

# Six-Transmembrane Epithelial Antigen of Prostate 4: An Indicator of Prognosis and Tumor Immunity in Hepatocellular Carcinoma

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**Purpose:** The six-transmembrane epithelial antigen of prostate 4 (STEAP4) has been linked to tumor progression via its involvement in inflammatory responses, oxidative stress, and metabolism. However, STEAP4 has rarely been studied in hepatocellular carcinoma (HCC). We explored STEAP4 expression associated with tumor prognosis to understand its role in tumor biology in HCC.

**Patients and Methods:** STEAP4 mRNA and protein expressions were primarily analyzed using bioinformatics tools based on The Cancer Genome Atlas database to understand the expression pattern, molecular mechanism, prognostic impact, and association with immune cell infiltration. We further investigated the association between STEAP4 protein expression and clinicopathological parameters and their predictive value in HCC patients using immunohistochemical staining of tissue microarrays.

**Results:** The expression of STEAP4 mRNA and protein in HCC tissues was significantly lower than in normal liver tissues. Reduced expression of STEAP4 was linked to advanced HCC stages, poor recurrence-free survival (RFS), and overall survival. Furthermore, reduced STEAP4 expression was a significant predictor of worse RFS in univariate and multivariate analyses in the immunohistochemical cohort. GO, KEGG, and GSEA analyses revealed that STEAP4 is related to numerous biological processes and pathways, including drug metabolism, DNA replication, RNA metabolism, and immune response. In terms of the immune system, the decreased level of STEAP4 was correlated with the immunosuppressive microenvironment.

**Conclusion:** Our data indicated that reduced STEAP4 expression was significantly associated with tumor aggressiveness and poor prognosis, possibly because of its link to various biological processes and induction of HCC immune evasion. Therefore, STEAP4 expression may serve as a potential prognostic biomarker for cancer progression and immunity, as well as a therapeutic target in HCC.

**Keywords:** immunohistochemistry, bioinformatics, Hepatocellular carcinoma, prognostic factor

## Introduction

Hepatocellular carcinoma (HCC) is the most prevalent primary liver carcinoma, accounting for > 80% of all cancers and the third leading cause of cancer-related deaths globally.<sup>1,2</sup> According to global cancer statistics in 2020, there have been 905,677 new cases of liver and intrahepatic bile duct cancers and 830,180 liver cancer-related deaths worldwide. Patients with chronic liver disease, such as hepatitis B (HBV) or C virus (HCV), are primarily affected.<sup>3</sup> Currently, the decreasing prevalence of HBV and HCV and the rising prevalence of metabolic conditions, such as obesity, diabetes, and nonalcoholic fatty liver disease, appear to have altered the primary risk factors for HCC in many countries.<sup>4</sup> Notably, the fundamental essence of the onset and progression of HCC, which requires a multistep process of genetic alterations in hepatocytes through persistent inflammatory stimulation, remains unchanged.<sup>5</sup>

As tumor immunology research has advanced, a relationship between chronic inflammation and carcinogenesis has been confirmed.<sup>6</sup> During chronic liver injury induced by various pathogens and toxins, the liver experiences

immunological dysfunction caused by excessive activation of pro-inflammatory cytokines, leading to hepatocarcinogenesis.<sup>7,8</sup> The complex relationships between immune cell subsets and effector molecules in the micro-environment are responsible for establishing an immunosuppressive or immune-active milieu that suppresses or promotes HCC progression, respectively. The six-transmembrane epithelial antigen of prostate 4 (STEAP4), also known as the six-transmembrane protein of prostate 2 or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced protein 9, is a member of the STEAP family and has multiple biological functions, including cell proliferation and apoptosis, oxidative stress, and molecular signaling pathways.<sup>9</sup> When compared to the other STEAP proteins, STEAP4 has the most extensive enzymatic activity and is involved in these processes by forming homo- or heterodimers. In addition, STEAP4 is involved in inflammatory regulation by modulating iron and copper homeostasis.<sup>10</sup> STEAP4 expression has been correlated with inflammatory signaling and the proliferation and progression of prostate cancer, highlighting its critical role in carcinogenesis.<sup>11,12</sup> Colitis-associated colon cancer has also been shown to facilitate inflammatory tissue injury resulting from mitochondrial iron dysfunction via STEAP4.<sup>13</sup> Furthermore, STEAP4 has been implicated in the mitochondrial iron pathway and has been pharmacologically targeted in human epidermal growth factor receptor 2 (HER2)+ breast cancer.<sup>14</sup> The role of STEAP4 in HCC is yet to be determined.

Unfortunately, most HCC cases are diagnosed at unresectable advanced stages, and the prognosis for these patients is poor.<sup>15</sup> Sorafenib and regorafenib were the only systemic drugs for advanced HCC prior to the worldwide approval of atezolizumab plus bevacizumab.<sup>16,17</sup> Currently, atezolizumab and bevacizumab are the first-line treatments for advanced HCC.<sup>18</sup> Despite the efficacy of immune checkpoint inhibitors (ICIs), a large cohort of patients remain unresponsive to ICI.<sup>18</sup> Identifying predictive biomarkers is one of the main challenges for proper patient selection. Thus, this study aimed to identify STEAP4-related biological characteristics and their interactions with the tumor microenvironment that influence HCC prognosis to evaluate its utility as a biomarker.

## Materials and Methods

### Analysis of the Expression of STEAP4 mRNA and Protein Using Bioinformatics Tools

Based on The Cancer Genome Atlas (TCGA) initiatives, the Tumor Immune Estimation Resource (TIMER) database was used to evaluate the differential expression of STEAP4 mRNA across various cancers and associated normal tissues.<sup>19</sup> The University of Alabama at Birmingham Cancer data analysis Portal (UALCAN) platform was used to compare mRNA expression and protein levels of STEAP4 between TCGA and The Genotype-Tissue Expression databases and to explore their relationship with clinicopathological features of HCC.<sup>20</sup> To determine the genetic alterations and their relationship with mRNA expression, we interactively analyzed multidimensional genomics data for cancer using the cBio Cancer Genomics Portal platform, which included data from over 5000 tumor samples from 20 studies.<sup>21</sup> We next analyzed the relationship between STEAP4 mRNA and survival rates, such as recurrence-free survival (RFS) and overall survival (OS), in patients with HCC using the Kaplan–Meier plotter.<sup>22</sup> Moreover, we explored the correlated diverse biological functions involving STEAP4 based on enrichment analysis for biological process (BP) and molecular function (MF) of gene ontology (GO) using the ClueGO plug-in in the Cytoscape software. Pathway enrichment analysis was carried out according to the Kyoto Encyclopedia of Genes and Genomes (KEGG).<sup>23</sup> In HCC, we explored the type of genetic mutations that affected STEAP4 expression. To illustrate the immune microenvironment of HCC with STEAP4, we used the TIMER 2.0 database,<sup>19</sup> Gene Expression Profiling Interactive Analysis (GEPIA) database,<sup>24,25</sup> and Tumor Immune System Interaction Database (TISIDB),<sup>26</sup> which provide the immune infiltration levels of multiple tumors in TCGA. Immune infiltration was estimated using CIBERSORT and Estimate methods.

### Patients and Sample Collection

The study population comprised 173 HCC patients treated at the Dong-A University Hospital (Busan, Korea) between 2010 and 2013. None of the patients received pre-operative neoadjuvant therapy. Surgical pathology reports and clinical information, including sex, age, and stage, were reviewed. HCC stage was categorized based on the eighth edition of the Cancer Staging Manual of the American Joint Committee on Cancer. The clinicopathological characteristics of the HCC patients are shown in Table 1. RFS was defined as the time interval between the first surgery and the last follow-up visit when the patient did not show any detectable recurrence. The clinical study protocol was approved by the Institutional

**Table I** Clinicopathological Characteristics According STEAP4 Expression in Hepatocellular Carcinoma immunohistochemical Cohort

Characteristics	Retained Expression (n, %)	Reduced Expression (n, %)	P value
Age, yr			0.448
< 60	32 (46.4)	60 (52.6)	
≥ 60	37 (53.6)	54 (47.4)	
Sex			0.171
Female	60 (87.0)	89 (78.1)	
Male	9 (13.0)	25 (21.9)	
Hepatitis virus			0.646
Absent	10 (14.5)	13 (11.4)	
Present	59 (85.5)	101 (88.6)	
Tumor size, cm			0.007
< 2	35 (50.7)	34 (29.8)	
≥ 2	34 (49.3)	80 (70.2)	
Gross type			0.029
Type 1	37 (53.6)	39 (34.2)	
Type 2	32 (46.4)	75 (65.8)	
Focality			0.025
Absent	63 (91.3)	89 (78.1)	
Present	6 (8.7)	25 (21.9)	
Edmondson-Steiner grade			0.006
I, II	28 (40.6)	25 (21.9)	
III, IV	41 (59.4)	89 (78.1)	
Underlying cirrhosis			0.359
Absent	8 (11.8)	17 (14.9)	
Present	60 (88.2)	97 (85.1)	
Steatosis			0.262
Absent	47 (68.1)	71 (62.3)	
Present	22 (31.9)	43 (37.7)	
Major vessel invasion			0.084
Absent	68 (98.6)	105 (92.9)	
Present	1 (1.4)	8 (7.1)	
Microvascular invasion			<0.001
Absent	61 (88.4)	74 (64.9)	
Present	8 (11.6)	40 (35.1)	

(Continued)

**Table 1** (Continued).

Characteristics	Retained Expression (n, %)	Reduced Expression (n, %)	P value
Necrosis			0.150
Absent	50 (72.5)	70 (61.4)	
Present	19 (27.5)	44 (38.6)	
pT category			0.014
pT1a	28 (40.6)	28 (24.6)	
pT1b	27 (39.1)	37 (32.5)	
pT2	9 (13.0)	31 (27.2)	
pT3	5 (7.2)	18 (15.8)	

Review Board of Dong-A University Hospital, Busan, Republic of Korea (DAUHIRB-16-023). The requirement for informed consent from patients was waived because of the use of archived formalin-fixed and paraffin-embedded tumor tissues. The study complies with the Declaration of Helsinki.

## Tissue Microarray Construction and Immunohistochemistry Protocol

Tissue microarray (TMAs) were used for immunohistochemical staining (IHC). We reviewed the hematoxylin and eosin-stained slides and selected the most representative areas of the tumors. Three cores of 3 mm diameter from different representative areas of the carcinoma and one from an adjacent non-neoplastic area were reconstructed into a tissue array and arranged into new recipient blocks using a tissue arrayer (Unitma Co., Ltd., Seoul, Korea). IHC staining for STEAP4 was performed using a BenchMark XT automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) and Ultraview Universal DAB Detection Kit (Ventana Medical Systems Inc.). After deparaffinization, rehydration, and antigen retrieval, diluted primary rabbit polyclonal anti-human STAMP2 antibody (Rosemont, IL, USA, 1:20) was added and incubated.

## Scoring System of Immunohistochemical Staining

TMA tissue sections were imaged using a virtual slide scanner (Pannoramic MIDI, 3d Histech, Hungary) at the Neuroscience Translational Research Solution Center (Busan, South Korea) and scored by two pathologists who were blinded to the clinicopathological features. Expression patterns and staining proportions were explored to evaluate STEAP4 expression. The expression pattern of STEAP4 in non-neoplastic hepatocytes was used as a reference point to determine abnormal expression changes in HCC. In molecular biology, protein aggregation is a phenomenon in which intrinsically disordered or misfolded proteins aggregate.<sup>27,28</sup> It has negative effects on cellular functions. Thus, reduced expression was determined when  $\geq 30\%$  of the tumor cells showed clumped cytoplasmic expression or when  $< 30\%$  of the tumor cells retained expression.

## Statistical Analysis

Statistical analyses were performed using the SPSS software (version 22.0; SPSS, Inc.; Chicago, IL, USA). The relationship between STEAP4 expression and clinicopathological characteristics was assessed using the chi-squared test. RFS based on STEAP4 expression was evaluated using the Kaplan–Meier method with the Log-rank test. Univariate survival analysis with individual covariates and multivariate survival analysis was performed using the Cox proportional hazards regression model to assess whether the expression level of STEAP4 was an independent predictor of disease relapse or survival in patients with HCC. In all tests, statistical significance was defined as  $p < 0.05$ .

## Results

### The mRNA and Protein Expression of STEAP4 in HCC

To determine the expression of STEAP4, the online databases TIMER 2.0 and UALCAN were utilized. PanCancer analysis revealed that STEAP4 expression was significantly lower in HCC tissues than in normal liver tissues ( $p < 0.001$ ) (Figure 1A). Analysis of expression patterns between HCC and normal liver tissues in UALCAN also showed that the mRNA expression level of STEAP4 was significantly lower in HCC tissues than in normal tissues ( $p < 0.001$ ) (Figure 1B). We then analyzed the protein expression levels of STEAP4 in patients with HCC using the UALCAN database. The STEAP4 proteomic expression level in HCC tissues was lower than that in normal tissues ( $p < 0.001$ ) (Figure 1C).

Additionally, we used the cBioPortal database, composed of data from four studies encompassing 1225 cases, to explore the genetic variation of STEAP4 (243 cases in INSERM Nat Genet 2015; 231 cases in AMC, Hepatology 2014; 379 cases in TCGA, PanCancer Atlas; 372 cases in TCGA, Firehose Legacy). As shown in Figure 1D, amplification and mutations were detected in STEAP4 in 1225 samples. The frequency of genetic alterations was 1.6%. The type and rate of STEAP4 alterations varied according to data type (Figure 1D). Amplification is a genetic variation with the highest incidence rate in the TCGA database.

To identify the type of alteration that affects STEAP4 expression in HCC, we explored the relationship of STEAP4 expression with copy number variation, somatic mutations, and methylation. STEAP4 expression was not significantly correlated with copy number variation or mutation type (Figure 1E). However, we found that the methylation level of STEAP4 in HCC was significantly negatively correlated with STEAP4 mRNA expression (Pearson's  $r = -0.28$ ,  $p < 0.001$ ) (Figure 1F).

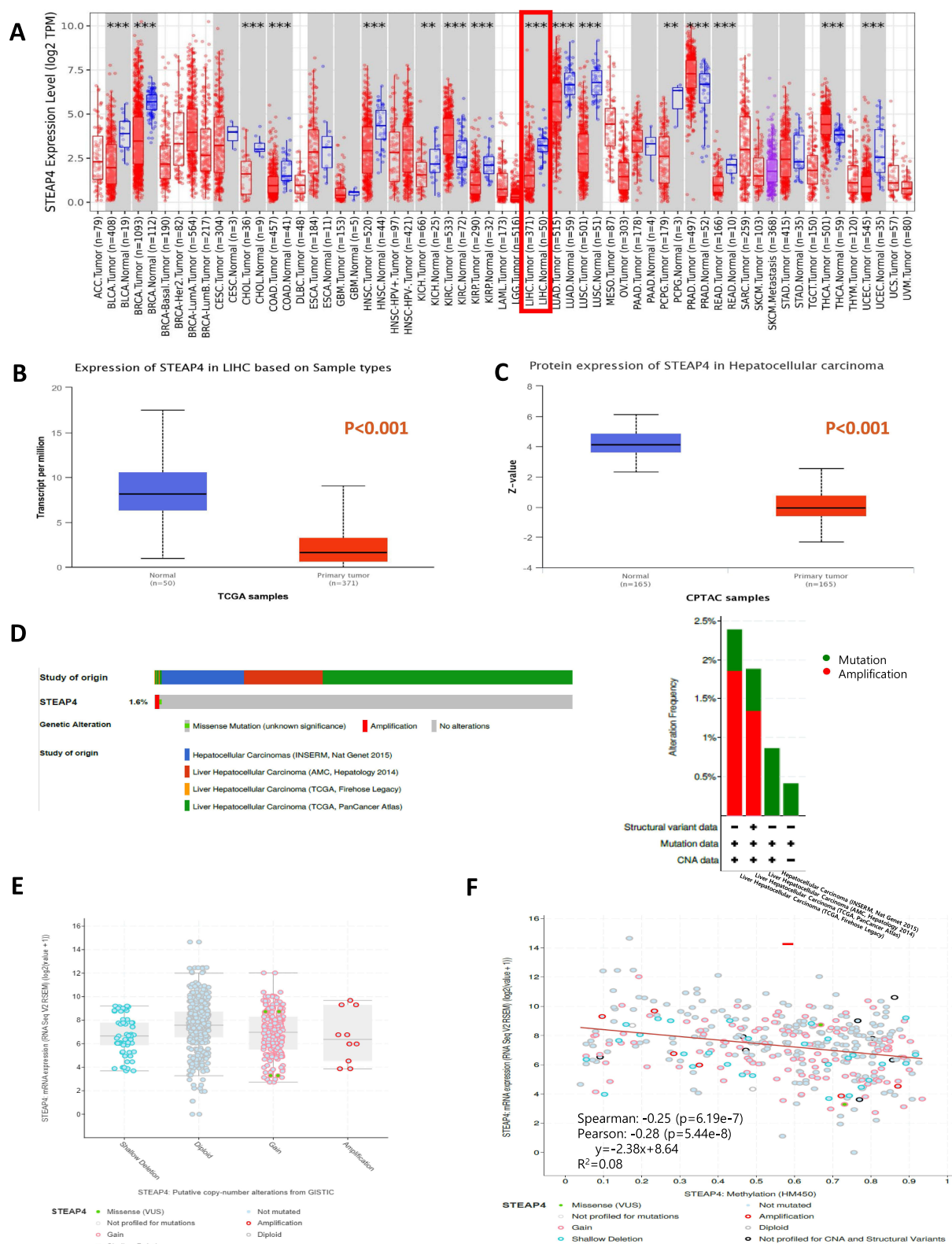
### The Prognostic Significance of STEAP4 Associated with Various Clinicopathologic Characteristics of HCC

We investigated the STEAP4 mRNA expression patterns in a histopathological variant of HCC using the UALCAN platform. STEAP4 expression tended to be lower in conventional HCC than in combined hepatocellular-cholangiocarcinoma cases (Figure 2A). Histological grading of HCC based on a modified Edmondson-Steiner system reflects its biological behavior, which means that higher grades correlate with a worse prognosis. The mRNA expression of STEAP4 was significantly decreased when the grade was higher than that of the normal tissue (Figure 2B). We also explored the correlation between pathologic stage and mRNA expression of STEAP4. STEAP4 mRNA expression level was significantly lower in pathologic tumor stage and clinical stages 3 and 4, compared to stages 1 and 2 ( $p = 0.05$  and  $p = 0.03$ , respectively) (Figure 2C and 2). Notably, the relatively low mRNA expression level of STEAP4 in HCC was closely related to the advanced status of the tumor. We then used the Kaplan–Meier plotter to determine the survival state of STEAP4 in HCC patients (Figure 2E and 2). Our results revealed that lower mRNA expression was remarkably correlated with poor RFS and OS ( $p < 0.001$ ) in patients with HCC.

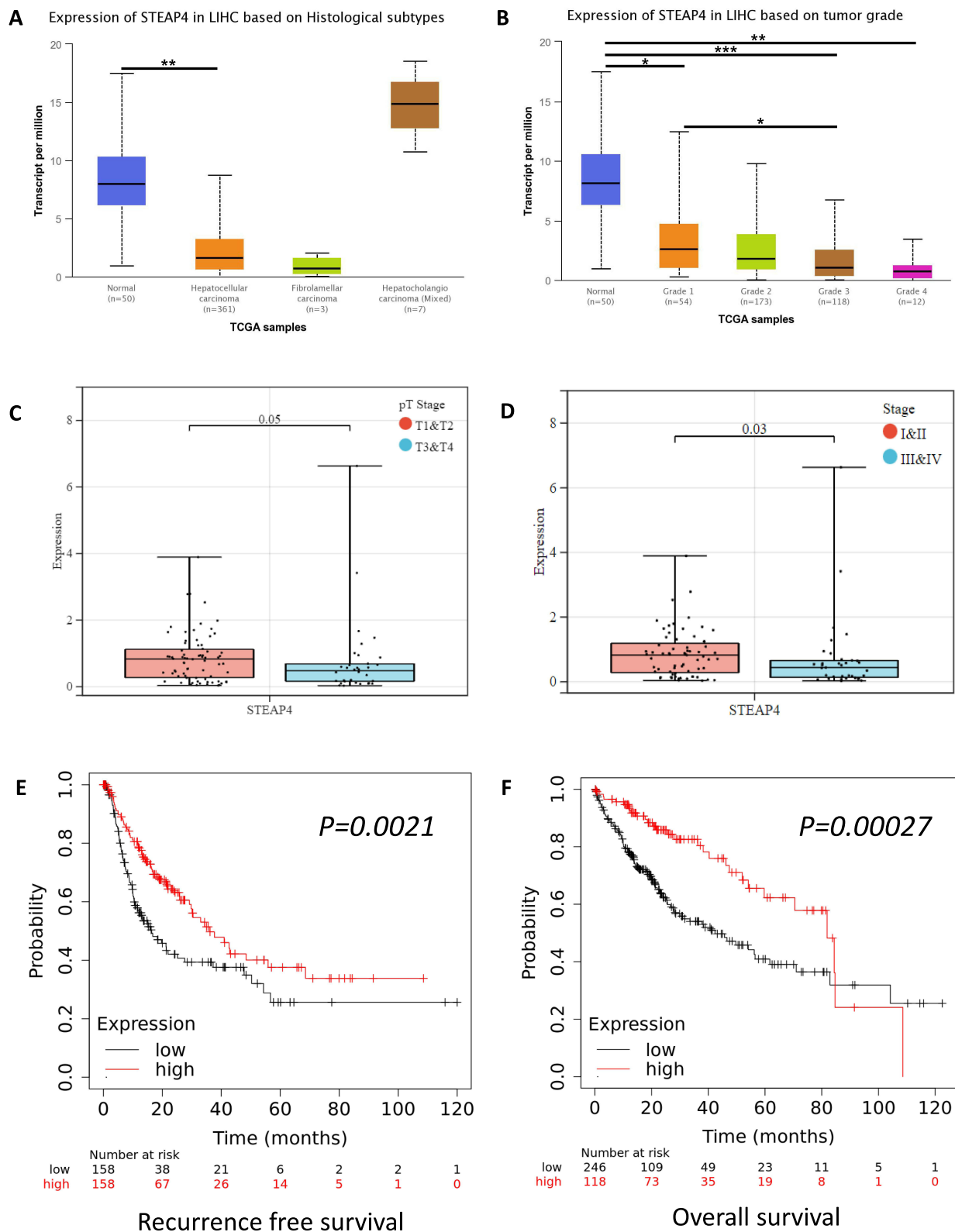
### Clinical Significance of STEAP4 Expression in IHC Analysis of HCC Cohort

We conducted an IHC analysis of the HCC cohort and compared it with adjacent non-neoplastic tissues. Normal tissue showed diffuse and even cytoplasmic expression (Figure 3A). The IHC analysis of the HCC cohort revealed cytoplasmic clumping (Figure 3B and 3). Reduced expression was found in 114 (62.3%) cases (Figure 3C). Normally, protein folding into a functional shape is an essential process in cell biology.<sup>27,28</sup> Failure of protein folding results in abnormal aggregates, leading to loss of function. Thus, the cytoplasmic clumping expression of STEAP4 observed in HCC was interpreted as a reduced expression related to the loss of function. Reduced STEAP4 expression was associated with HCC size  $\geq 2$  cm ( $p = 0.007$ ), gross tumor type 2 ( $p = 0.029$ ), tumor multifocality ( $p = 0.025$ ), high-grade Edmondson-Steiner grade ( $p = 0.006$ ), presence of microvascular invasion ( $p < 0.001$ ), and higher pathologic T stage ( $p = 0.014$ ) (Table 1).

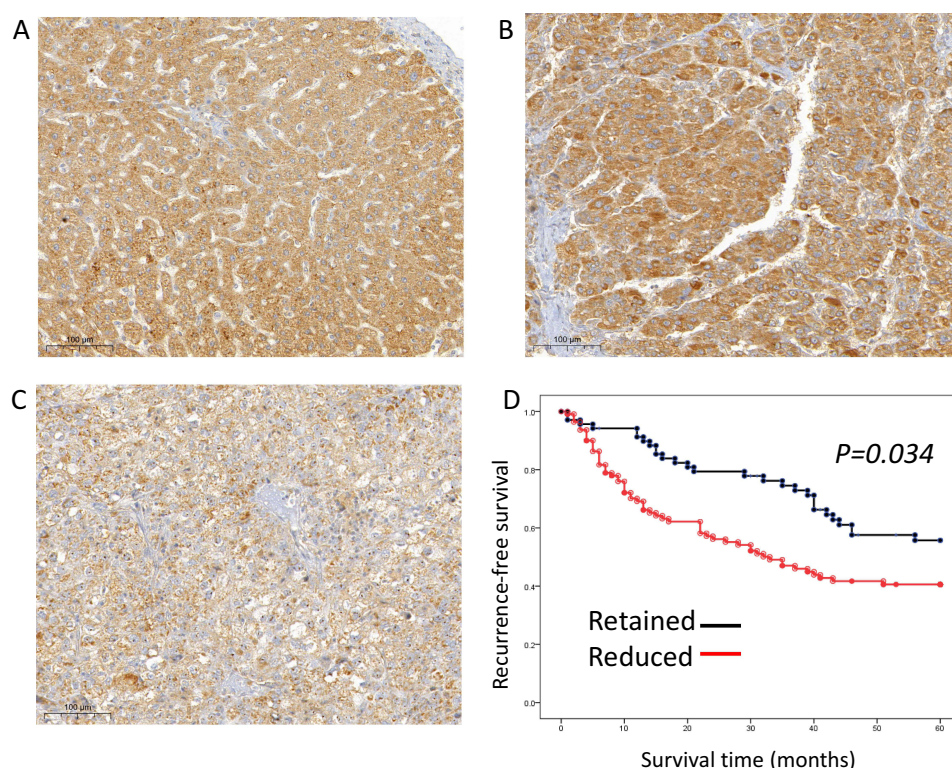
Kaplan–Meier analysis demonstrated that reduced STEAP4 expression was significantly associated with poor RFS ( $p = 0.034$ , Figure 3D). In the multivariate analysis, reduced STEAP4 expression (hazard ratio [HR] = 1.739,  $p = 0.023$ ), higher Edmondson-Steiner grade (HR = 1.975,  $p = 0.003$ ), higher pathologic T stage (HR = 2.273,  $p = 0.007$ ), and



**Figure I** The expression of STEAP4 in patients with hepatocellular carcinoma (HCC) in the TCGA database. **(A)** STEAP4 expression levels in different cancer and normal tissues were analyzed using the TIMER database (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). **(B and C)** Plot chart showing the mRNA and protein expression levels of STEAP4 in HCC compared with normal control using the UALCAN database. **(D)** The OncoPrint of the STEAP4 gene alteration showing various genetic alterations with different colors in HCC samples using cBioPortal database. The bar diagram represents STEAP4 alteration frequencies according to HCC cohorts. **(E)** Box plot showing the relationship between STEAP genetic alteration and expression level. **(F)** Scatter plot showing the relation of STEAP4 expression with methylation level.



**Figure 2** The correlation of STEAP4 mRNA expression with clinical characteristics and prognosis. The expression of STEAP4 in hepatocellular carcinoma was analyzed according to (A) Histological subtype, (B) Tumor grade, (C) Pathologic tumor stage, and (D) Clinical stage (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). The effect of STEAP4 mRNA expression on (E) Recurrence-free survival and (F) Overall survival was analyzed using Kaplan–Meier plotter database.



**Figure 3** STEAP4 protein expression analysis of cases with hepatocellular carcinoma (HCC) in immunohistochemical analysis. **(A)** In the non-neoplastic tissue, STEAP4 expression showed a diffusely even pattern. **(B and C)** In the HCC sample, STEAP4 expression showed the clumping pattern in cytoplasm and membranous reaction; retained expression **(B)** and reduced expression **(C)** ( $\times 400$ ). **(D)** A reduced expression of STEAP4 was significantly associated with shorter RFS ( $p = 0.034$ ).

presence of microvascular invasion ( $HR = 2.379$ ,  $p = 0.023$ ) were independent prognostic factors for shorter RFS in HCC (Table 2). These findings suggested that reduced STEAP4 expression influences HCC progression.

## STEAP4-Related Biological Pathway

To further illustrate the potential role of STEAP4 in the tumor biology of HCC, we divided the TCGA-LIHC samples into low and high groups based on the median STEAP4 mRNA expression and the differential expression profile was conducted to identify the differentially expressed genes (DEG) according to the criteria of  $P$ -value  $< 0.01$  and a fold-change (FC)  $> 1$ . The volcano plot was presented in Figure 4A. The heatmap depicted the top 50 genes that were positively and negatively related to STEAP4 expression in HCC (Figure 4A). We then systematically evaluated the potential biological functions of the DEGs utilizing the GO term and KEGG pathway enrichment analyses. KEGG analysis revealed relationships between different signaling pathways and metabolic processes in cells that control different physiological functions (Figure 4B). According to the GO enrichment analysis, STEAP4 participated in inflammation and cell growth and differentiation, both of which are often associated with cancer progression (Figure 4C). An analysis of GSEA was conducted to determine which biological processes and signaling pathways are enriched for STEAP4. The data showed that STEAP4 with low expression levels was primarily enriched in DNA and RNA replication and repair (Figure 4D). A negative enrichment was found for the genes involved in cytochrome P450 drug metabolism (Figure 4E).

## Relation of STEAP4 Expression with the Somatic Mutation of HCC

Genomic analysis was conducted to identify the molecular characteristics associated with various STEAP4 expression levels. As shown in Figure 5A, the top 20 somatic mutations in HCC are summarized by group. There are several hub genes that influence HCC carcinogenesis, such as TP53, CTNNB1, and AXIN1.<sup>29,30</sup> In both groups, the tumor suppressor

**Table 2** Univariate and Multivariate Analyses of Recurrence-Free Survival in the Hepatocellular Carcinoma

Variable	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age, yr <60 vs ≥60	0.695 (0.440–1.097)	0.118		
Hepatitis virus Absent vs present	1.524 (0.709–3.275)	0.280		
Gross type Type 1 vs 2	0.934 (0.576–1.514)	0.780		
Focality Absent vs present	1.418 (0.716–2.808)	0.316		
Edmondson-Steiner grade I, II vs III, IV	1.886 (1.145–3.107)	0.013	1.975 (1.259–3.099)	0.003
pT stage I vs 2, 3	2.157 (1.139–4.083)	0.018	2.273 (1.250–4.132)	0.007
Microvascular invasion Absent vs present	2.714 (1.356–5.434)	0.005	2.379 (1.301–4.351)	0.005
STEAP4 expression Retained vs reduced	1.708 (1.043–2.798)	0.034	1.739 (1.081–2.797)	0.023

**Abbreviations:** HR, hazard ratio; CI, confidence interval; STEAP4, six-transmembrane epithelial antigen of prostate 4.

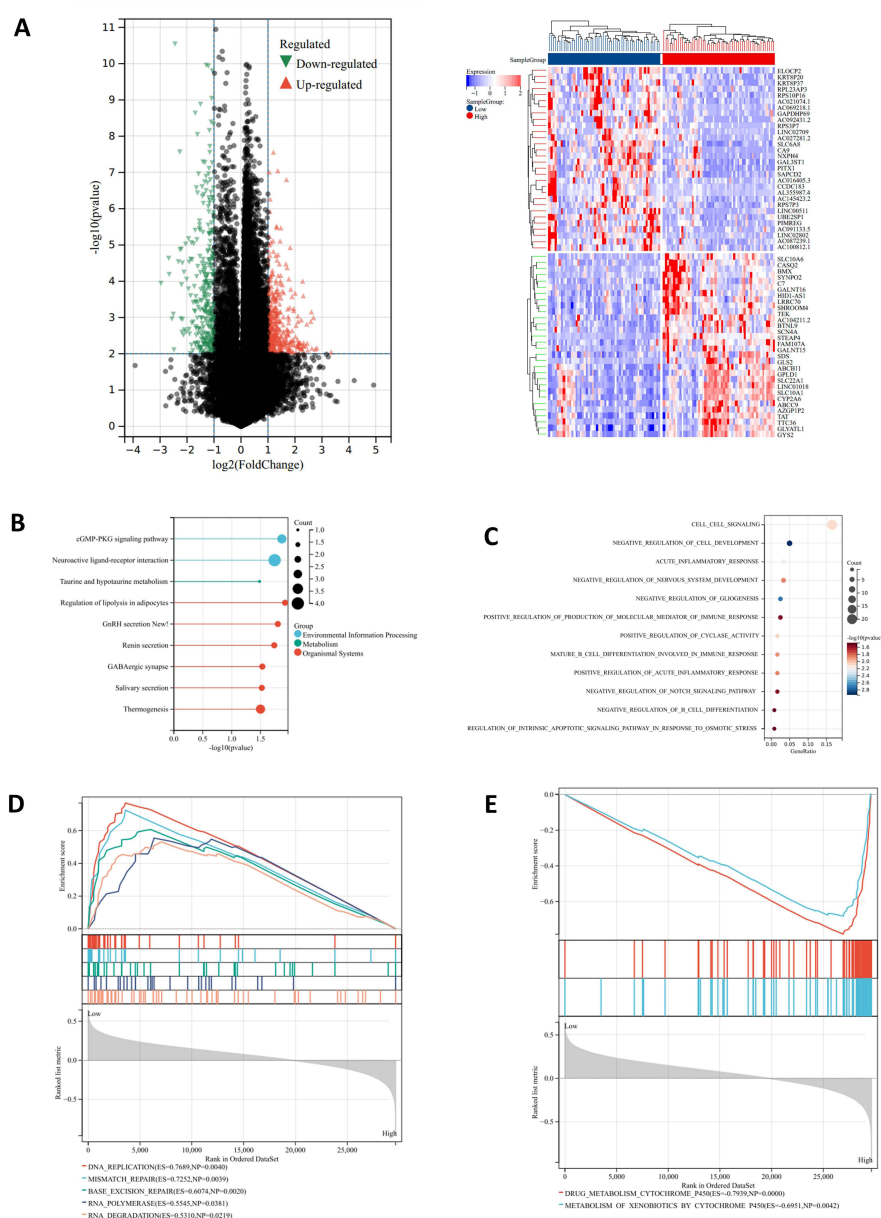
TP53, protein-encoding gene TTN, and proto-oncogene CTNNB1 were significantly mutated. However, TP53 and CTNNB1 were more altered in the low STEAP4 expression group compared to the high STEAP4 expression group. Mutations in TP53 are associated with hepatocarcinogenesis and clinical outcomes.<sup>41,42</sup> CTNNB1 and AXIN1 are also linked to the development of HCC through the Wnt signaling pathway. Thus, mutations related to specific molecules were associated with clinical outcomes.

## Analysis of the Role of STEAP4 in HCC Tumor Microenvironment

The tumor microenvironment (TME) has been implicated in genetic damage responses.<sup>43</sup> The STEAP4 expression in HCC was analyzed to comprehend its relationship with immune cell infiltration. According to the ESTIMATE algorithm, we found a positive correlation between STEAP4 expression and stromal ( $p = 3.2 \times 10^{-6}$ ) and estimate ( $p = 1.4 \times 10^{-3}$ ) scores with an increasing tendency (Figure 5B). The results suggested that STEAP4 expression may be involved in regulating the tumor microenvironment.

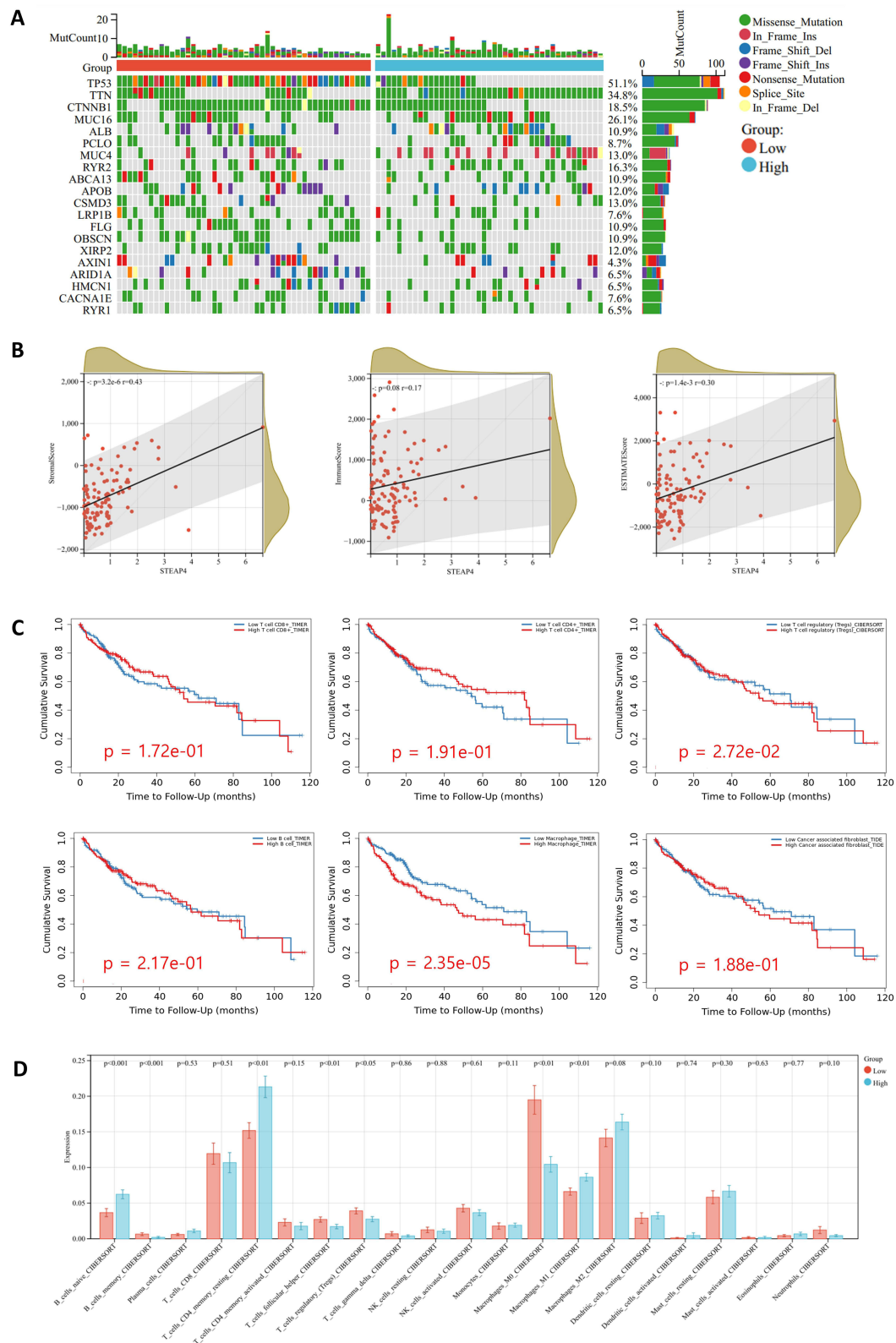
Tumor-infiltrating lymphocyte (TIL) is a reliable indicator of a host antitumor immune response and plays a crucial role in the development and spread of tumors. Thus, we examined how it relates to immune cell infiltration and the prognosis of HCC patients. Based on a detailed analysis of TME-infiltrated immune cells in the TIMER database, Treg ( $p = 2.72 \times 10^{-2}$ ) and macrophage ( $p = 2.35 \times 10^{-5}$ ) were associated with HCC prognosis (Figure 5C). The difference in TIL infiltration between the groups of HCC patients with high and low STEAP4 expression revealed that, of the 21 lymphocyte subsets, 4 TILs (naive B cells, T cells CD4 memory resting, macrophages M1, and resting mast cells) were elevated in the high STEAP4 expression cohort compared to the low expression cohort (Figure 5D). In contrast, T regs and macrophages M0 were noticeably elevated in the low STEAP4 group compared to the high cohort (Figure 5D).

In order to understand the relationship between STEAP4 expression and the abundance of TILs, TIMER2.0 was used. A significant negative correlation was seen between STEAP4 and tumor purity ( $r = 0.307$ ,  $p = 5.76 \times 10^{-9}$ ), Treg cells ( $r = -0.244$ ,  $p = 4.46 \times 10^{-6}$ ), macrophages M0 ( $r = -0.17$ ,  $p = 1.56 \times 10^{-3}$ ), and macrophage M2 ( $r = -0.218$ ,  $p = 4.34 \times 10^{-5}$ ) (Figure 6A).

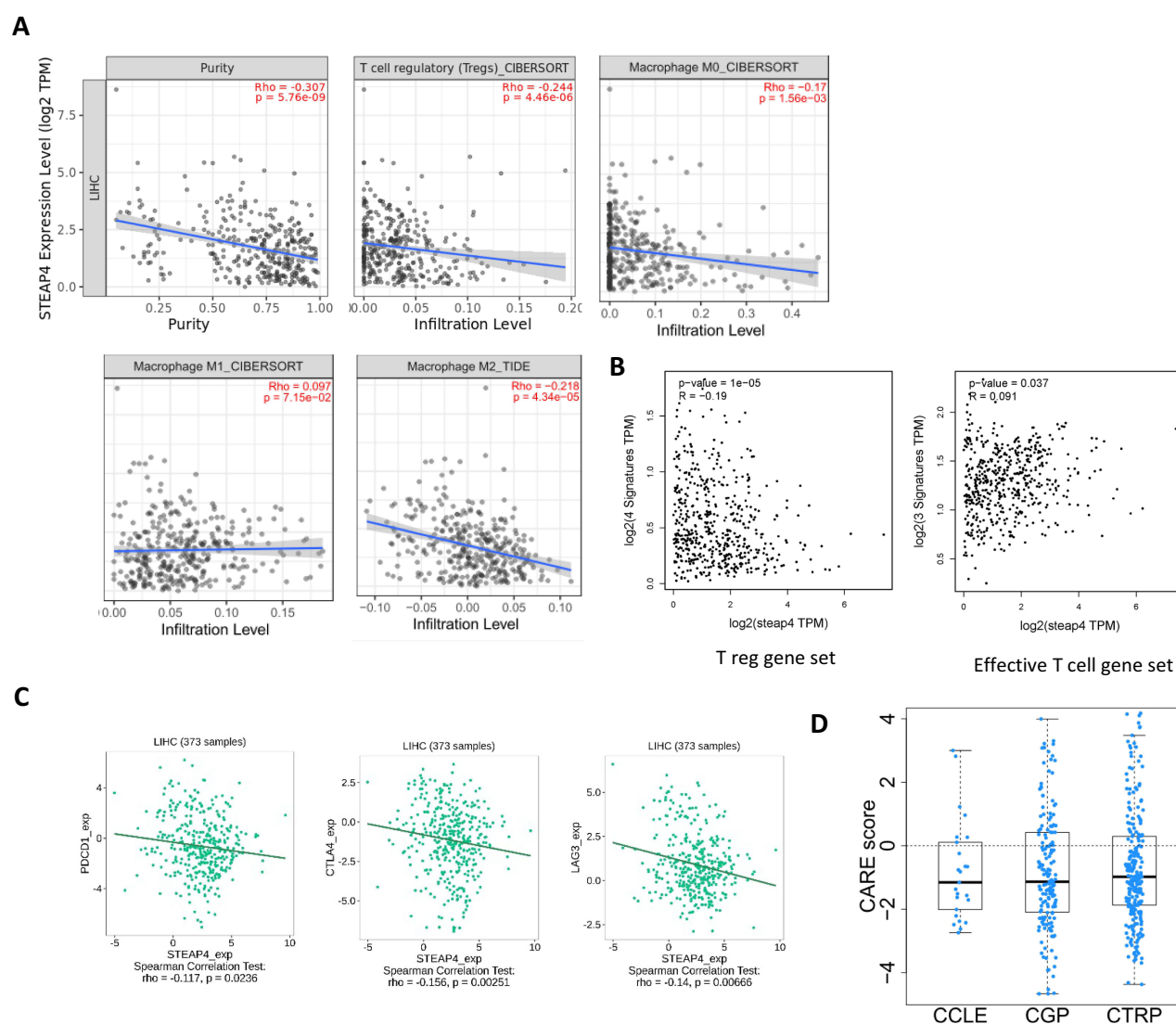


**Figure 4** Gene enrichment analysis of STEAP4 in hepatocellular carcinoma (HCC). **(A)** The volcano plots of DEGs represented the upregulated and downregulated genes in red and blue and Heatmap depicted the top 50 positively and negatively correlated genes of STEAP4 in HCC. **(B)** The results of Kyoto Encyclopedia of Genes and Genomes pathway analysis of upregulated genes enrichment. **(C)** The gene ontology of STEAP4 was analyzed according to biological functional enrichment. **(D and E)** The results of gene set enrichment analysis based on Kyoto Encyclopedia of Genes and Genomes databases.

We then used the GEPIA2 database to examine the expression of STEAP4 and T cell activity-related gene panels. As shown in **Figure 6B**, it was negatively correlated with Treg cell signature genes, such as FOXP3, CTLA4, CCR8, and TNFRSF9 ( $R = -0.19$ ,  $p = 1e-05$ ), and positively correlated with effective T cell signature gene sets like FCGR3A, FGFBP2, and CX3CR1 ( $R = 0.091$ ,  $p = 0.037$ ). As part of the tumor microenvironment, immune checkpoints are also crucial for regulating and directing the immune response. Based on the TISIDB database, we analyzed the correlation between STEAP4 expression and immune checkpoints (ICs) in HCC to estimate the association. There was a negative correlation between STEAP4 expression and PDCD1 (PD-1) ( $p=0.0232$ ), CTLA4 ( $p=0.00251$ ), and LAG3 ( $p=0.00666$ ) (**Figure 6C**). The findings further support the hypothesis that STEAP4 plays an important role in regulating immunity in TME.



**Figure 5 (A)** The significantly somatic mutated genes in the STEAP4 low and high groups. **(B)** The relationship between STEAP4 expression and immune score, stromal score, and ESTIMATE score **(C)** Survival analysis of CD4, CD8, Treg, B cell, macrophage, and cancer-associated fibroblast. **(D)** Correlation between STEAP4 and 21 kinds of tumor microenvironment infiltrating immune cells analyzed using CIBERSORT.



**Figure 6** (A) Correlation between STEAP4 and immune cells such as T regs, M0, M1, and M2 macrophages (B) Correlation between STEAP4 expression and Treg gene set. (C) The relationship between STEAP4 and immune checkpoints such as PDCD1, CTLA4, and LAG3. (D) The negative correlation between CARE scores and many compounds found in the Cancer Cell Line Encyclopedia (CCLE), Genomics of Drug Sensitivity in Cancer (GDSC), and Cancer Therapeutics Response Portal (CTRP) databases.

## STEAP4 Expression and Therapy Responsiveness

GSEA analysis suggested that STEAP4 expression is correlated with drug metabolism. In consideration of the correlation between tumor progression and drug resistance, we utilized the CARE algorithm to analyze the relationship between STEAP4 and drug resistance. Based on data from the Cancer Cell Line Encyclopedia (CCLE), Genomics of Drug Sensitivity in Cancer (GDSC), and The Cancer Therapeutics Response Portal (CTRP).<sup>44</sup> We found that STEAP4 expression is negatively correlated with CARE scores for various compounds (Figure 6D). These findings suggest that STEAP4 expression plays a role in drug resistance. STEAP4 expression and drug metabolism are related, as demonstrated in the GSEA analysis.

## Discussion

Based on previous studies, we selected STEAP4 as a target to investigate its role in HCC. As a metalloredutase involved in cellular iron and copper homeostasis, STEAP4 modulates inflammation.<sup>10</sup> Previous studies have revealed that STEAP4 is overexpressed and related to invasion and metastasis in various cancers, including prostate, lung, and

colon cancers;<sup>3,31,32</sup> STEAP4 is also associated with the immune response within the tumor microenvironment.<sup>10</sup> However, it is unclear whether STEAP4 affects carcinogenesis and immune reactions in HCC patients. Hence, we analyzed data from multiple databases and a cohort of HCC patients to determine the effect of STEAP4 on HCC. Our analysis revealed that the expression of STEAP4 mRNA and protein levels were lower in HCC tissues compared to non-neoplastic tissues and that the expression pattern identified by IHC was distinct. Prognosis analysis based on the Kaplan–Meier plotter and HCC cohort showed that low expression of STEAP4 mRNA and protein and reduced expression of STEAP4 by the HCC cohort on IHC were associated with poor clinical outcomes. Furthermore, analysis by the histological grade and tumor stage revealed that reduced STEAP4 expression was significantly related to tumor progression. Using multiple databases, we also found that STEAP4 can promote certain immune subsets and proliferation-associated biological processes suggesting that STEAP4 has a significant prognostic value for HCC patients.

Transport of the electron from NADPH to reduce the extracellular Fe<sup>3+</sup> to Fe<sup>2+</sup> and Cu<sup>2+</sup> to Cu<sup>1+</sup> is the primary function of STEAP4.<sup>10</sup> Thus, STEAP4 is involved in fatty acid and glucose metabolism, inflammation, and oxidative stress through cellular iron and copper homeostasis. STEAP4 appears to be located in the Golgi apparatus network and plasma membrane of cells.<sup>33</sup> However, cellular expression patterns and functional roles of STEAP4 in the liver are not well understood. Notably, recent studies have highlighted that hepatic disease is induced by a deficiency in STEAP4, unlike diseases of the prostate and colon. Kim et al found that the downregulation of hepatic STEAP4 mRNA and protein expression was linked to disturbances in fatty acid and glucose metabolism associated with iron accumulation.<sup>34</sup> Moreover, it increased the presence of HBV-encoded X protein, which is known to play a pivotal role in the pathogenesis of virus-induced HCC.<sup>35</sup> In line with these *in vitro* results, we demonstrated that decreased STEAP4 mRNA and protein expressions were associated with tumor progression. IHC analysis of HCC tissues, compared with non-neoplastic tissues, showed that STEAP4 protein expression was significantly different showing a clumping pattern. We interpreted this as a reduced expression. This reduced expression was associated with aggressive tumor behavior, such as large tumor size, type 2 gross type, multiple presentations of HCC, microvascular invasion, and a higher pathologic T stage. Furthermore, we demonstrated that reduced STEAP4 levels were an independent prognostic factor for inferior outcomes in HCC.

Given the aggressive behaviors associated with low STEAP4 expression, it was a compelling study to examine underlying factors correlated with poor survival. To identify potential functional changes associated with reduced STEAP4 expression, we performed bioinformatic analysis, including gene ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, to better understand the molecular mechanisms underlying this relationship. GO analysis showed that the listed biological processes were related to cell development and response to stress, signaling pathways, and immune response. Based on these results, STEAP4 expression appeared to be related to immune response as well as to cell signaling and development. GSEA results showed enrichment in DNA replication, RNA processing, and drug metabolic pathways leading us to speculate that STEAP4 has potential links to drug metabolism as well as DNA and RNA maintenance and regulation. It is crucial to maintain, replicate, and regulate DNA and RNA for tumor proliferation to occur.<sup>45</sup> According to Hanahan et al,<sup>46</sup> maintaining proliferation signals is one of the most important signals that cell acquired as they progress from normal to malignant growth.

Furthermore, as shown in Figure 5A, TP53, CTNNB1, and AXIN were more altered in the low STEAP4 expression group. TP53, CTNNB1, and AXIN1 are dominant mutational drivers in HCC.<sup>29</sup> Cell cycle and apoptosis are controlled by TP53, which is associated with poor clinical outcomes in HCC.<sup>36,47</sup> It is also believed that CTNNB1 and AXIN1 promote HCC by aberrantly enhancing Wnt/ $\beta$ -catenin signaling.<sup>37,48</sup> Several other studies have also shown that STEAP4 is associated with HCC progression.<sup>14,31,38</sup> As mRNA levels increased in prostate cancer, STEAP4 overexpression increased the number and size of prostate cancer cell lines.<sup>38</sup> STEAP4 knockdown interferes with the lipopolysaccharide-induced proliferation of prostate cancer cells.<sup>31</sup> Orfanou et al suggested that STEAP4 silencing inhibits the proliferation of HER2+ breast cancer cells.<sup>14</sup> Recent studies have demonstrated that STEAP4 inhibits the proliferation and metastasis of HCC cells as a tumor suppressor gene.<sup>39</sup> Thus, our results demonstrated for the first time that low STEAP4 expression may promote the HCC through its involvement in the cell proliferating pathways, although the specific mechanism needs to be further verified.

Cancer cells are characterized by uncontrolled proliferation. Furthermore, the alteration of the tumor microenvironment is considered a significant component in tumor development.<sup>40</sup> In the present study, we examined the changes of

immune subsets based on STEAP4 expression level. According to the ESTIMATE algorithm, the stromal score and estimate score were lower in low expression group. It was revealed that tumor purity was greater in the lower expression group, regardless of the degree of immune cell infiltration. Hence, it was speculated that the composition of the immune subsets might be different depending on STEAP4 expression. In our study, we found that higher infiltration levels of Tregs and macrophages were associated with poor prognosis in HCC. We demonstrated that STEAP4 expression negatively influenced Treg and M2 macrophages and positively affected M1 macrophage infiltration. We further explored the relationship between STEAP4 expression and T-cell activity. As expected, exhausted T cell signatures were negatively correlated with STEAP4 expression levels and effective T cell signatures were positively correlated. It has been reported that patients with higher levels of Treg infiltration in HCC have a poor prognosis associated with defective cytotoxic T lymphocytes (CTLs).<sup>49,50</sup> Macrophages also have been recognized as an important component of the tumor microenvironment that contributes to the development and progression of cancer.<sup>51</sup> Several studies demonstrated that HCC patients with low infiltration of M1 and high infiltration of M2 macrophage levels have a poor prognosis.<sup>51,52</sup> Furthermore, we found that immune checkpoints, such as CTLA4, LAG3, and PD-L1, were also inversely correlated with STEAP4 expression in HCC suggesting that STEAP4 may be involved in regulating the TME and immune response in HCC.

## Conclusion

In summary, we examined the expression, prognosis, immune cell involvement in TME, and drug sensitivity related to STEAP in HCC patients. Although the molecular mechanisms and signaling pathways of STEAP4 in influencing the composition of the immune subset, immune escape, and tumor progression in HCC require further investigation, we first demonstrated that STEAP4 can be a potential prognostic marker for HCC patients and has considerable immunotherapeutic potential for HCC patients.

## Abbreviations

BP, biological process; CCLE, Cancer Cell Line Encyclopedia; CTLs, cytotoxic T lymphocytes; CTRP, Cancer Therapeutics Response Portal; DEG, differentially expressed genes; FC, fold-change; GDSC, Genomics of Drug Sensitivity in Cancer; GEO, Gene Expression Omnibus; GEPIA, Gene Expression Profiling Interactive Analysis; GO, gene ontology; GSEA, Gene Set Enrichment Analysis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; HR, hazard ratio; IC, immune checkpoint; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDSC, myeloid-derived suppressor cells; MF, molecular function; RFS, recurrence-free survival; STEAP4, six-transmembrane epithelial antigen of prostate 4; TCGA, The Cancer Genome Atlas; TIL, tumor-infiltrating lymphocyte; TIMER, Tumor Immune Estimation Resource; TISIDB, Tumor Immune System Interaction Database; TMA, Tissue microarray; TME, tumor microenvironment; TNF- $\alpha$ , tumor necrosis factor-alpha; UALCAN, University of Alabama at Birmingham Cancer data analysis Portal.

## Data Sharing Statement

All genetic data were obtained from The Cancer Genome Atlas (TCGA) (<https://cancergenome.nih.gov/>) and Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) databases.

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

## Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1D1A3B03935135). Additional this research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant No.: 2022R1C1C1003307).

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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