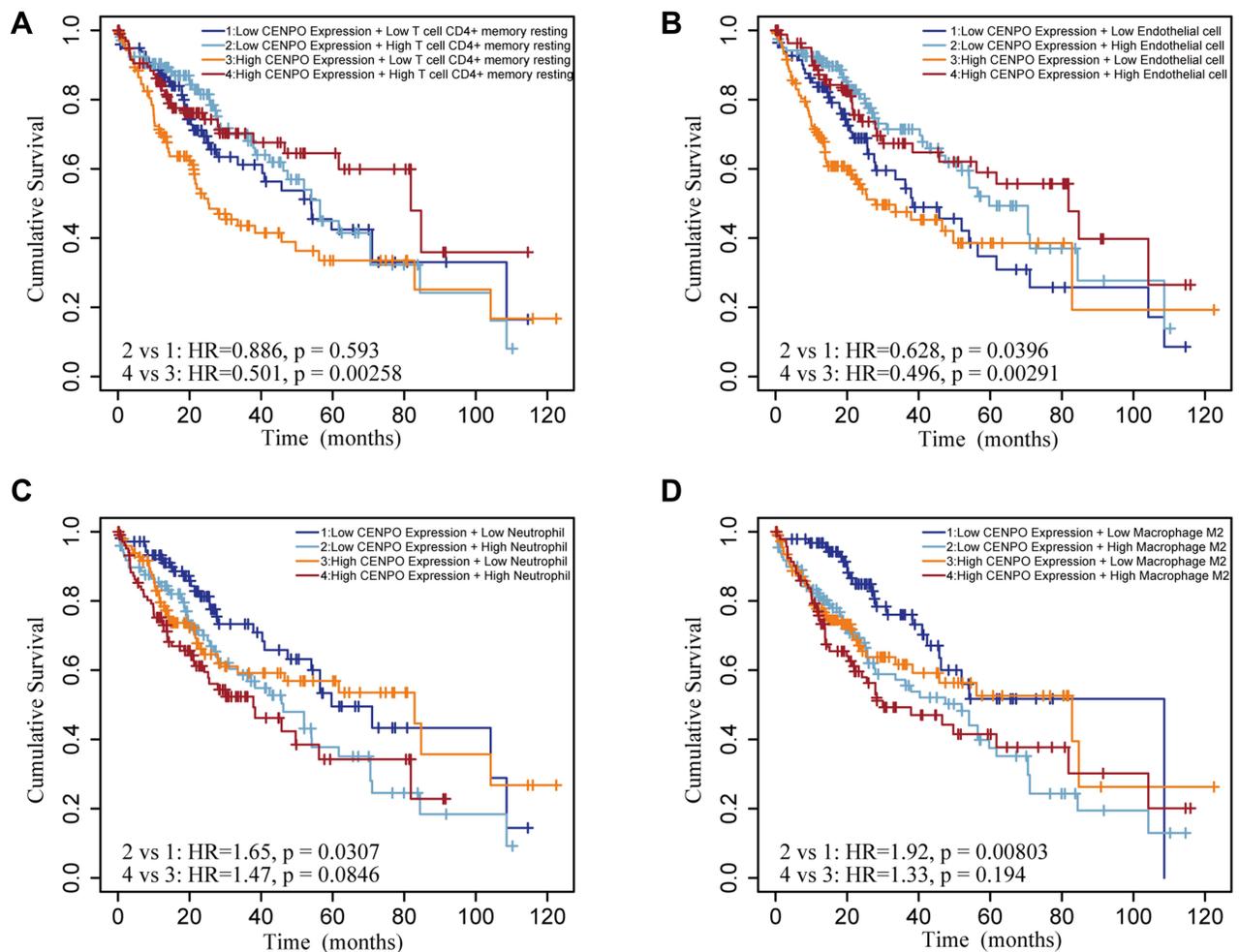
Immunotherapy is a standard feature of care for patients with advanced HCC; however, it is accompanied by complications, such as immunological tolerance. Several immunological checkpoints linked to immune escape have been identified, among which, PD1, PD-L1, and CTLA-4 are most prominent. Using the TIMER2.0 database, we investigated the relationships of *CENPO* with PDCD1, CD274, and CTLA4, to learn more about the role of *CENPO* in HCC immunological tolerance. *CENPO* expression in HCC was strongly and positively correlated with CTLA4 ( $r = 0.395$ ,  $p < 0.001$ ), PDCD1 ( $r = 0.389$ ,  $p < 0.001$ ), and CD274 ( $r = 0.364$ ,  $p < 0.001$ ) levels (Figure 7A–C). These findings suggest that *CENPO* is a potential target for immunotherapy in HCC.



**Figure 4** *CENPO* expression was significantly correlated with the abundance of 15 immune cell infiltrates. (A) Th2 cells, (B) T helper cells, (C) T follicular helper cells (TFH), (D) natural killer (NK) CD56<sup>bright</sup> cells, (E) activated dendritic cell (aDC), (F) dendritic cells (DCs), (G) neutrophils, (H) cytotoxic cells, (I) Th17 cells, (J) plasmacytoid DCs (pDCs), (K) CD8 T cells, (L) gamma delta T (Tgd), (M) regulatory T cells (Tregs), (N) NK cells, and (O) mast cells.



**Figure 5** Analysis of the abundance of various immune cell infiltrates between different CENPO expression cohorts. (A) Th2 cells, (B) T helper cells, (C) T follicular helper cells (TFH), (D) natural killer (NK) CD56<sup>bright</sup> cells, (E) activated dendritic cell (aDC), (F) dendritic cells (DCs), (G) neutrophils, (H) cytotoxic cells, (I) Th17 cells, (J) plasmacytoid DCs (pDCs), (K) CD8 T cells, (L) gamma delta T (Tgd), (M) regulatory T cells (Tregs), (N) NK cells, and (O) mast cells.



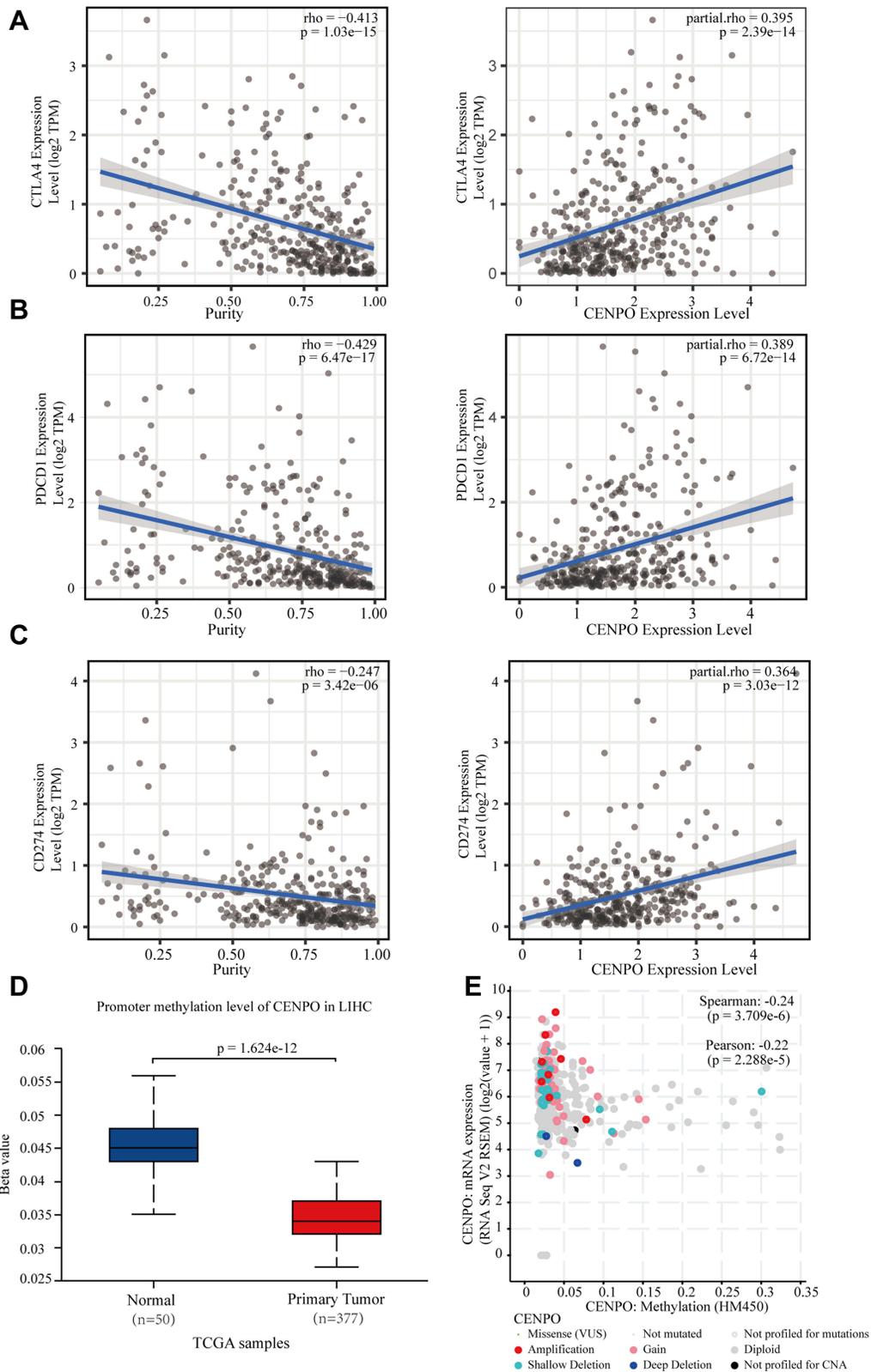
**Figure 6** Prognosis of HCC according to *CENPO* expression combined with immune cell infiltration based on TIMER2.0 database analysis. (A) resting memory CD4<sup>+</sup>T cells, (B) endothelial cells, (C) M2 macrophages, and (D) neutrophils.

## Relationship Between *CENPO* Expression and Methylation in HCC

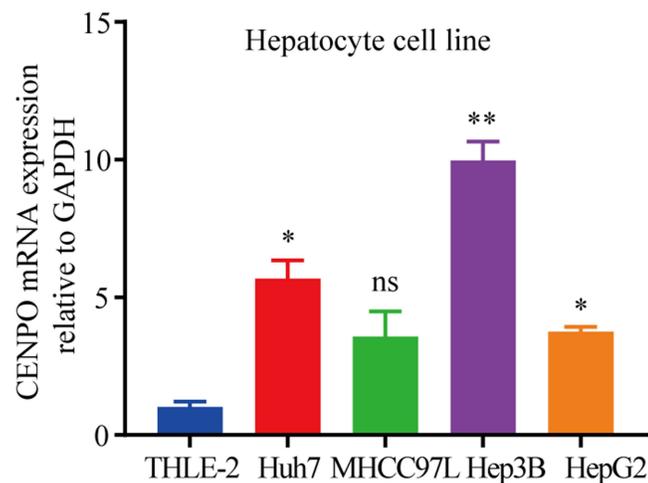
Tumor occurrence is generally associated with abnormal gene expression. To further investigate the mechanisms underlying abnormal *CENPO* expression in HCC, we analyzed the relationship between *CENPO* expression and genomic methylation. A total of 50 normal liver tissue and 377 liver cancer samples from the UALCAN database were included in the analysis of methylation levels at the *CENPO* promoter region. The results showed that methylation levels at the *CENPO* promoter region were significantly lower in the HCC group than those in normal liver tissues ( $p < 0.001$ ) (Figure 7D). Further, genomic level analysis of changes in *CENPO* in 1009 liver cancer samples from four groups of HCC studies selected through the cBioPortal database showed that its expression was negatively correlated with methylation (Spearman's  $r = -0.24$ ,  $p < 0.001$ ; Pearson's  $r = -0.22$ ,  $p < 0.001$ ) (Figure 7E). These findings suggest that methylation changes may control *CENPO* expression in HCC.

## Analysis of *CENPO* mRNA Expression in Cell Lines

To further explore the expression of *CENPO* in HCC cell lines, we examined the expression of *CENPO* mRNA in human HCC Huh7, MHCC97L, Hep3B, HepG2 cell lines, and normal liver THLE-2 cells by RT-qPCR. The results showed that the expression of *CENPO* mRNA was significantly higher in Huh7, Hep3B, and HepG2 cells than in normal hepatocytes. In MHCC97L cells, *CENPO* mRNA expression tended to be higher, although the difference was not statistically significant (Figure 8).



**Figure 7** Associations between *CENPO* expression and (A) CTLA4, (B) PD-1, and (C) PD-L1 in the TIMER 2.0 database. (D) *CENPO* promoter methylation levels in HCC. (E) Correlation analysis of *CENPO* expression and methylation.



**Figure 8** Expression of CENPO mRNA in HCC cell lines. The results were normalized with GAPDH. ns, not significant; \* $p < 0.05$ , \*\* $p < 0.01$ .

## Discussion

HCC remains a major cause of cancer-related deaths worldwide.<sup>18</sup> The number of men with liver cancer in China is second only to that with lung cancer, whereas the number of women with liver cancer is second only to that with breast cancer.<sup>19</sup> Patients with HCC are often diagnosed when the disease is at an advanced stage, and many patients continue to die from disease recurrence and distant metastasis after surgery. Therefore, there is an urgent need to find more effective biomarkers for early diagnosis and surveillance of the development of liver cancer.

With the development of molecular biology techniques, over 100 members of the CENP family of centromere proteins have been discovered.<sup>20</sup> CENPs can be subdivided into subgroups (CENP-C, CENP-L/M, CENP-H/I/K/M, CENP-T/W, CENP-O/P/Q/R/U, and CENP-S/X) based on their functions.<sup>21,22</sup> Mislocalization or abnormal expression of these proteins or complexes can result in abnormal cell division and the formation of chromosome aneuploidies, and such alterations are frequently linked to cell cycle disruption and cancer development.<sup>23,24</sup> With advances in bioinformatics and molecular biology, an increasing number of centromere proteins have been identified as involved in the regulation of HCC. CENPA,<sup>25</sup> CENPE,<sup>26</sup> CENPF,<sup>27</sup> CENPH,<sup>28</sup> CENPK,<sup>29</sup> CENPL,<sup>30</sup> CENPM,<sup>31</sup> CENPN,<sup>32</sup> and CENPU<sup>33</sup> are highly expressed in HCC and their levels correlate with patient prognosis; however, to our knowledge, there have been no studies on the role of CENPO in HCC. In this study, we explored the possible mechanisms underlying the function of CENPO in HCC and its suitability as a diagnostic and prognostic biomarker.

In our pan-cancer analyses, we observed upregulation of *CENPO* mRNA expression in the majority of cancer types. By integrating data from multiple databases, we demonstrated that *CENPO* expression is consistently upregulated in HCC. Further, our RT-qPCR data confirmed that *CENPO* mRNA expression in liver cancer tissues was higher than that in healthy liver. Furthermore, we investigated whether *CENPO* expression was associated with the clinicopathological characteristics of HCC and found significant associations between *CENPO* and AFP levels, T stage, pathological stage, histological grade, and vascular invasion, and a negative correlation with age. Survival analysis showed that patients with high *CENPO* expression had worse OS, DSS, and PFI compared to those with low *CENPO* expression. Moreover, univariate and multivariate Cox analysis showed that *CENPO* was an independent predictor of factors influencing overall survival in HCC. In addition, ROC curve analysis suggest that *CENPO* can serve as a diagnostic biomarker in HCC. Overall, *CENPO* could be applied as a new marker to diagnose HCC and predict patient prognosis.

The tumor immune microenvironment comprises a complex system characterized by immunosuppression as well as immune escape, induced by multiple immune cells within the tumor.<sup>34</sup> The percentages of immune cell types infiltrating the tumor immune microenvironment can significantly influence the malignant biological behavior of tumors. Other members of the centromere protein family (CENPF<sup>35,36</sup> and CENPM<sup>31</sup>) are also associated with infiltration of multiple immune cell types in HCC. GO analysis demonstrated that the main biological processes involving CENPO include humoral immune responses

and complement activation. We further demonstrated that *CENPO* expression was associated with HCC infiltration of multiple immune cells. We speculate that *CENPO* may influence the tumor microenvironment by altering the ratio of immune cells, which in turn promotes HCC progression, because HCC is a chronic inflammation-driven tumor, and disruption of the immune microenvironment is an important cause of HCC progression.<sup>37</sup>

Our study shows that *CENPO* expression is significantly and positively correlated with various immune cell types, particularly Th2 cells. Previous studies have shown that skewing of the Th1/Th2 cell ratio toward Th2 cells may be associated with HCC metastasis.<sup>38</sup> Further, Th2 cells can induce the polarization of M1 into M2 macrophages, which ultimately leads to suppression of the host immune system, thus promoting cancer development.<sup>39</sup> There is also evidence that energy metabolism between Th2 cells and the tumor microenvironment is interdependent, and can influence tumor development.<sup>40</sup> Our data show that *CENPO* expression is positively correlated with abundance of immune cells, including TFH cells which can be switched to Th1 or Th2 cells to regulate humoral immunity.<sup>41</sup> Hence, upregulation of *CENPO* expression may contribute to HCC occurrence and progression by affecting immune infiltration. In light of these findings, we used the TIMER2.0 database to investigate the effects of *CENPO* and immune cells on HCC prognosis. Our results suggest that *CENPO* expression, along with CD4<sup>+</sup> memory resting phase T cell, endothelial cell, M2 macrophage, and neutrophil levels, may influence the outcome of patients with HCC. TIMER2.0 database analysis of the relationship between *CENPO* and immune checkpoint-related molecules revealed a positive correlation, indicating that *CENPO* likely promotes HCC via immune regulation. Together, these results suggest that *CENPO* may influence HCC development by altering immune cell infiltration; however, the tumor microenvironment is highly complex and other immune cell classes in the tumor microenvironment are also likely to influence tumor cell survival. Hence, further experiments to investigate the association between *CENPO* and immune cells are required.

Finally, we explored the upstream mechanisms underlying the high *CENPO* expression detected in HCC. Tumors are frequently the result of abnormal gene expression. Methylation status or regulation by upstream non-coding RNA can alter gene expression, which may influence tumor progression.<sup>42,43</sup> Attachment of a methyl group to the fifth carbon of the cytosine ring to generate 5-methylcytosine (5-mC) is referred to as DNA methylation, and 5-mC modification can modulate transcription both in vitro and in vivo.<sup>44</sup> Here, we observed lower methylation levels of the *CENPO* promoter region in HCC compared to healthy tissue, and correlation analysis showed that *CENPO* expression in HCC was negatively correlated with methylation. We hypothesize that *CENPO* expression in HCC may be influenced by methylation modification, thus contributing to HCC development; however, our data could only be associated with the influence of methylation on *CENPO* levels, and we did not investigate other potential mechanisms of *CENPO* expression regulation, such as the effects of various non-coding RNAs.

Although this study was the first to reveal the correlation between *CENPO* and HCC, our study has limitations. First, the majority of the datasets used in this study were obtained directly from public databases; hence, further validation using in vivo and in vitro experimental and clinical data is needed. Second, longer follow-up and larger patient populations are necessary to confirm the validity of *CENPO* as a predictor of OS, DSS, and PFI. Finally, additional investigations are required to determine the cause of high *CENPO* expression in HCC.

## Conclusion

Our findings clarify pan-cancer *CENPO* mRNA expression levels, including in HCC tissues and cell lines. *CENPO* expression was associated with multiple clinicopathological features of HCC, and high *CENPO* expression was associated with poor patient prognosis. Downstream pathway analysis suggests that *CENPO* may influence HCC progression by regulating tumor immune cells and participating in pathways related to the cellular G2M checkpoint. Our data also suggest that *CENPO* may be associated with immune escape in HCC. Further investigation of the potential mechanisms upstream of HCC showed that differential expression of *CENPO* in HCC may be controlled by changes in methylation status, suggesting that methylation may be involved in HCC development.

## Abbreviations

CENPA, centromere protein A; CENPE, centromere protein E; CENPF, centromere protein F; CENPH, centromere protein H; CENPK, centromere protein K; CENPL, centromere protein L; CENPM, centromere protein M; CENPN, centromere protein N; CENPO, centromere protein O; CENPU, centromere protein U; DEGs, differentially expressed

genes; DSS, disease-specific survival; GEO, Gene Expression Omnibus; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; GTEX, Genotype-Tissue Expression; HCC, hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; OS, overall survival; PFI, progression-free interval; TCGA, The Cancer Genome Atlas.

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## Disclosure

The authors report no conflicts of interest in this work.

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