

The Prognostic Significance of CTSV Expression in Patients with Hepatocellular Carcinoma

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Purpose: Cathepsin V (CTSV) is a cysteine protease peptidase, which is typically upregulated in cancer and is associated with various oncogenic processes, such as angiogenesis, proliferation, apoptosis, and invasion. The study explored the role of CTSV in hepatocellular carcinoma (HCC) and its potential as a potential biomarker.

Patients and Methods: This study collected tumor and peritumoral archived specimens from 180 HCC patients who underwent surgical resection at Zhongshan Hospital, Fudan University (Shanghai, China) between 2009 and 2010. We extracted data from the TCGA and GEO databases and conducted differential expression analysis, univariate Cox regression, and Kaplan-Meier analysis. Ultimately, we determined that CTSV may emerge as a potential biomarker. Then, immunohistochemical staining for CTSV was performed on tumors and adjacent tissues of HCC patients, and a Cox proportional hazards model was constructed to evaluate the prognostic significance of CTSV expression levels. Applied functional enrichment analysis to reveal the underlying molecular mechanisms. Utilized ssGSEA enrichment analysis and TIMER2.0 algorithm to explore the correlation between CTSV expression and immune cells in HCC. In vitro and in vivo experiments were conducted using human liver cancer cell lines to further validate the clinical application value of CTSV.

Results: In this study, we observed that CTSV expression was notably elevated in HCC ($P < 0.001$), and identified a significant association between elevated CTSV expression and reduced overall survival rates in patients. In vitro and in vivo experiments indicated that CTSV knockdown could significantly inhibit the proliferation, migration, and invasion of liver cancer cells, and it was found that the combination of CTSV knockdown with PD-1 inhibitors might enhance the therapeutic effect of PD-1 inhibitors in HCC.

Conclusion: CTSV serves as a standalone negative prognostic indicator and possesses clinical significance in HCC.

Keywords: Cathepsin V, HCC, biomarker, immune infiltrating cells, PD-1 inhibitor

Introduction

Liver malignancies rank among the prevalent forms of cancer globally. The global cancer burden report indicates that in 2020, there were approximately 906,000 new cases of hepatocellular carcinoma (HCC) and 830,000 deaths globally. In China, liver cancer accounted for approximately 410,000 new instances and was responsible for around 391,000 fatalities, positioning it fifth in incidence among all malignancies and second in terms of mortality rates.¹ Various elements can precipitate the onset of HCC, such as infections with the hepatitis B and C viruses, contact with carcinogenic substances and toxins, and hereditary influences.² An increasing body of research indicates that the pathogenesis of HCC is linked to the activation of cancer-promoting genes, such as FGF19, VEGFA, TERT, CNND1,

CTNNB1, and TP53.³ However, the mechanisms by which these oncogenes, when mutated or overexpressed, mediate the acceleration of HCC occurrence and development are still not fully understood.

Cathepsins represent a class of proteolytic enzymes that are predominantly found within lysosomes, encompassing members such as Cathepsins A, B, C, D, E, F, G, H, L, K, O, S, and W, which are essential for maintaining normal tissue equilibrium.⁴ There are various enzyme subtypes of tissue proteases, including serine proteases, aspartic proteases, and cysteine proteases. Cathepsins, a family of lysosomal cysteine proteases, are typically upregulated in cancer and are associated with various tumorigenic processes, including the stimulation of new blood vessel formation, cell multiplication, programmed cell death, and the spread of cancer cells from the primary tumor site.^{5,6} Cathepsin V (CTSV), belonging to the peptidase C1 family, is the gene that codes for a lysosomal cysteine protease.⁷ CTSV is a multifunctional cysteine protease that mainly aggregates in lysosomes, participates in the liberation of antigenic peptides and the maturation of Major Histocompatibility Complex (MHC) class II molecules, as well as the flipping of elastin fibrils and the lysis of intracellular and extracellular matrix. It is mainly expressed in the heart, thymus, brain, skin, and cornea.^{8–10} Skrzypczak's research has identified a positive correlation between the expression levels of CTSV and those of proteins that play a role in the regulation of the cell cycle and cellular proliferation, which may be related to the progression of endometrial cancer.¹¹ Sereesongsaeng discovered that CTSV was capable of reducing the expression of the GATA3 protein in estrogen receptor (ER)-positive breast cancers through a mechanism that involved the promotion of its degradation by the proteasome. This suppression correlated with a more favorable clinical outcome, suggesting that high levels of CTSV expression were significantly and positively linked to an improved prognosis in ER-positive breast cancer.¹² They further found that targeting CTSV had disrupted cell cycle progression by regulating histone stability, suggesting that CTSV might represent a potential therapeutic target for the treatment of ER-positive breast cancer.¹³ Yang L performed immunohistochemical assays on tissues from lung cancer patients, comparing cancerous samples with their normal counterparts. The analysis revealed a marked increase in the expression levels of CTSV within the cancerous tissues. Moreover, this elevated expression of CTSV was found to be associated with a poorer prognosis for affected individuals.¹⁴ Xia Y and peers discovered that an overabundance of CTSV could trigger the activation of inflammatory signaling pathways in bladder cancer by upregulating the NF- κ B pathway, which promoted cancer cell proliferation. The addition of an NF- κ B inhibitor could inhibit this process, revealing that CTSV could represent a promising target for therapeutic intervention in bladder cancer.¹⁵ Lin's research revealed that Praeruptorin B was highly effective at curbing the migratory and invasive properties of human renal cancer cells. Additionally, Lin discovered that this compound also led to a reduction in both the transcriptional and translational levels of CTSV within these cells.¹⁶ The existing body of research implies that CTSV could have a substantial impact on the progression of various tumor types. Nonetheless, its specific contribution to the development and progression of HCC remains less explored. Therefore, additional studies are necessary to elucidate its role in HCC.

The purpose of this study was to assess the potential of CTSV as a prognostic marker in HCC and to explore its correlation with a range of clinical outcomes. In this study, we utilized immunohistochemical techniques to assess the levels of CTSV expression within tumor samples and investigated the correlation between CTSV expression levels and the clinical features and outcomes of the patients. We employed the Kaplan-Meier method and the COX proportional hazards model to evaluate the prognostic significance of CTSV. Furthermore, we used functional enrichment analysis to reveal the underlying molecular mechanisms. Additionally, leveraging ssGSEA enrichment analysis and the TIMER2.0 database, we investigated the correlation between CTSV expression and the extent of immune cell infiltration within HCC. Finally, *in vitro* and *in vivo* experiments were conducted using hepatocellular carcinoma cell lines to further validate the clinical application value of CTSV.

Materials and Methods

Raw Data

The RNA sequencing data and corresponding clinical data from the TCGA database were downloaded from the GDC database (<https://portal.gdc.cancer.gov/>). Additionally, the RNA expression matrix from the GEO database GSE84402 dataset was obtained through the NCBI database (<https://www.ncbi.nlm.nih.gov/>).

Human Samples

For this research, we collected preserved tumor and surrounding (peritumor) tissue samples from 180 individuals diagnosed with HCC who had undergone surgical resection at Zhongshan Hospital, Fudan University, located in Shanghai, China, during the period from 2009 to 2010. Each participant provided their written consent to participate, and the study was reviewed and approved by the Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China). The guidelines outlined in the Declaration of Helsinki were followed.

Bioinformatics Analysis

Differential Gene Expression Analysis

Differential expression analysis was performed using the DESeq2 package in R software (version 4.3.1). To identify differentially expressed genes (DEGs), we established stringent criteria that required $|\log_2\text{Fold Change}| \geq 1$, along with a false discovery rate (FDR) threshold of < 0.05 . The pheatmap package in R software was utilized to generate heatmaps of DEGs.

Kaplan-Meier Analysis

Survival curves were plotted employing the Kaplan-Meier method, and their differences were assessed with the Cox regression. Analyses were performed utilizing the survival and survminer packages in R software, and statistical significance was determined with a threshold of a two-tailed p-value below 0.05.

Cox Proportional Hazard Model

Conducted univariate Cox regression analysis using the survival package in R software. Displayed the top 10 genes in the univariate Cox analysis, ranked by p-value from smallest to largest, in the chart.

Functional Enrichment Analysis

In R software, the clusterProfiler, enrichplot, and ggplot2 packages were used to perform GO and KEGG enrichment analysis on the results of gene differential analysis. Only results with p.adjust less than 0.05 were considered significantly enriched. In R software, Gene Set Enrichment Analysis (GSEA) was conducted using the GSVA and ggpvr packages to evaluate the activation or repression status of specific biological pathways or functions. Only gene sets with p.adjust less than 0.05 and FDR less than 0.05 were considered significant.

Tumor-Infiltrating Immune Cells Profile

The abundance changes of different immune cell subsets in the tumor microenvironment were estimated using the CIBERSORT algorithm. Single Sample Gene Set Enrichment Analysis (ssGSEA) was another computational method used to assess the composition and infiltration level of immune cells in the tumor microenvironment. The TIMER 2.0 database was primarily used to analyze the correlation between gene expression and the status of immune cell infiltration (<http://timer.comp-genomics.org/>).

Immunohistochemical (IHC) Staining

Collected HCC and corresponding adjacent non-cancerous tissues, fix them with 10% formalin, and embed them in paraffin to create 4- μm thick slices. Constructed a tissue microarray (TMA) in triplicate using these slices for immunohistochemistry (IHC) staining.¹⁷ After antigen retrieval and deparaffinization, the microarray was sequentially incubated with secondary antibodies conjugated to primary antibodies (CTSV, Cat.ab124656, Abcam) and horseradish peroxidase (HRP). Subsequently, two pathologists conducted an independent evaluation of the staining results utilizing semi-quantitative approach, while concurrently ensuring the privacy of patient clinical data.¹⁸ The ultimate score was calculated as the product of staining intensity and staining area, categorized into four levels. Staining intensity was scored as follows: 0 for no staining, 1 for weak (1–25%), 2 for moderate (26–50%), and 3 for strong (51–100%) staining. Staining area was measured as a percentage (0–100%). Based on these scores, expression levels were defined as follows: negative (0% staining area) and weak (1–25% staining area) were considered low expression, while moderate (26–50% staining area) and strong (51–100% staining area) were considered high expression.

Cell Lines

This study utilized two human HCC cell lines: PLC/PRF/5 and MHCC97H. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 mg/mL penicillin-streptomycin, and maintained in an incubator at 37°C with 5% CO₂. Short hairpin RNA (shRNA) constructs (shNC, shCTSV#1, or shCTSV#2) were used to knock down the expression of CTSV in both PLC/PRF/5 and MHCC97H cells.

Quantitative Real-Time PCR (qRT-PCR)

Extracted RNA from cells using TRIzol reagent (Invitrogen) and further purified the RNA through chloroform and isopropanol precipitation. Then, used the All-in-One First-Strand Synthesis Master Mix (with dsDNase) for reverse transcription and cDNA synthesis. Employed the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) for real-time PCR analysis to quantitatively measure mRNA relative expression levels.

Each sample was treated with GAPDH as an internal control, and the PCR primer sequences were as follows: CTSV forward, 5'-CGTGACGCCAGTGAAGAATCA-3' and reverse, 5'-CGCTCAGTGAGACAAGTTTCC-3'; GAPDH forward, 5'-ACTCCACTCACGGCAAATTC-3' and reverse, 5'-TCTCCATGGTGGTGAAGACA-3'. Calculated and normalized the relative transcription levels of CTSV using the $2^{-\Delta\Delta Ct}$ method to quantify qRT-PCR products.

Western Blot Assay

Lysed cells with lysis buffer to extract proteins, then mixed the samples with loading buffer and underwent heat denaturation. Separated the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures, and transferred them onto a polyvinylidene fluoride (PVDF) membrane. After transfer, blocked the membrane with 5% skim milk at room temperature for 1 hour, then incubated with the primary antibody (CTSV, Cat.ab124656, Abcam) at 4°C overnight. The next day, washed the membrane with Tris-buffered saline with Tween (TBST) three times, each for 10 minutes, then incubated with the appropriately diluted horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1–2 hours. Finally, incubated the membrane with a chemiluminescent substrate in the dark and performed visualization detection of the protein bands.

Cell Proliferation Assay

Used the Cell Counting Kit-8 (CKK-8) to evaluate cell proliferation. Seeded cells into a 96-well plate with 1000 cells per well in 100 μ L of culture medium. At the designated time points (24, 48, 72, and 96 hours) of cell culture, added 10 μ L of CKK-8 reagent solution to each well, and then incubated at 37°C with 5% CO₂ for 1 hour. Measured the absorbance at 450 nm (OD₄₅₀) using a microplate reader, and corrected for background absorption by measuring the reference wavelength as needed.

Clonogenic Assay

Seeded the cells into a six-well culture plate and cultured them at 37°C and 5% CO₂ for 7–14 days. Then, the cells were gently washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde solution for 30 minutes, and stained with 0.1% crystal violet for 15 minutes. Finally, counted and recorded the number of cell colonies to evaluate the proliferation ability of the cells.

Transwell Assay

The Transwell assay was utilized to evaluate the migration and invasion capabilities of cells. For invasion assays, the Transwell chambers were coated with extracellular matrix gel; this step was not required for migration assays. Cell suspensions were prepared in serum-free medium and seeded into the upper chamber, while the lower chamber was filled with RPMI 1640 medium containing 10% FBS. After incubation for 48 hours at 37°C and 5% CO₂, cells that had migrated or invaded to the lower chamber were fixed with 4% formaldehyde and stained with 0.1% crystal violet. Subsequently, the un-migrated cells in the upper chamber were removed using a cotton swab, and the lower chamber was gently washed to prepare for counting. The stained cells in the lower chamber were then counted using a microscope and image analysis software to assess the migration or invasion capabilities of cells.

Animal Studies

In vivo tumorigenesis assay, 6-week-old male C57BL/6J mice were first induced with tumors by subcutaneous injection of MHCC97H cells (1×10^6). After tumor formation, mice were randomly assigned to four groups (five mice per group) for continuous treatment. For the PD-1 inhibitor treatment group, mice were intraperitoneally injected twice a week with 100 μ g of PD-1 inhibitor. Subsequently, tumor response and animal condition were monitored, and data were ultimately collected for analysis. Used a vernier caliper to measure the tumor size and calculated the tumor volume as $0.5 \times \text{length} \times \text{width}^2$. All experiments were conducted in accordance with the guidelines for laboratory animal care and use, and were approved by the Institutional Animal Care and Ethics Committee of Zhongshan Hospital (ID: 2021–30).

Statistical Analysis

Numerical values were expressed as the mean $\pm$ standard deviation. Disparities among different groups were evaluated using Student's *t*-test, one-way ANOVA, Dunn's test or the Mann–Whitney *U*-test, selected based on the data's characteristics. Categorical variables were represented as n (%), and the compositional ratios amongst groups were contrasted utilizing Pearson's χ^2 or Fisher's exact test.

Results

CTSV May Emerge as a Potential Biomarker for Detecting HCC

Firstly, we conducted a screening process to identify DEGs (Figure 1). The TCGA-LIHC dataset was screened by comparing 369 HCC tissues with 50 surrounding tissues, while the GSE84402 dataset involved comparing 14 HCC tissues with 14 corresponding non-cancerous tissues. Gene differential expression analysis was performed on both datasets, resulting in the identification of DEGs. The TCGA-LIHC dataset analysis revealed 1664 DEGs, with 1411 up-regulated and 233 down-regulated (Figure 2A and B). The GSE84402 dataset analysis identified 1773 DEGs, with 689 up-regulated and 1084 down-regulated (Figure 2C and D). Venn diagrams were used to intersect the DEGs from both datasets, yielding a total of 477 DEGs. Among these, 321 were up-regulated, while 156 were down-regulated (Figure 2E and F). Cox's hazard model was constructed using the shared DEGs from the TCGA-LIHC and GSE84402 datasets. Displayed the top 10 genes from the univariate Cox analysis, ranked by p-value from smallest to largest, with CTSV at the top of the list (Figure 2G). The results indicated that CTSV (hazards ratio [HR] = 1.307, 95% confidence interval [CI]: 1.167–1.465, $p < 0.001$) could be regarded as a standalone prognostic indicator for determining overall survival (OS) rates in individuals with HCC. Results from the Kaplan-Meier analysis indicated that elevated levels of CTSV expression were significantly associated with reduced OS and disease-specific survival (DSS) in patients (Figure 2H and I). Therefore, we determined that CTSV may serve as a promising biomarker for the prognostic evaluation of HCC patient outcomes.

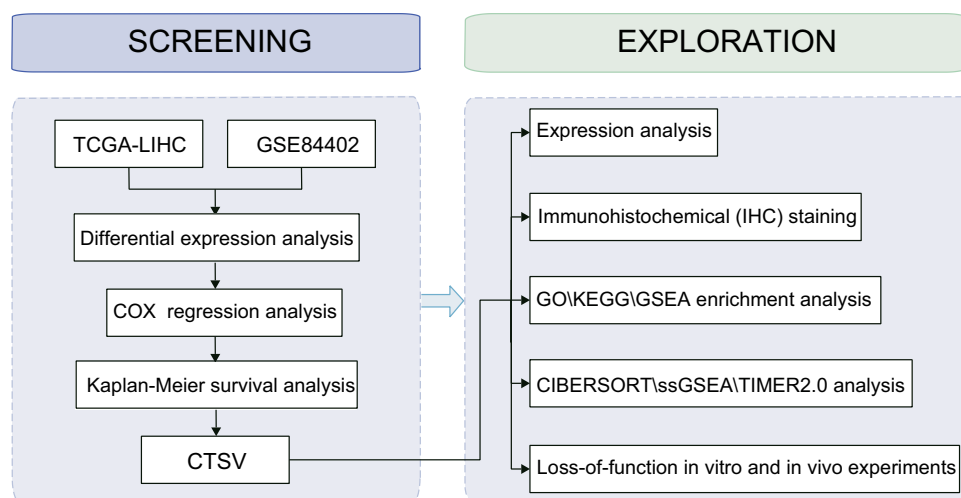


Figure 1 Workflow of the study design.

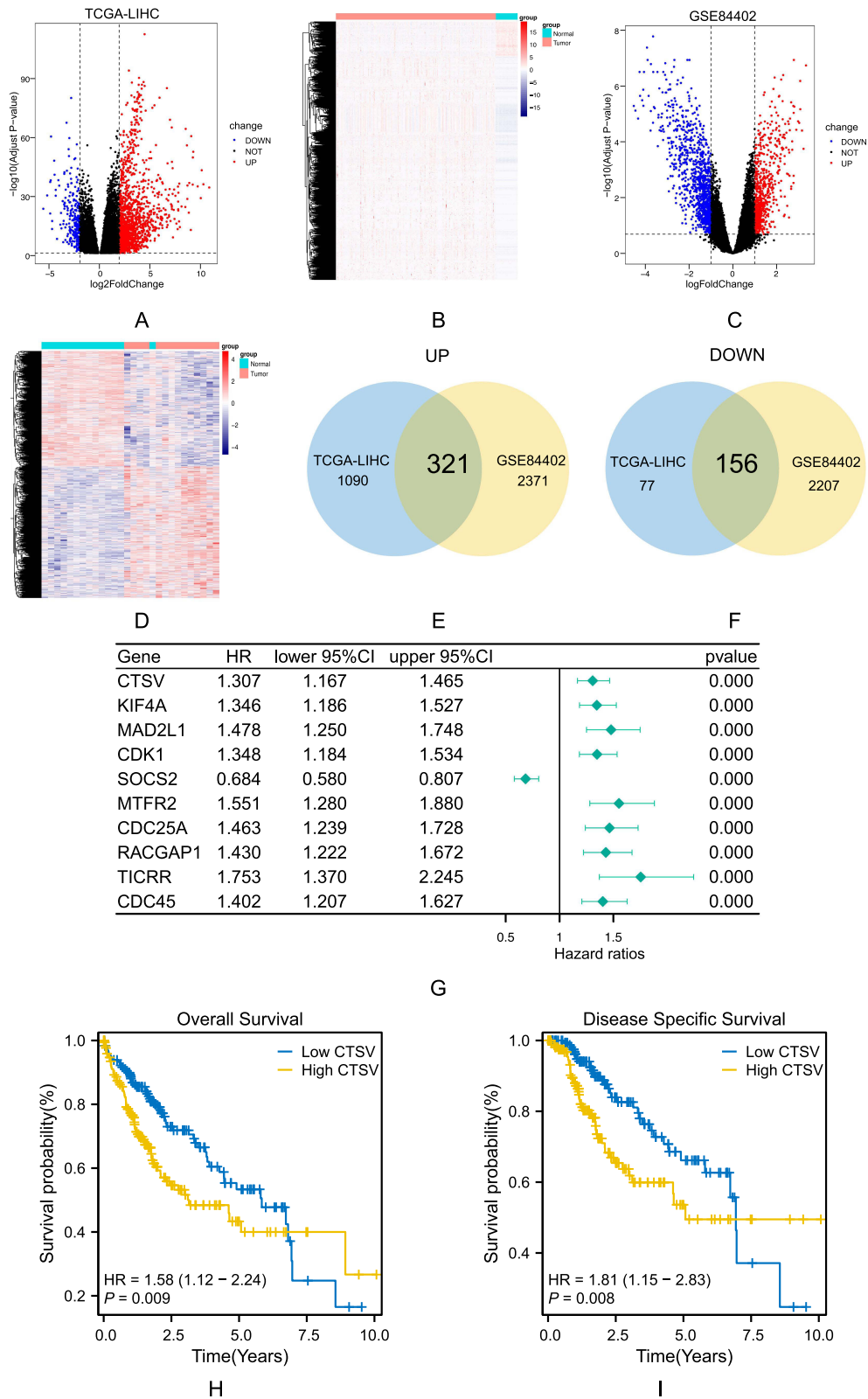


Figure 2 Screening for differentially expressed genes. **(A)** Volcano map of differential analysis in TCGA. **(B)** Heatmap of differential analysis in TCGA. **(C)** Volcano map of differential analysis in GSE84402. **(D)** Heatmap of differential analysis in GSE84402. **(E)** Up-regulated DEGs obtained from the intersection of the Venn plots comparing the TCGA and GSE84402 datasets. **(F)** Down-regulated DEGs obtained from the intersection of the Venn plots comparing the TCGA and GSE84402 datasets. **(G)** COX proportional hazards model constructed using shared DEGs from the TCGA and GSE84402 datasets. **(H)** Relationship between CTSV expression level and overall survival (OS) of HCC patients. **(I)** Relationship between CTSV expression levels and disease specific survival (DSS) in HCC patients.

CTSV Expression Correlates with Both the Clinicopathological Attributes and the Prognostic Outcomes for HCC Patients

To gain a deeper insight into the role of CTSV in HCC, we analyzed RNA sequencing data from the TCGA-LIHC and GSE84402 databases to assess the expression levels of CTSV. The findings revealed that HCC tissues exhibited a markedly elevated expression of CTSV when compared to their normal tissue counterparts (Figure 3A and B). In addition, an analysis of 50 sets of matched samples provided confirmation that the expression of CTSV was elevated in the cancerous tissues as compared to their adjacent normal tissue (Figure 3C). By using multiple hypothesis tests (Dunn's test) to analysis the expression of CTSV in various clinical pathological characteristics, it was found that the levels of CTSV expression within HCC tissues are notably associated with patient age and gender (Figure 3D and E), and observed the expression of CTSV in cancer tissues at different stages (Figure 3F and G). To further verify this correlation, immunohistochemical (IHC) staining was performed on CTSV in the tumor and adjacent tissues of HCC patients (Figure 3H and I). The results indicated that elevated levels of CTSV were observed in 38.9% (n=70) of HCC tissues, while only 11.1% (n=20) were observed in adjacent tissues. Kaplan-Meier analysis based on IHC staining results showed that elevated levels of CTSV expression were found to correlate with a reduced OS rate in patients (Figure 3J). Moreover, Cox proportional hazard regression analysis was conducted on clinical parameters of HCC patients (Figure 3K). The results demonstrated robust correlations between CTSV expression levels and critical markers of HCC progression, such as vascular invasion, TNM stage, BCLC stage, microvascular invasion (MVI), liver cirrhosis, tumor encapsulation, and tumor size. However, no significant associations were observed between CTSV expression and factors such as hepatitis B surface antigen (HBsAg) status, alpha-fetoprotein (AFP) levels, or patient age. Thus, it was deduced that the elevated levels of CTSV expression correlate with the aggressive progression of HCC.

Functional Enrichment Analysis of CTSV in HCC

GO and KEGG analyses for functional enrichment denoted that genes associated with CTSV were predominantly involved in processes of immune recognition and the proliferation of immune cells (Figure 4A and B). GSEA enrichment analysis indicated that CTSV-related genes were mainly enriched in immune response regulation, immune response activation, and cell cycle pathways, with the majority of the enriched genes being down-regulated (Figure 4C–E). Additionally, these genes were enriched in pathways targeting E2F, with the majority of the enriched genes being up-regulated (Figure 4F).

The Levels of CTSV Expression in HCC are Linked to the Infiltration of Immune Cells

CIBERSORT, ssGSEA enrichment analysis, and TIMER2.0 were utilized to clarify the role of CTSV within the tumor microenvironment (TME) and to observe its correlation with immune cell infiltration status. The analysis by CIBERSORT revealed the relative abundance of different immune cell subsets in the tumor microenvironment and the ssGSEA findings demonstrated a significant correlation between CTSV expression in HCC and the infiltration of nine distinct immune cell types (Figure 5A and B). Specifically, the expression levels of CTSV in HCC showed a significant positive correlation with CD4⁺ T cells and Th2 cells. Conversely, a significant negative correlation was observed with CD8⁺ T cells, CD56bright natural killer (NK) cells, memory CD8⁺ T cells, eosinophils, neutrophils, Th1 cells, and Th17 cells. Utilizing the TIMER2.0 algorithm, we conducted a correlation analysis of immune cell infiltration in HCC. The results indicated a significant correlation between the expression of CTSV and the infiltration of four distinct immune cell types, namely CD4⁺ T cells, CD4⁺ Th2 cells, CD8⁺ T cells, and NK cells (Figure 5C). In alignment with the findings from ssGSEA analysis, TIMER2.0 analysis has also shown that the expression of CTSV exhibits the most significant correlation with CD4⁺ T cells and CD4⁺ Th2 cells, as evidenced by the smallest p-values obtained. These results implied that the level of CTSV expression might have influenced the state of the TME.

CTSV Possesses Certain Clinical Application Values

To further verify the credibility of the bioinformatics analysis results, we initially conducted in vitro and in vivo functional assays. We infected PLC/PRF/5 and MHCC97H cells with lentivirus carrying CTSV-specific short hairpin

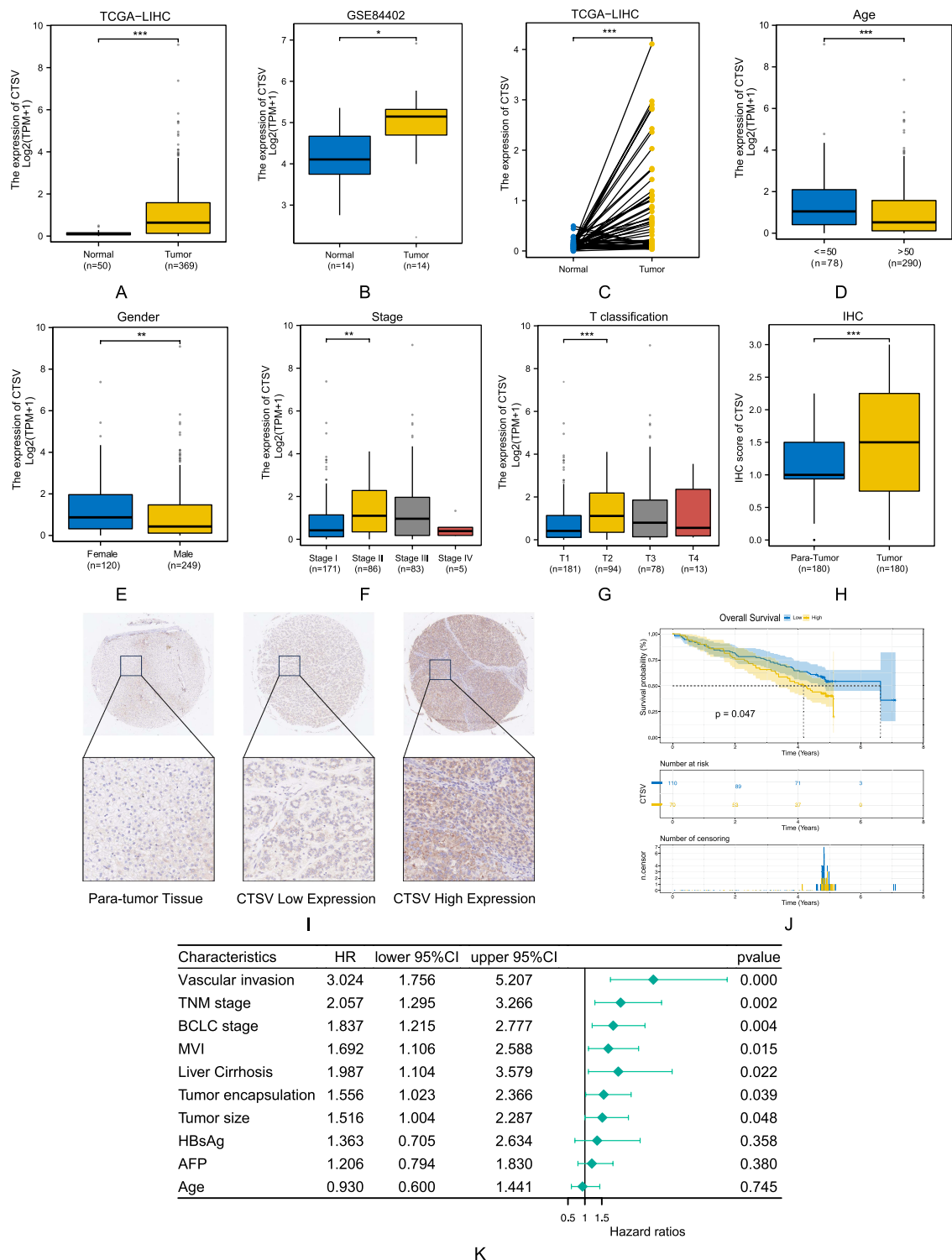


Figure 3 Overexpression of CTSV is associated with poor prognosis in HCC patients. The transcriptome sequencing data and clinical features of HCC were obtained from the TCGA database (<http://www.cbiportal.org>) and the NCBI database (<https://www.ncbi.nlm.nih.gov>) to analysis the CTSV mRNA expression levels. The protein levels of CTSV in 180 clinical samples were further analyzed using immunohistochemical staining. **(A)** In the TCGA-LIHC dataset, the CTSV mRNA expression level in tumor tissue was significantly higher than in normal tissue. **(B)** In the GSE84402 dataset, the CTSV mRNA expression level in tumor tissue was significantly higher than in normal tissue. **(C)** In 50 tumor-normal paired cases, CTSV mRNA levels were higher in most tumor tissues compared to adjacent normal tissues. **(D and E)** The differential expression levels of CTSV were analyzed in relation to age and gender in HCC. **(F)** The CTSV mRNA levels were evaluated at different stages of HCC progression. **(G)** CTSV expression was confirmed in tumor tissues at different stages according to TNM staging criteria, compared with normal tissues. **(H&I)** Immunohistochemical staining **(I)** and quantitative analysis **(H)** were used to detect the protein level of CTSV in HCC. **(J)** Kaplan-Meier analysis of OS in patients with HCC. **(K)** Multivariate Cox regression analysis was conducted on clinical parameters. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

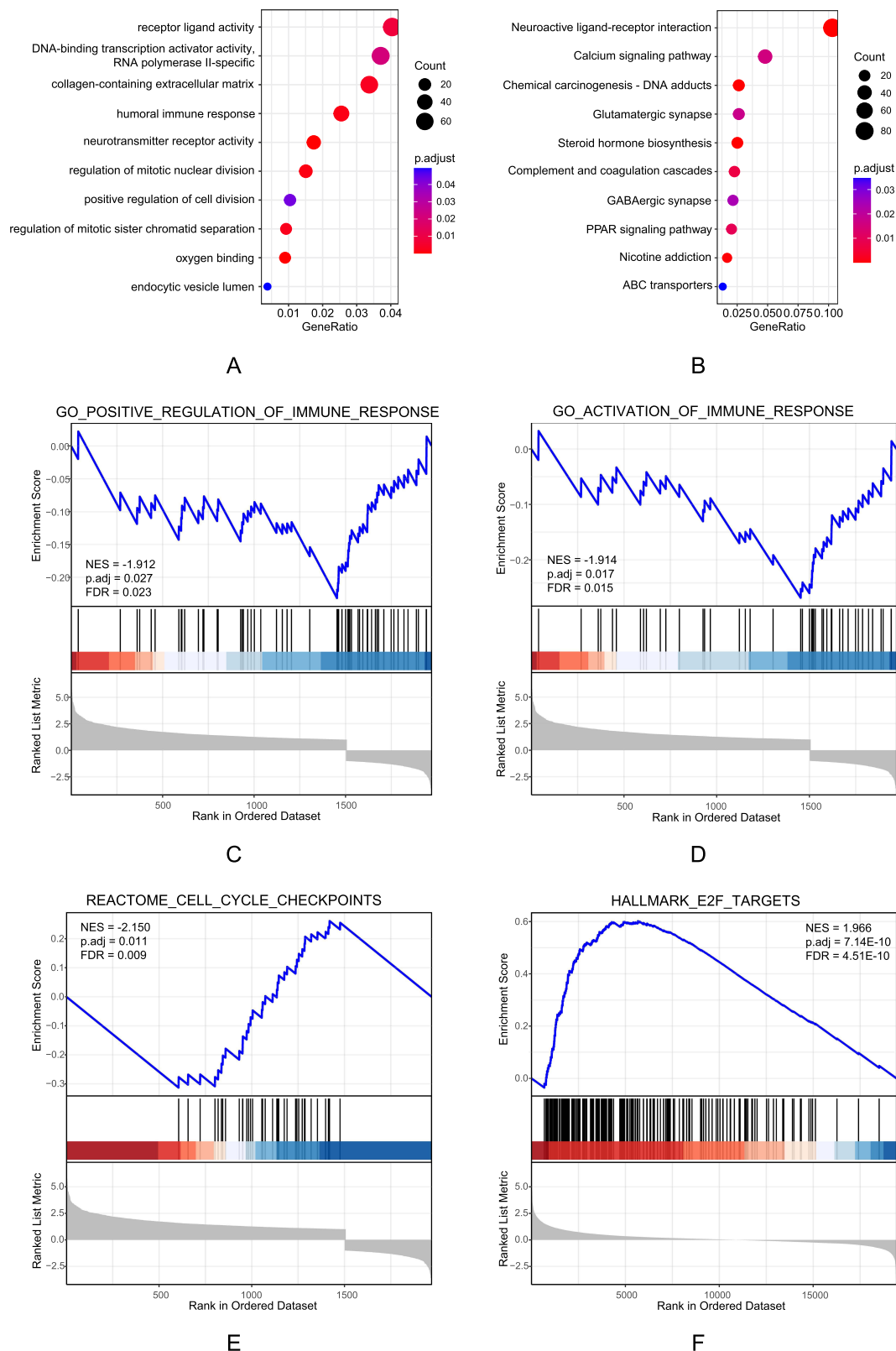


Figure 4 GO, KEGG, and GSEA enrichment analyses of CTSV co-expressed genes. **(A)** The GO enrichment analysis was conducted for CTSV-related genes. **(B)** The KEGG enrichment analysis was conducted for CTSV-related genes. **(C)** Enriched in the immune response regulatory pathway, with the majority of the enriched genes having been down-regulated. **(D)** Enriched in the immune response activation pathway, with the majority of the enriched genes having been down-regulated. **(E)** Enriched in the cell cycle pathway, with the majority of the enriched genes having been down-regulated. **(F)** Enriched in the targeted E2F pathway, with the majority of the enriched genes having been up-regulated.

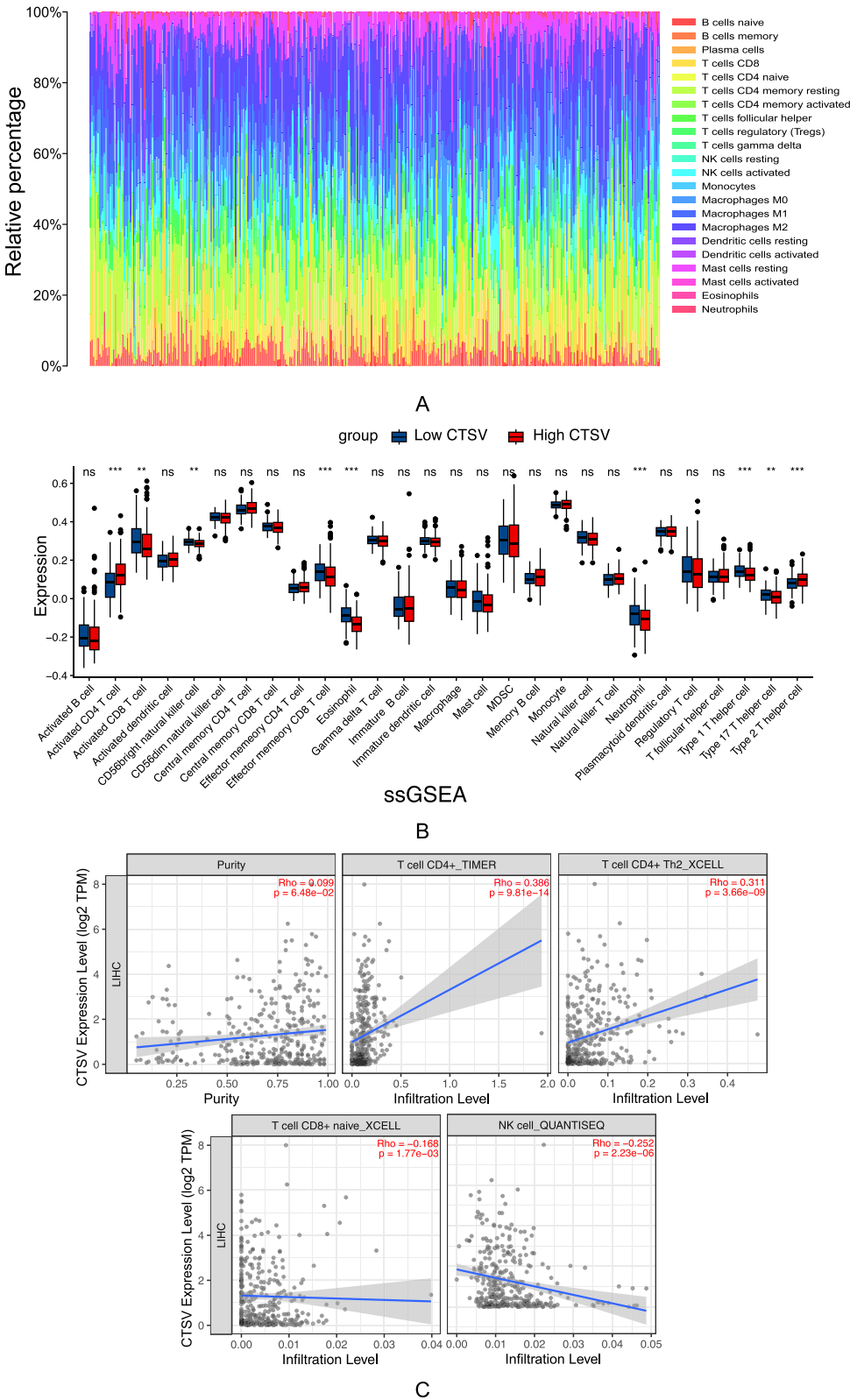


Figure 5 CTSV expression in HCC correlates with immune cell infiltrations. **(A and B)** CIBERSORT and ssGSEA enrichment analyses were conducted to assess the proportion of infiltrating immune cells in TCGA-LIHC samples. **(A)** The CIBERSORT analysis heatmap showed the relative abundance of different immune cell types in the samples. **(B)** The ssGSEA enrichment analysis indicated the correlation between CTSV and 28 levels of immune cell infiltration. **(C)** The TIMER2.0 database was used to analyze the correlation between CTSV expression levels and immune cell infiltration levels.

RNA to facilitate loss-of-function studies. qRT-PCR and Western blot confirmed that both mRNA levels and protein expression of CTSV were significantly reduced in both PLC/PRF/5 and MHCC97H cells (Figure 6A). Subsequently, the impact of CTSV on cell growth was further investigated through CCK-8 and clonogenic assays. The study results showed that cell proliferation was significantly decreased in CTSV knockdown cells compared to control cells (Figure 6B), and they produced fewer and smaller colonies (Figure 6C). Then, Transwell assays were used to determine whether CTSV knockdown would affect the migration and invasion capabilities of HCC cells. The findings indicated that CTSV knockdown had reduced the migration ability of HCC cells, and invasiveness was also significantly decreased (Figure 6D), suggesting that CTSV knockdown had a significant inhibitory effect on HCC cell metastasis. Finally, animal experiments were conducted to validate the relationship between CTSV and anti-tumor immunity. We established a subcutaneous HCC mouse model derived from MHCC97H and treated it with PD-1 inhibitor combination therapy. During the treatment process, shCTSV, both shNC and shCTSV combined with PD-1 inhibitors significantly limited the growth of subcutaneous tumors (Figure 6E). Moreover, the treatment group that received shCTSV combined with PD-1 inhibitors had the smallest tumors at the end of the experiment (Figure 6F). In conclusion, inhibiting CTSV expression could effectively suppress the growth of HCC cells and CTSV played a key role in inhibiting HCC cell metastasis. In addition, it was found that CTSV truly can affect the immune microenvironment, and the combination of shCTSV and PD-1 inhibitors might enhance the therapeutic effect of PD-1 inhibitors in HCC.

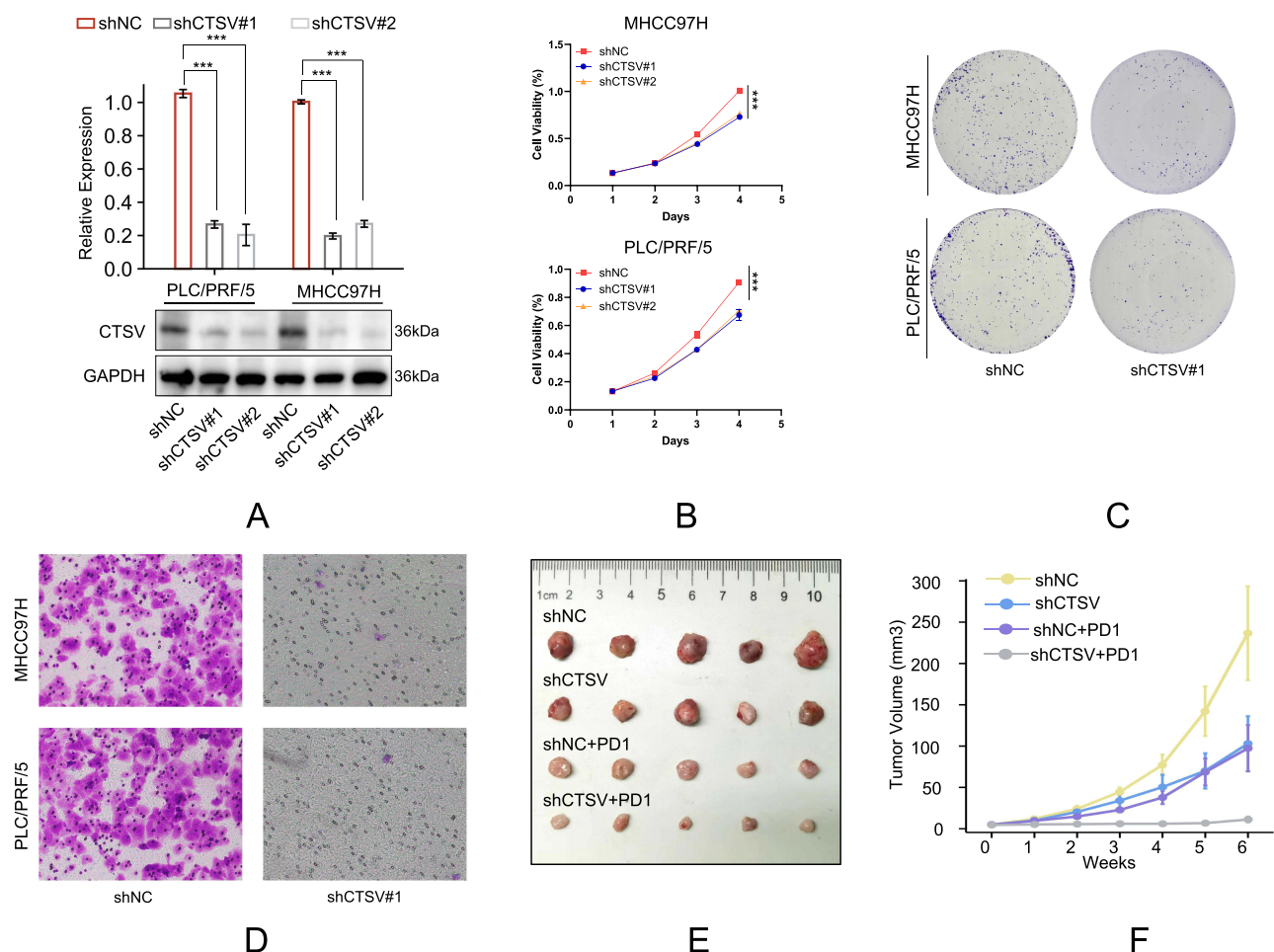


Figure 6 Further experiments have demonstrated that CTSV has certain clinical application value. PLC/PRF/5 and MHCC97H cells were infected with lentivirus encoding shNC, shCTSV#1, or shCTSV#2, and CTSV knockdown was confirmed for subsequent functional assays. (A) Confirmed the relative expression of CTSV knockdown using qRT-PCR and Western blotting. (B) Measured the proliferation efficiency of CTSV knockdown cells using the CCK-8 method. (C) Assess cell proliferation capacity by forming colonies through clonogenic assay. (D) Evaluate cell migration ability and invasiveness using the Transwell assay. (E and F) Subcutaneously injected MHCC97H shCTSV or shNC cells into 6-week-old male C57BL/6J mice for in vivo tumorigenesis assays, which displayed the appearance (E) and volume changes (F) of subcutaneous tumors in each group during the treatment process.

Discussion

HCC represents the predominant histological subtype of malignant liver tumors, constituting approximately 80–90% of all diagnosed cases of primary liver cancer.¹⁹ The latest statistical data indicates that patients diagnosed with early-stage liver cancer have a 5-year survival rate exceeds 50%, whereas those with advanced liver cancer have a rate below 20%. The early diagnosis rate of liver cancer is only 20–40%, with approximately 50% of patients receiving an initial diagnosis when the disease is already advanced, thereby missing the opportunity for surgical intervention.²⁰

Currently, the predominant therapeutic approaches for treating HCC encompass surgical excision, orthotopic liver transplantation, thermal ablation therapies, and various interventional radiology techniques. For patients diagnosed with early-stage liver cancer, surgical excision is often the treatment of choice. Additionally, the US Food and Drug Administration (FDA) has approved radiofrequency ablation (RFA) and transarterial chemoembolization (TACE) as standard treatments for patients with advanced HCC.²¹ With the ongoing advancement of research, molecular targeted therapies combined with immunotherapy are increasingly being utilized in the clinical oncology treatment of patients with advanced hepatic cancer,²² such as the combination of lenvatinib and PD-1 inhibitors. However, less than one-third of patients benefit from the treatment, and some may develop significant drug resistance within six months after initiating therapy.²³

CTSV is a cysteine protease peptidase with 78% homology to cathepsin L (CTSL), also known as cathepsin L2 (CTSL2). Its coding gene contains 8813 base pairs, including 8 exons and 7 introns, and is located on chromosome 9q22.2.²⁴ CTSV becomes active under acidic conditions, demonstrates moderate substrate specificity, and is susceptible to inhibition by a range of cysteine protease inhibitors.²⁵ Recently, Liu et al conducted an analysis to screen for cell cycle-related genes (CCRGs) in HCC samples by calculating the ssGSEA scores derived from cell cycle signatures, and then identified CTSV as a potential prognostic marker for multiple cancers using LASSO regression.²⁶

Different from Liu J's research, we conducted an analysis to determine the correlation between CTSV mRNA levels and HCC by extracting data from the TCGA and GEO databases, as well as by performing IHC staining of CTSV in the cancerous and neighboring tissues extracted from HCC patients. The findings revealed that levels of CTSV expression within HCC tissues were markedly elevated in comparison to those in the non-cancerous tissues. High CTSV expression was significantly correlated with vascular invasion, TNM stage, BCLC stage, MVI, liver cirrhosis, tumor encapsulation, and tumor size. Results from the Kaplan-Meier method demonstrated a significant correlation between elevated CTSV expression and diminished OS and DSS in patients. The results implied that CTSV plays a role in the advancement and spread of HCC and could potentially be utilized as a biomarker to assess the prognosis of HCC.

Subsequent analysis was performed to explore the possible roles of CTSV in HCC by examining the functional enrichment of genes that are co-expressed with CTSV. The results indicated that in both GO and KEGG enrichment analyses, CTSV-related genes were significantly enriched in immune recognition and immune cell proliferation. In GSEA enrichment analysis, CTSV-related genes were significantly enriched not only in gene sets associated with immune response regulation, immune response activation, and cell cycle, but also in targeted gene sets such as E2F. The former was predominantly enriched in the down-regulated portion of the gene sets, while the latter was predominantly enriched in the up-regulated portion.

The advent of immunotherapy offers a promising new avenue for cancer treatment, yet its impact on HCC has been restricted, with only a subset of patients responding favorably to Immune Checkpoint Inhibitors.²⁷ The TME is paramount in nurturing the growth of the tumor and in aiding the spread of cancer to other parts of the body.²⁸ An increasing number of research indicates that both the innate immune system, encompassing macrophages, dendritic cells, and NK cells, and the adaptive immune system, including T and B lymphocytes, significantly contribute to the development and progression of tumors within the TME.²⁹

In this study, we employed ssGSEA enrichment analysis and the TIMER2.0 algorithm to explore the correlation between CTSV expression and the infiltration of immune cells within the TME. The objective of this analysis was to clarify the importance of CTSV within the TME and to delve deeper into its potential functions in the local immune response specific to HCC. Our findings indicated a significant positive association between the expression levels of CTSV and the presence of CD4+ T cells and Th2 cells. Conversely, a significant negative correlation was observed between CTSV expression and the levels of CD8+ T cells and NK cells. Previous research demonstrated that CD8+ T cells recognized molecules such as PD-L1, PD-L2, CD80, and CD86 on the outer layer of cancerous cells. They provoked programmed cell death in tumor cells by releasing

substances like granzymes, perforin, and granzyme B, which led to the fusion with tumor cell membranes.³⁰ NK cells released cytotoxic granules such as perforin and granzymes, as well as employed antibody-dependent cellular cytotoxicity (ADCC) mechanisms to kill target cells. Moreover, they secreted cytokines and chemokines that promoted adaptive immune responses in T and B cells, thereby enhancing the killing efficiency against tumor cells.³¹ The aforementioned findings implied that CTSV might have significantly modulated the infiltration of immune cells within the TME of HCC, thereby facilitating the local immune evasion process of HCC.

Finally, *in vitro* and *in vivo* experiments were conducted using liver cancer cell lines to further validate the clinical application value of CTSV. The *in vitro* and *in vivo* experiments had shown that lentiviral-specific targeting of shRNA to induce knockdown of the CTSV could significantly inhibit the proliferation, migration, and invasion of liver cancer cells. That is to say, suppressing the expression of CTSV could effectively inhibit the growth of HCC cells, and CTSV played a key role in inhibiting the metastasis of HCC cells. In addition, it was found that the combination of CTSV knockdown with PD-1 inhibitors might enhance the therapeutic effect of PD-1 inhibitors in HCC.

Conclusion

CTSV appears to be a potential biomarker of HCC that is associated with poor clinical and pathologic features and may contribute to poor patient prognosis. CTSV knockdown can effectively inhibit the growth of HCC cells, and CTSV played a key role in inhibiting the metastasis of HCC cells. It may stimulate the cell cycle of tumor cells while suppressing local anti-tumor immune responses, thereby promoting the growth and progression of HCC.

Data Sharing Statement

All data relevant to the study are included in the article or uploaded as supplementary information. Data are available from the GDC database (<https://portal.gdc.cancer.gov/>) and series GSE84402 in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). Other data analyzed in this study are included in the article and supplementary materials.

Ethics Approval and Informed Consent

The study was reviewed and approved by the Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China) (ID: B2021-143R). Participants gave informed consent to participate in the study before taking part. The guidelines outlined in the Declaration of Helsinki were followed.

Consent for Publication

All contributing authors have reviewed and consented to the submission of the manuscript, confirming the absence of any conflicts of interest related to the content of the paper. Upon acceptance, we commit to adhering to the publication's licensing terms. Collectively, we have reached a consensus on the target journal for submission, have granted approval for the final version proposed for publication, and accept responsibility for the entirety of the research work presented.

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Disclosure

The authors confirm that there are no competing interests associated with this research.

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