Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(6): 190
https://doi.org/10.31083/j.fbl2706190

Original Research

Gene Signature and Prognostic Value of Ubiquitin-Specific Proteases Members in Hepatocellular Carcinoma and Explored the Immunological Role of *USP36*

Weijie Sun[1,†], Jiapei Shen[1,†], Jiaying Liu[1], Kexing Han[1], Leilei Liang[2,*], Yufeng Gao[1,*]

[1]Department of Infectious Diseases, The First Affiliated Hospital of Anhui Medical University, 230022 Hefei, Anhui, China
[2]Department of Gynecologic Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 100021 Beijing, China
*Correspondence: aygyf@ahmu.edu.cn (Yufeng Gao); liangleilei10006@163.com (Leilei Liang)
[†]These authors contributed equally.
Academic Editor: Cheng-Maw Ho
Submitted: 29 March 2022 Revised: 6 May 2022 Accepted: 26 May 2022 Published: 15 June 2022

Abstract

Background: Ubiquitination is one of the most common post-translational modifications in cells and dysregulation is closely associated with the development of cancer. However, a comprehensive analysis of the role of ubiquitination in hepatocellular carcinoma (HCC) is still lacking. In this study we analyzed expression and prognostic value of Ubiquitin-Specific Proteases (USPs) in HCC, and the immunological role of *USP36* in HCC. **Methods**: Expression data, prognostic data, and DNA methylation data in cases of HCC were obtained from the cancer genome atlas (TCGA). Overexpression of *USP36* in HCC was confirmed in the gene expression omnibus (GEO) database and verified by quantitative PCR in 10 pairs of HCC samples. ULCAN was used to analyze the correlation between *USP36* and clinicopathological features. TIMER2.0 and DriverDBv3 were used to analyze the *USP36* mutational profile. GSEA analysis explored the potential signaling pathways of *USP36* affecting HCC. The immune and stromal scores of HCC samples were calculated using the ESTIMATE algorithm. TIMER1.0 was used to explore the correlation between *USP36* and immune cell infiltration. Finally, we analyzed the correlation of *USP36* expression with immune checkpoint molecules and determined the IC50 values of 6 chemotherapeutic drugs using the pRRophetic software package. **Results**: Most USPs are abnormally expressed in HCC, among which *USP36* and *USP39* are most closely associated with HCC prognosis. We also found that *USP36* is associated with *TP53* mutational status. GSEA analysis indicated that USP36 may affect HCC progression through the dysregulation of various pathways such as ubiquitin-mediated proteolysis. *USP36* expression positively correlated with both macrophage infiltration levels and multiple immune checkpoint molecules. Finally, chemosensitivity analysis indicated that chemosensitivity was lower in cells within the *USP36* high expression group. **Conclusions**: Most USPs are abnormally expressed in HCC. Overexpression of *USP36* in HCC is closely related to poor prognosis. In particular, the unique immunological role of *USP36* may have potential clinical application value.

Keywords: Ubiquitin-Specific Proteases (USPs); *USP36*; HCC; immune; bioinformatics analysis

1. Introduction

Primary liver cancer (PLC) displays high morbidity and mortality in patients with this disease. The latest cancer statistics report shows that 905,677 new cases are diagnosed, and 830,180 patients die from this disease, every year. These figures place PLC as the sixth highest incidence and third highest mortality rate among all cancers [1]. The 3-year survival rate of patients diagnosed with primary liver cancer is approximately 50% [2]. Hepatocellular carcinoma (HCC) is the major form of PLC observed clinically. In early stages, curative treatments for HCC, such as tumor resection, ablation, and liver transplantation can be used for treatment of this disease [3]. However, a large proportion of HCC patients initially present with advanced disease and thus have limited therapeutic options. Systemic drug therapy is one of the approaches to treat advanced HCC, but first-line treatments for advanced HCC, such as sorafenib or lenvatinib, only extend survival by approximately 3 months [4]. Despite tremendous advances in treatment in recent years, the overall prognosis of liver cancer remains poor. Therefore, it is crucial to explore novel biomarkers with prognostic value for prognosis HCC patients.

Ubiquitination is an important post-translational modification that involves the coordinated activity of ubiquitin activating enzymes (termed E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3). These molecules are responsible for covalently conjugating single or multiple ubiquitin molecules to target proteins [5]. Ubiquitination alters important properties of target proteins, including target protein activity, cellular localization, half-life, receptor endocytosis, and protein-protein interactions [5–7]. Similar to other post-translational modifications, ubiquitination is a reversible process that is dynamically regulated by ubiquitinases and deubiquitinases (DUBs). Thus, dysregulation of the ubiquitination process can lead to impairment of many important cellular processes and contribute to serious diseases, including cancer [8].

DUBs cleave ubiquitin chains from protein substrates, and owing to structural differences within their catalytic domains, DUBs can be divided into 5 families: Ubiquitin C-Terminal Hydrolases (UCHs), Ubiquitin-Specific Proteases (USPs), Machado-Joseph Disease Protein Domain Proteases (MJDs), Ovarian Tumor Proteases (OTUs), and JAMM Motif Proteases [9]. Among these, USPs is the largest family and has the most diverse structures. Moreover, USPs also belong to the cysteine protease family [9]. Growing evidence suggests that DUBs act as important regulators of immune response, making the development of new drugs targeting USPs a promising therapeutic avenue for the treatment of cancer [10]. Loosdregt *et al*. [11] found that *USP7* can control Treg cell number and function by regulating Foxp3 expression in this type of T lymphocyte. Liu *et al*. [12] found that *USP18* plays a key role in T cell activation and Th17 generation, and further affects NF-κB and NFAT activation. Zou *et al*. [13] observed that inhibition of *USP15* induces tumor cell apoptosis and promotes antitumor T cell activation. These findings suggest that DUBs, especially USPs, can be considered promising targets for cancer immunotherapy. However, there is currently a lack of comprehensive studies on USPs in HCC, especially in their potential impact on immunotherapy. To our knowledge, this study is the first to explore the expression and prognostic value of 39 USPs members in liver cancer. Furthermore, we also focused on the immunological role of a representative USP member, specifically, *USP36*.

2. Materials and Methods

2.1 Data Download

RNA-seq data, clinical information, and DNA 450k methylation data obtained from HCC tumors were downloaded from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/), and missing data were removed from the data set. In addition, RNA-seq data of HCC patients were downloaded from three data sets (GSE14520, GSE36376 and GSE64041) in the public database Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/).

2.2 Tissue Samples

Tumor and adjacent normal tissues from 10 primary HCC patients the First Affiliated Hospital of Anhui Medical University. Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (No. Quick-PJ2020-16-23). All patients provided written informed consent and complied with the Declaration of Helsinki.

2.3 RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from frozen tissue using an RNA extraction kit (ShangHai YiShan Biotechnology), and cDNA was synthesized and amplified using PrimeScript™ RT Master Mix (Takara Bio). USP36 mRNA was quantified by TB-Green qPCR (Takara Bio) and normalized to GAPDH. The *USP36* primer sequence used in this study is: Forward: 5′-TCTGCCAAGAAGGTCCTTTTACA-3′; Reverse: 5′-TGGCGACTAGCTCCCTCTG-3′.

2.4 Expression and Prognostic Value of USPs

Using the Limma package, differential gene expression analysis was performed to determine if USPs expression differed between HCC and normal tissue groups. In this study, we initially focused on 48 USPs (*USP1-USP48*). In this group, we found that 4 USPs members (*USP9*, *USP17*, *USP23* and *USP27*) were missing in the database, and the average expression of 5 USPs members (*USP6*, *USP26*, *USP29*, *USP41* and *USP44*) was lower than 0.2. As a result of these initial efforts, we analyzed the differential expression of 39 USPs. Survival analysis was conducted to determine their relative prognostic value using Kaplan-Meier analysis. Prognostic endpoints included overall survival (OS), disease-free survival (DFS), disease-specific survival (DSS), and progression free survival (PFS).

2.5 Correlation between USP36 Expression and Clinicopathological Features

UALCAN (http://ualcan.path.uab.edu/analysis.html) is a portal for straightforward exploration, analysis, and visualization of data derived from TCGA [14]. We used UALCAN to explore the expression of *USP36* in different genders, ages, lymph node metastasis, tumor grade and stage, and *TP53* mutation status. Significant differences in gene expression levels between groups were calculated by *t*-test. $p < 0.05$ indicates statistical significance.

2.6 Mutation and Methylation Profiles of USP36

We explored *USP36* gene mutation spectrum using TIMER2.0 (http://timer.comp-genomics.org/) and DriverDBv3 (http://driverdb.tms.cmu.edu.tw/). These analysis packages can be obtained at no charge, and provide the mutation modules of genes [15,16]. Using the DNA 450k methylation data of liver cancer samples downloaded from the TCGA database, the degree of methylation was determined by calculating the β value of each potential methylation site within the *USP36* gene. $\beta \geq 0.6$ represents a fully methylated gene, $\beta \leq 0.2$ represents fully unmethylated gene, and anything in between represents partial gene methylation.

2.7 Gene Set Enrichment Analysis (GSEA)

The potential *USP36* signal transduction pathways that affect HCC progression were analyzed using gene set enrichment analysis GSEA (version 4.2.3). The 5 pathways most significantly enriched in the high-expression group and the low-expression group are shown.

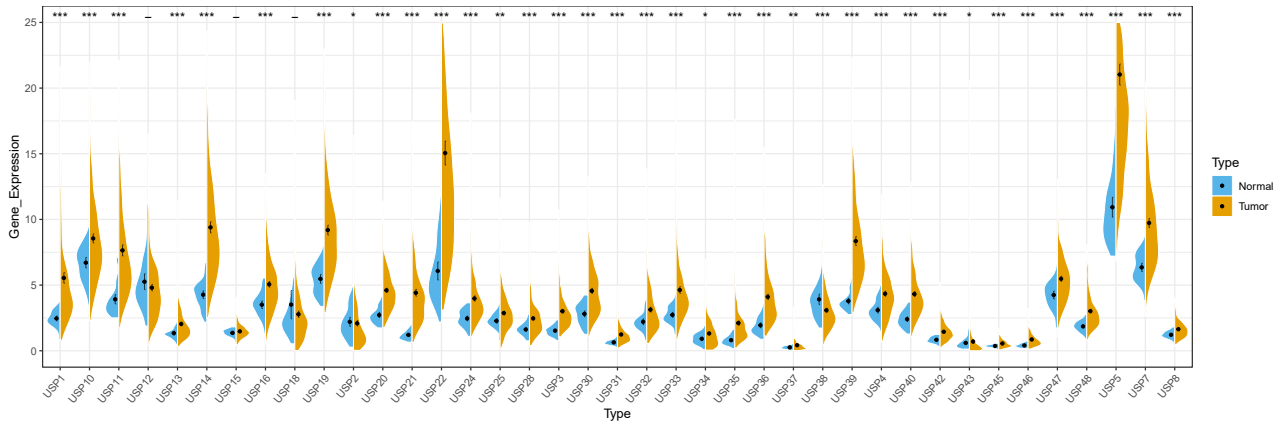

Fig. 1. Differential expression of USPs members in HCC. "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$.

2.8 Correlation between USP36 Expression and Tumor Microenvironment

We used the HCC gene expression signature to first infer the proportion of stromal and immune cells in HCC tumor samples using the ESTIMATE algorithm [17], and predicted the Immunescore and Stromalscore in the tumor tissue. In addition, we also used TIMER 1.0 (https://cistrome.shinyapps.io/timer/) to explore a potential correlation between *USP36* expression and immune cell infiltration. TIMER 1.0 is an open platform that can be used to explore tumor immune interactions [18]. Due to a unique correlation between *TP53* and *USP36*, we also investigated immune cell infiltration in HCC tumors with *TP53* mutation.

2.9 Chemotherapy Drug Sensitivity Analysis

We calculated the 50% inhibitory concentration (IC50) of 6 commonly used chemotherapeutic drugs using the pRRophetic package to evaluate the sensitivity of HCC samples to chemotherapeutic drugs. Wilcoxon signed-rank test was used to compare the difference in IC50 between high and low expression groups.

3. Results

3.1 Expression and Prognostic Value Of USPs Members in HCC

Among the 39 USPs family members, except for *USP12*, *USP15* and *USP18*, the remaining 36 USPs are abnormally expressed in HCC. Specifically, 34 USPs (*USP1*, *USP3*, *USP4*, *USP5*, *USP7*, *USP8*, *USP10*, *USP11*, *USP13*, *USP14*, *USP16*, *USP19*, *USP20*, *USP21*, *USP22*, *USP24*, *USP25*, *USP28*, *USP30*, *USP31*, *USP32*, *USP33*, *USP34*, *USP35*, *USP36*, *USP37*, *USP39*, *USP40*, *USP42*, *USP43*, *USP45*, *USP46*, *USP47* and *USP48*) are highly expressed in HCC, and 2 USPs (*USP2* and *USP38*) display reduced expression in HCC, results of this analysis are shown in Fig. 1.

We also analyzed the relationship between abnormally expressed USPs and HCC patient prognosis. According to median expression of these USPs, HCC patients were divided into a high expression group and a low expression group, and Kaplan-Meier survival analysis was used to analyze survival among HCC patients in these two groups. The results showed that only 10 of 35 abnormally expressed USPs, specifically *USP1*, *USP11*, *USP13*, *USP14*, *USP21*, *USP24*, *USP36*, *USP39*, *USP46* and *USP48*, affected the overall survival (OS) of HCC patients with high expression of these USPs indicating poorer OS (Fig. 2A).

In addition to OS, we also explored other important prognostic indicators such as disease-free survival (DFS), disease-specific survival (DSS), and progression-free survival (PFS). The results showed that expression of four USPs (*USP19*, *USP36*, *USP39* and *USP44*) affect HCC patient DFS. Except for high expression of *USP44* which correlated with favorable DFS, high expression of the remaining three USPs was associated with poorer DFS (Fig. 2B). Expression of seven USPs, specifically *USP1*, *USP13*, *USP21*, *USP36*, *USP39*, *USP42* and *USP48*, is correlated with altered HCC patient DSS. This analysis indicated that high expression of all of these USPs indicate poorer HCC patient DSS (Fig. 2C). Similarly, seven USPs, *USP1*, *USP3*, *USP13*, *USP14*, *USP21*, *USP24*, *USP32*, *USP36*, *USP39*, *USP42* and *USP48*, affected HCC patient PFS. Specifically, high expression of all of these USPs correlated with poorer PFS (Fig. 2D). Further Kaplan-Meier analysis of expression of other USPs in HCC patients was conducted (**Supplementary Fig. 1**).

These analyses lead us to conclude that USPs expression is commonly closely related to HCC patient prognosis, and *USP36* and *USP39* can simultaneously affect OS, DFS, DSS and PFS in these patients.

3.2 Clinical Relevance of USP36

Several studies have suggested an important role for *USP39* in HCC [19–21], but *USP36* remains largely unexplored in this tumor type. It has been reported that *USP36*

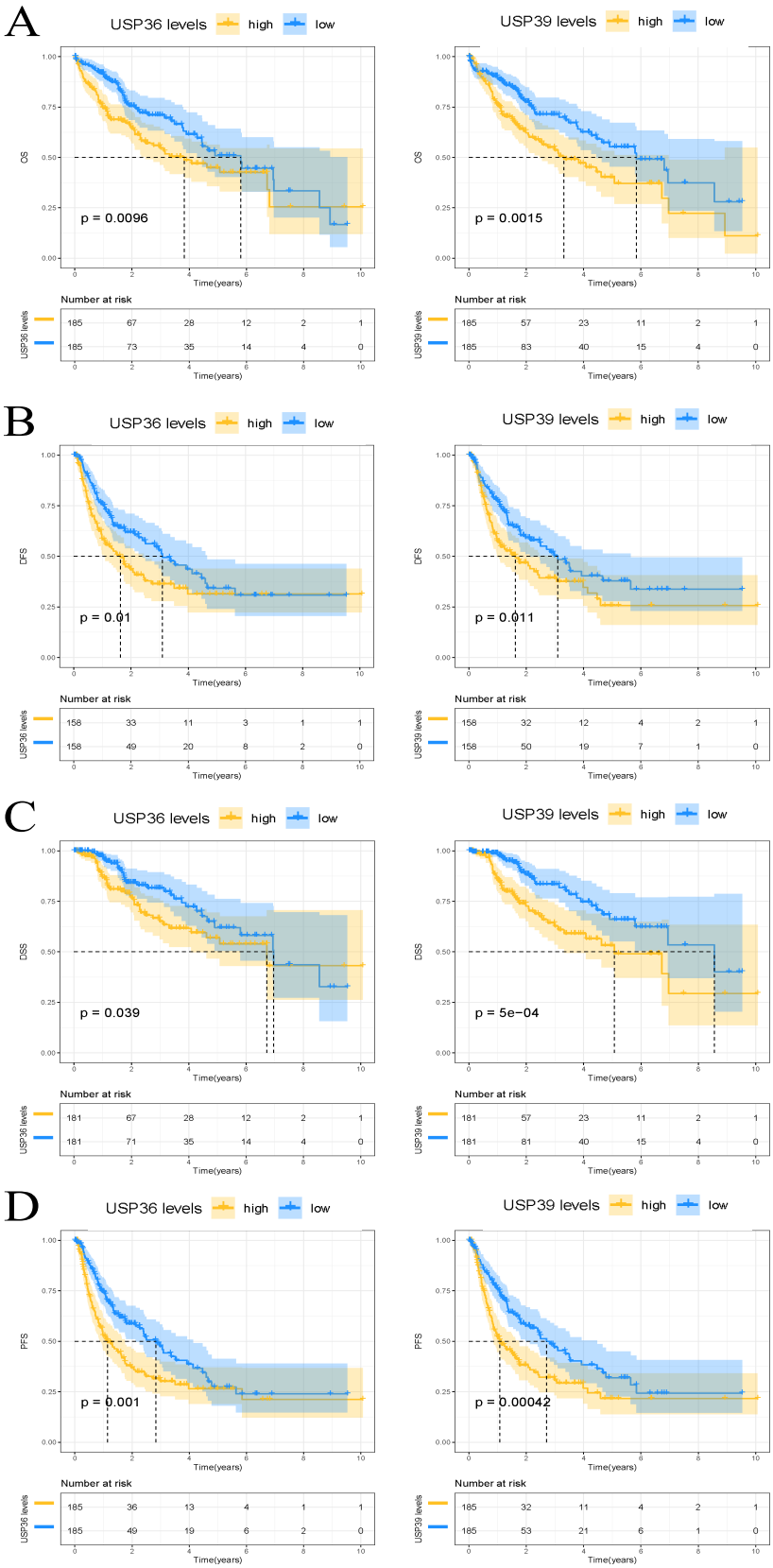

Fig. 2. Kaplan-Meier plot for prognostic analysis of *USP36* and *USP39* in HCC. OS (A), DFS (B), DSS (C) and PFS (D) of HCC patients grouped by median expression of USPs members. OS, overall survival; DFS, disease-free survival; DSS, disease-specific survival; PFS, progression free survival.

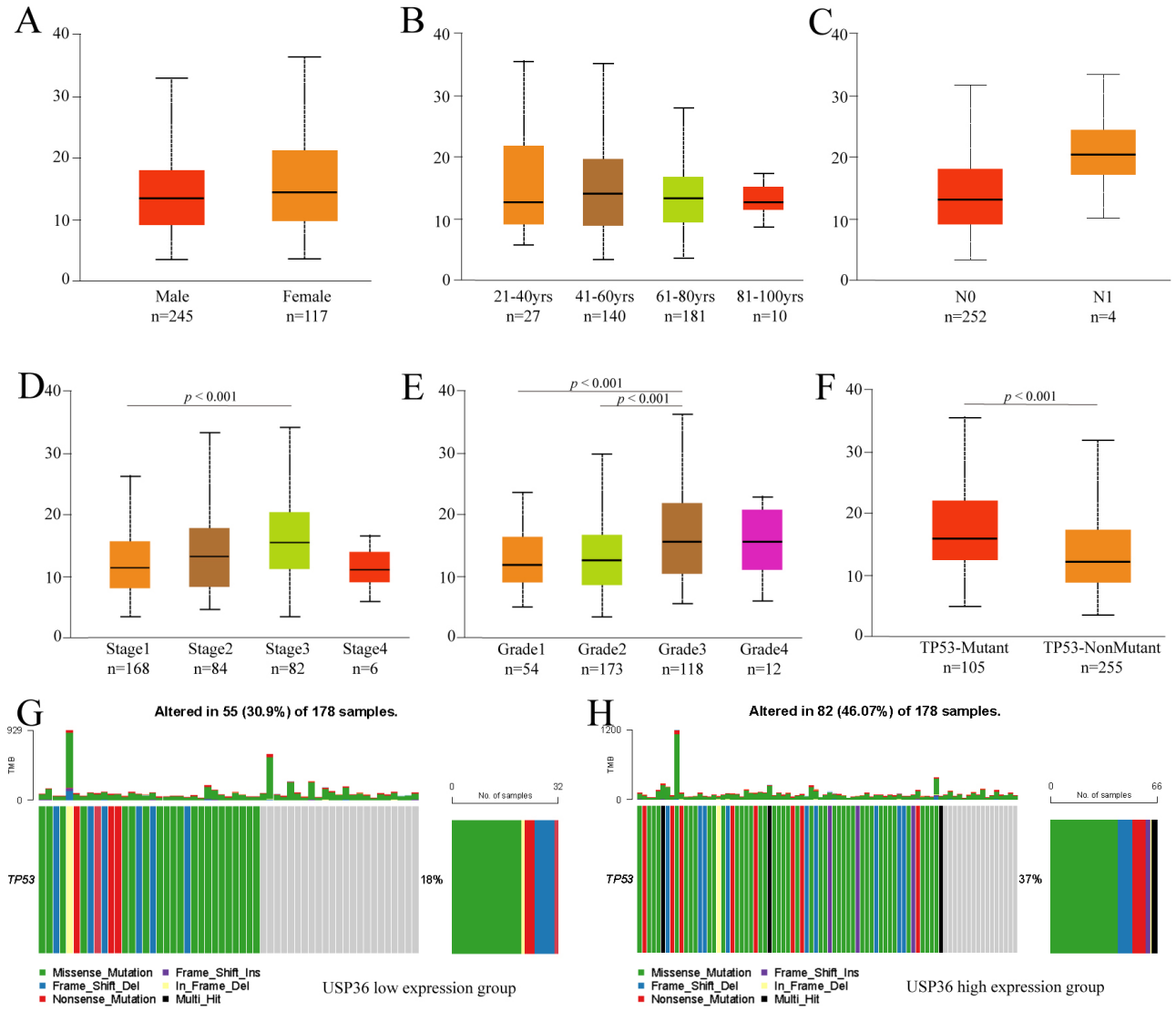

Fig. 3. Subgroup analysis of clinicopathological features of *USP36* in HCC. Expression of *USP36* in different gender (A), age(B), lymph node metastasis status (C), tumor stage (D), tumor grade (E) and *TP53* mutation status (F) grouping. *TP53* mutation in *USP36* high expression group (G). *TP53* mutation in *USP36* low expression group (H).

participates in the progression of non-small cell lung cancer and ovarian cancer [22,23]. Importantly, *USP36* can increase *c-myc* stability by altering ubiquitination of this important oncogenic transcription factor [24]. A prominent pro-tumorigenic effect of *c-myc* in HCC is supported by observations that overexpression of *c-myc* in mouse liver drives induction of HCC [25]. Therefore, we speculated that *USP36* has strong hepatocarcinogenic potential and, given the unique expression pattern of *USP36* within HCC tumors as well as its biological effects on *c-myc* levels, we selected *USP36* for further investigation. We verified the expression of *USP36* in 3 GEO datasets (GSE14520, GSE36376 and GSE64041) and by qRT-PCR in 10 matched pairs of HCC tumors and adjacent pathologically normal tissues. The results indicated that expression of *USP36* was consistent with the results obtained from our analysis of the TCGA database (**Supplementary Fig. 2**).

To further explore the clinicopathological parameters of *USP36* in HCC, we performed subgroup analysis using UALCAN. This analysis uncovered no differences in *USP36* expression when patient data was grouped by gender, age, or lymph node metastasis (Fig. 3A–C). The results did show, however, that *USP36* was highly expressed in Stage3 when compared to Stage1 HCC tumors ($p < 0.001$) (Fig. 3D), and that *USP36* expression levels were significantly higher in Grade3 compared with Grade1 and Grade2 tumors ($p < 0.001$) (Fig. 3E). Notably, we observed that *USP36* was significantly overexpressed in HCC tumors with mutant *TP53* ($p < 0.001$) (Fig. 3F), and subsequently examined the mutational profile of *TP53* in different *USP36* expression subgroups (Fig. 3G–H). The results of this analysis indicated that *TP53* mutations were significantly higher

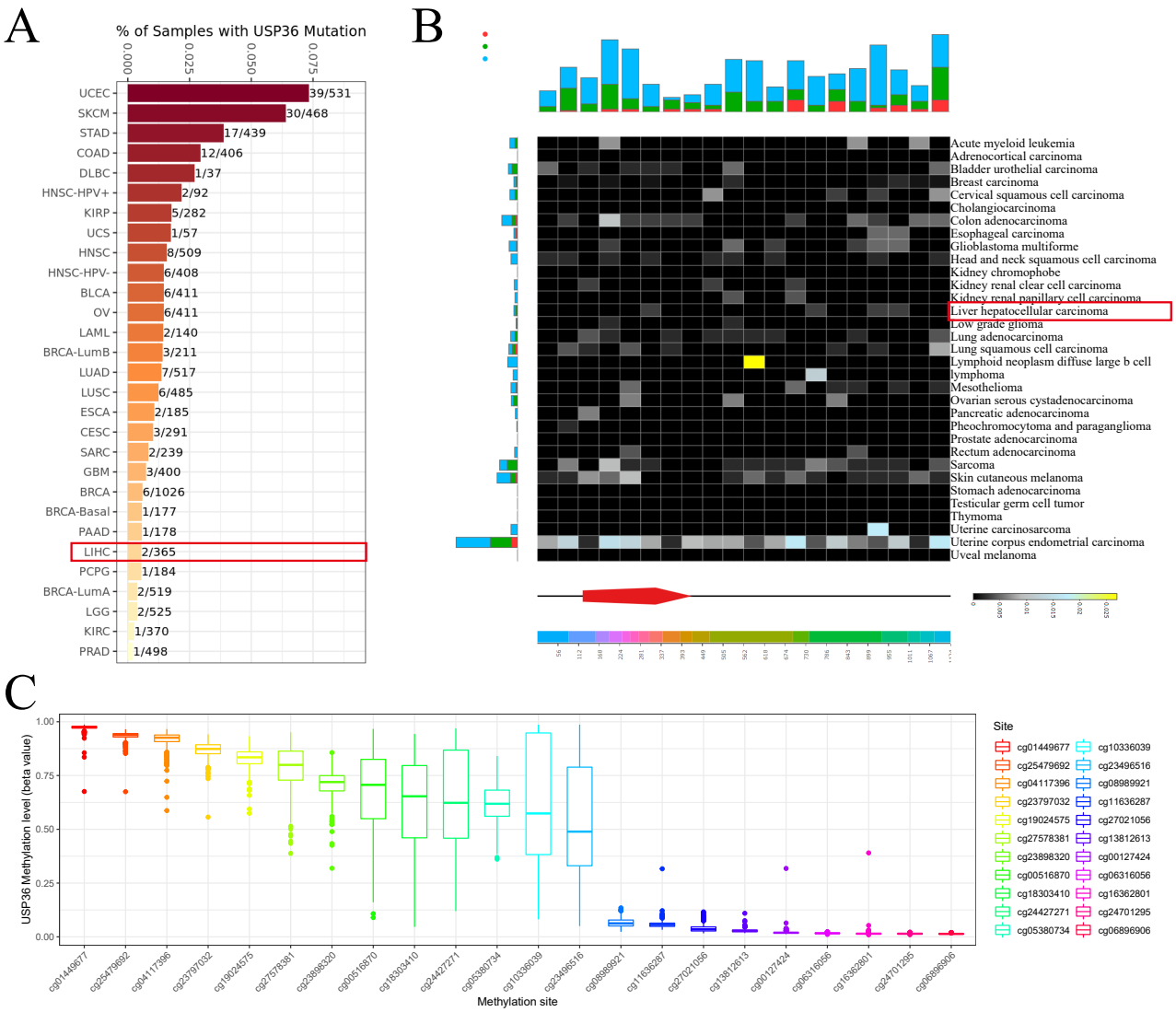

Fig. 4. Mutation and methylation analysis of *USP36* in HCC. (A) The TIMER 2.0 database shows the *USP36* mutation in various tumors. (B) The mutation status of *USP36* in various tumors according to the DriverDBv3 database. (C) Methylation analysis of each methylation site of *USP36* in HCC.

in the *USP36* high expression group than that present in the *USP36* low expression group. Most *TP53* mutation types are missense mutations.

3.3 Mutational and DNA Methylation Profiles Associated with USP36 Expression

To comprehensively analyze the mutational spectrum of *USP36*, we explored the mutation status of *USP36* in all cancer types in the TCGA database using TIMER2.0 and DriverDBv3. Results obtained using TIMER2.0 showed that *USP36* had the highest mutational frequency in uterine corpus endometrial carcinoma (UCEC) (39/531), and the least *USP36* mutations were noted in prostate adenocarcinoma (PRAD) (1/498), whereas the mutation frequency of *USP36* in HCC was 2/365 (Fig. 4A). The DriverDBv3 database indicated that the mutational rate of *USP36* in HCC was 0.003 (Fig. 4B). We next analyzed the DNA methylation level within the *USP36* gene in HCC tumors. The results of this analysis showed that the *USP36* gene was methylated at multiple CpG dinucleotides (11/22) (Fig. 4C). Detailed methylation beta values for each sample provided in **Supplementary Table 1**.

3.4 Gene Set Enrichment Analysis (GSEA) of USP36 in HCC

Given the importance of *USP36* within the USP family, we next decided to explore potential mechanisms of *USP36* dysregulation in HCC. We divided HCC patients into high and low-expression groups according to median *USP36* mRNA expression (median: 3.75302) in the HCC cohort in TCGA and subsequently conducted GSEA. The five most significantly affected pathways in tumors

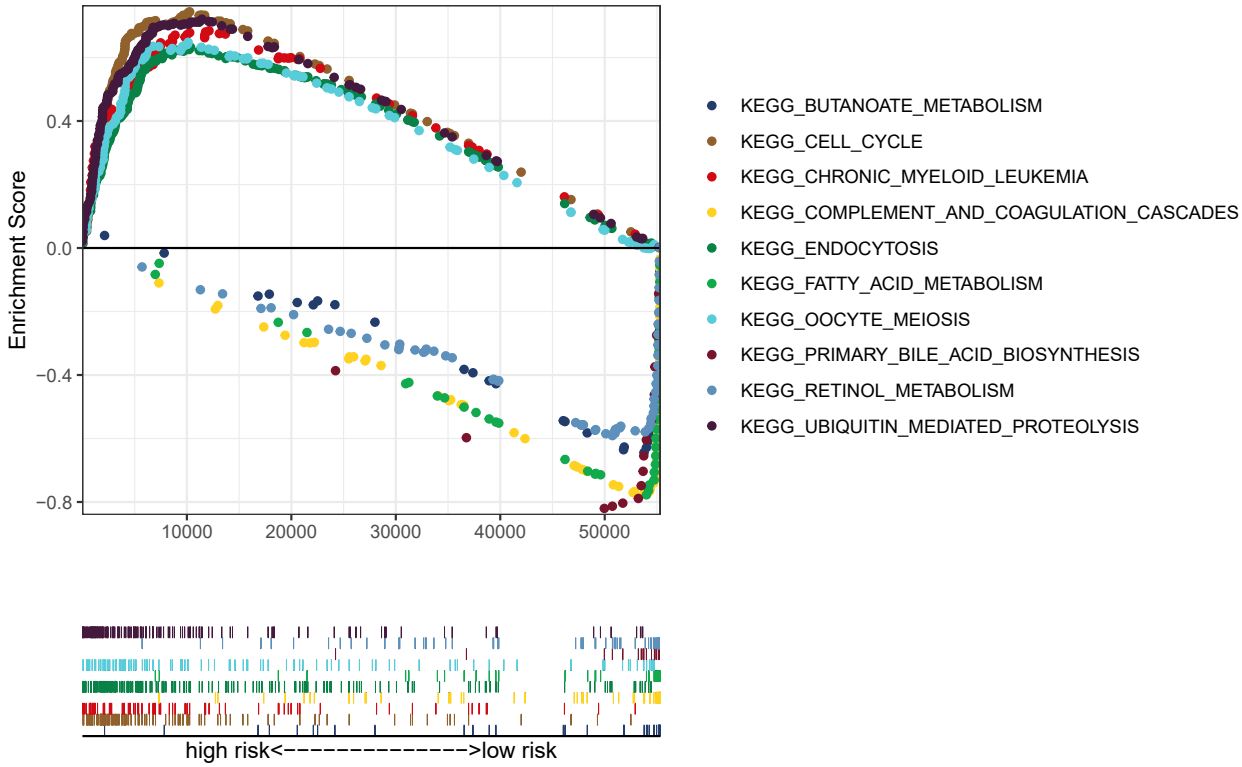

Fig. 5. Gene Set Enrichment Analysis of *USP36* in HCC. The five pathways most significantly enriched in the *USP36* high-expression group and low-expression group.

with high *USP36* expression included KEGG CELL CYCLE, KEGG CHRONIC MYELOID LEUKEMIA, KEGG ENDOCYTOSIS, KEGG OOCYTE MEIOSIS and KEGG UBIQUITIN MEDIATED PROTEOLYSIS. In the *USP36* low-expression group, the five most significantly altered pathways included KEGG BUTANOATE METABOLISM, KEGG COMPLEMENT AND COAGULATION CASCADES, KEGG FATTY ACID METABOLISM, KEGG PRIMARY BILE ACID BIOSYNTHESIS and KEGG RETINOL METABOLISM. Results of GSEA are shown in Fig. 5.

3.5 Correlation between USP36 and Immune Infiltrating Cells and Its Potential as an Indicator of Immunotherapeutic Response in HCC

Since ubiquitination and the immune microenvironment are closely related [8,26], we next explored the relationship between *USP36* and the tumor immune microenvironment. As a first step, we analyzed the correlation between *USP36* expression and the tumor ESTIMATE score. Results showed a weak negative but statistically significant correlation between *USP36* expression and Immunescore (Relevance = –0.20, p = 7.3e-05) (Fig. 6A) as well as Stromalscore (Relevance = –0.24, p = 3.5e-06) (Fig. 6B). In addition, we further explored the correlation of *USP36* expression with the tumor infiltration of six types of immune cells. The results showed that the expression of *USP36* was positively correlated with infiltration of B cells (Cor = 0.285, p = 7.69e-08), CD8 + T cells (Cor = 0.170, p = 1.56e-03), CD4 + T cells (Cor = 0.368, p = 1.71e-12), macrophages (Cor = 0.402, p = 1.18e-14), neutrophils (Cor = 0.385, p = 1.17e-13), and dendritic cells (Cor = 0.316, p = 2.46e-09) (Fig. 6C).

We subsequently examined the relationship between *TP53* mutation and immune cell infiltration in HCC. The results showed that B cells and macrophages were significantly increased in HCC tumors that contain *TP53* mutations ($p < 0.05$) (Fig. 6D).

3.6 Correlation Analysis of USP36 Expression with Immune Checkpoint Molecules and Chemotherapy Drugs

In addition, given the substantial progress in cancer treatment in the resent past, immunotherapy targeting immune checkpoint molecules is a promising therapeutic approach for liver cancer patients. We analyzed the correlation between *USP36* expression and 48 immune checkpoint molecules using Spearman correlation analysis. The results showed a positive correlation between *USP36* and 16 immune checkpoint molecules, specifically, *NRP1*, *TNFSF4*, *CTLA4*, *CD28*, *CD276*, *CD80*, *PDCD1*, *ICOSLG*, *TMIGD2*, *VTCN1*, *HHLA2*, *TNFSF18*, *TNFSF9*, *TNFSF25*, *TNFSF15*, *CD86* and *TNFSF9*. Of note, 2 immune checkpoint molecules, *IDO2* and *TMIGD2*, were negatively correlated with *USP36* expression (Fig. 7A).

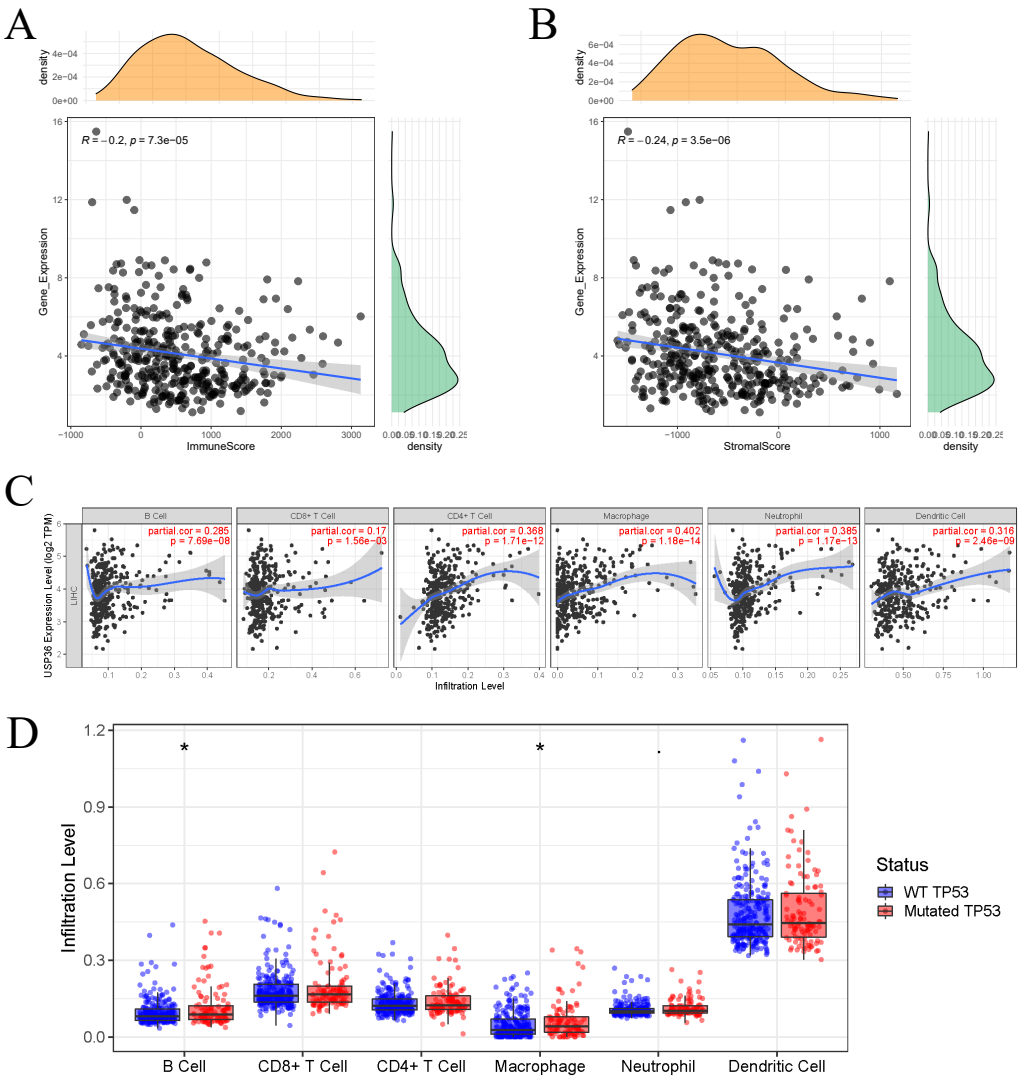

Fig. 6. Correlation between *USP36* expression and immune microenvironment. The Spearson test analyzed the correlation between *USP36* expression and Immunescore (A), Stromalscore (B). Correlation between *USP36* expression and infiltration levels of six types of immune cells (C). Levels of immune cell infiltration in different *TP53* mutation states (D).

Finally, we explored the relationship between *USP36* expression and sensitivity to commonly used HCC chemotherapeutics. The results of this analysis showed that high expression of *USP36* was associated with higher IC50 concentrations for sorafenib, rapamycin, docetaxel, and methotrexate (Fig. 7B).

4. Discussion

Ubiquitination is one of the most common and important post-translational protein modifications [27,28], and the ubiquitin-proteasome system is involved in the degradation of more than 80% of proteins in cells [29]. In addition, ubiquitination is also involved in diverse processes such as regulation of apoptosis, cell cycle progression, endocytosis, and transcription [5,27]. Ubiquitination and deubiquitination have been observed to be dysregulated in cancer and these processes play multiple roles in cancer-related pathways [30]. Interestingly, ubiquitination is a dynamically reversible process. DUB can effectively downregulate the ubiquitination of key target proteins, and thus further serve to control cell signaling events and cell fate.

At present, a large number of studies have shown that dysregulation of USPs are common molecular events in a variety of cancer types [31] and several studies have sought to clarify the role of USPs in liver cancer. For example, *USP7* can stabilize the E3 enzyme *TRIP12* and form a complex that promotes HCC cell growth and is significantly associated with HCC malignant phenotype [32]. *USP22* promotes hypoxia-induced HCC stemness by regulating *HIF-1α* [33]. However, we are unaware of a systematic study focused on the role of USPs family members in hepatocellular carcinoma and that this prompted us to seek to better understand an underlying mechanism(s) of USPs in HCC

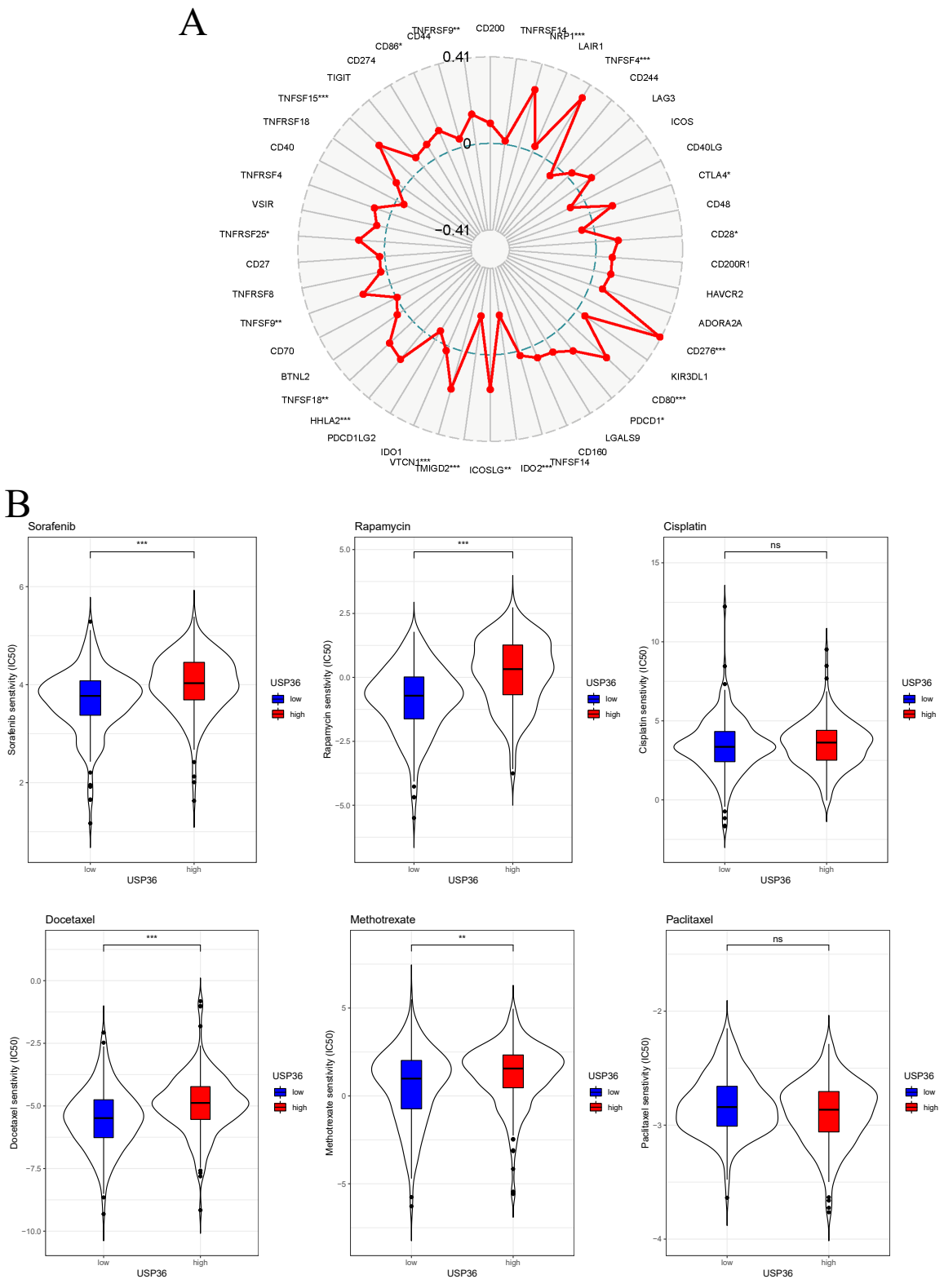

Fig. 7. Correlation analysis of *USP36* expression with immune checkpoint molecules and chemotherapeutic drug sensitivity. (A) Spearson test to analyze the correlation between *USP36* expression and 48 immune checkpoint molecules (B) Difference analysis of the sensitivity of 6 chemotherapeutic drugs in *USP36* high expression group and low expression group. "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$.

pathobiology.

Our analysis showed that the vast majority (36/39) of USPs were differentially expressed in HCC, suggesting that aberrant protein ubiquitination is prevalent in HCC. We further analyzed the prognostic value of these abnormally expressed USPs in HCC and found that more than ten USPs were linked to HCC prognosis. Importantly, this analysis indicated that *USP36* and *USP39* can simultaneously, and independently, affect OS, DFS, DSS and PFS in HCC patients, and support our conclusion that increased expression of these two genes are poor prognostic factors in HCC.

Previous studies explored a potential *USP39* mechanism of action in HCC. For example, *USP39* and *TRIM26* co-regulate *ZEB1* ubiquitination which controls HCC progression [19]. *USP39* promotes HCC progression by deubiquitinating *SP1* protein and stabilizing *SP1* [21]. In addition, high expression of *USP39* was significantly associated with age, tumor status, advanced pathological stage, T stage and higher histological grade in HCC [34].

Previous studies have shown that *USP36* can participate in the progression of a variety of cancer types [22,23], and *USP36* can stabilize the protein stability of the hepatoma promoting protooncogene *c-myc* through dysregulation of its ubiquitination [24,25]. However, *USP36* has not been studied in HCC. This lead us to explore the clinical significance and potential mechanism of *USP36* in HCC. We found that in HCC, *USP36* was highly expressed in three GEO databases (GSE14520, GSE36376, GSE64041) and validated this finding in 10 matched HCC tumor/normal tissue pairs. These studies revealed that *USP36* mutations are uncommon in HCC samples, but that abnormal gene methylation may be one of the reasons for altered *USP36* expression in this tumor type.

We also explored the correlation between *USP36* and 6 clinicopathological features. Results obtained showed no significant difference in *USP36* expression linked to different genders, ages, or lymph node metastasis. However, a significant correlation was noted with tumor stage and tumor grade as increased *USP36* expression is more common in higher tumor stage and grade in HCC. This finding prompts us to speculate that *USP36* may be involved in HCC progression and, more importantly, expression of *USP36* was significantly increased in HCC tumors with *TP53* mutation. Previous studies have shown that *TP53* is the most frequently mutated gene in HCC and is associated with poor prognosis [35–37]. Moreover, previous studies have explored a potential interaction between USPs and *TP53* gene mutations. For example, the *USP22*/*HIF1*α positive feedback loop promotes glycolysis and stemness after *TP53* mutation in HCC [33], and *USP10* promotes cancer cell proliferation in the presence of *TP53* mutations [38]. Taken together, these findings support the notion that *USP36* may synergize with *TP53* mutations to promote HCC progression. However, whether *USP36* plays an important role in *TP53*-mutated HCC is a scientific question worthy of attention in future studies.

We also analyzed the potential signaling pathways that are affected by *USP36* in HCC by using GSEA. Results indicated that the *USP36* high-expression group there was significantl enrichment in KEGG UBIQUITIN MEDIATED PROTEOLYSIS, KEGG ENDOCYTOSIS, and KEGG CELL CYCLE and other ubiquitination-related and cancer-related pathways [39,40]. Studies have shown that ubiquitination is closely related to the cell cycle and endocytosis and that this dysregulation can contribute to cancer progression [41–43]. Therefore, we speculate that *USP36* may affect HCC progression by affecting certain key cyclin expression or receptor endocytosis by dysregulating deubiquitination.

Tumor cells and immune cells, as well as many other cellular and acellular components, make up the complex tumor microenvironment (TME). The TME is highly heterogeneous and dynamic, and can ultimately impact the course of tumor progression [44]. Many studies have shown that TME plays a significant role in HCC and is a very promising potential therapeutic target [45–49]. Previous studies have shown that ubiquitination plays a crucial role in various physiological processes, including innate and adaptive immunity [8]; thus, the crosstalk between ubiquitination and TME deserves further exploration. It has been reported that *USP36* plays an important role in microbial immunity [50] leading us to explore a potential correlation between *USP36* and the TME. We used TIMER1.0 to explore the correlation between *USP36* expression and six immune cells found in the HCC TME. Notably, *USP36* expression showed the strongest correlation with macrophages in the TME. Previous studies have shown that hepatic macrophages contribute to the formation of HCC by suppressing antitumor immunity [51], and that high macrophage infiltration is generally linked to poor HCC prognosis [52]. The potential role of macrophages in HCC is consistent with our analysis of the possible tumor promoting effect of *USP36* in this tumor type, suggesting that *USP36* may promote HCC by affecting TME components. In addition, our results suggest that *USP36* is positively correlated with 16 immune checkpoint molecules, including *PDCD1* and *CTLA4*. Moreover, the IC50 of chemotherapy drugs such as sorafenib, rapamycin, docetaxel and methotrexate were significantly increased in the *USP36* high expression group. In conclusion, these results provide some theoretical basis for the use of *USP36* expression to help guide therapeutic regimen choices in HCC patients.

However, we must acknowledge some limitations in the current study. First, although we used the authoritative TCGA database, some members of the USPs were not included in the discussion of this study. Second, most of the results of this study were obtained by bioinformatics methods, and there were no *in vitro* and *in vivo* experiments and clinical data to verify. In the future, we will focus on the role of USPs members not included in the discussion

in HCC. Third, our analyses are all based on the TCGA database, which may reduce the reliability of our findings.

In conclusion, we comprehensively analyzed the expression and prognostic value of USPs in HCC, and analyzed the clinicopathological features of *USP36* as well as the potential mechanism of action in detail. We found that *USP36* expression is closely related to the tumor microenvironment, which has potential value in clinical application. Future studies should focus on the oncogenic mechanism of *USP36* in HCC as a means to independently validate our findings regarding *USP36* in this cancer type.

5. Conclusions

This study is the first to comprehensively analyze the gene characteristics and prognostic value of USPs members in HCC, and focus on the potential mechanism and immunological role of *USP36* in HCC. These findings provide new horizons for future research.

Abbreviations

ESTIMATE, Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data; qRT-PCR, Real-Time Quantitative Reverse Transcription PCR.

Author Contributions

YG and LL contributed to the study design. YG and WS were responsible for the writing and proofreading of the article. JS is responsible for data analysis and download. JL and KH took care of the experimental part and graphic editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (No.Quick-PJ2020-16-23).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2706190.

References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021; 71: 209–249.

[2] Heimbach JK, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, *et al*. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatology. 2018; 67: 358–380.

[3] Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nature Reviews Gastroenterology & Hepatology. 2019; 16: 589–604.

[4] Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, *et al*. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018; 68: 723–750.

[5] Hershko A, Ciechanover A. The UBIQUITIN SYSTEM. Annual Review of Biochemistry. 1998; 67: 425–479.

[6] Zheng N, Shabek N. Ubiquitin Ligases: Structure, Function, and Regulation. Annual Review of Biochemistry. 2017; 86: 129–157.

[7] Sun S. Deubiquitylation and regulation of the immune response. Nature Reviews Immunology. 2008; 8: 501–511.

[8] Popovic D, Vucic D, Dikic I. Ubiquitination in disease pathogenesis and treatment. Nature Medicine. 2014; 20: 1242–1253.

[9] Nijman SMB, Luna-Vargas MPA, Velds A, Brummelkamp TR, Dirac AMG, Sixma TK, *et al*. A Genomic and Functional Inventory of Deubiquitinating Enzymes. Cell. 2005; 123: 773–786.

[10] Liu Y, Penninger J, Karin M. Immunity by ubiquitylation: a reversible process of modification. Nature Reviews Immunology. 2005; 5: 941–952.

[11] van Loosdregt J, Fleskens V, Fu J, Brenkman A, Bekker CJ, Pals CGM, *et al*. Stabilization of the Transcription Factor Foxp3 by the Deubiquitinase USP7 Increases Treg-Cell-Suppressive Capacity. Immunity. 2013; 39: 259–271.

[12] Liu X, Li H, Zhong B, Blonska M, Gorjestani S, Yan M, *et al*. USP18 inhibits NF-κB and NFAT activation during Th17 differentiation by deubiquitinating the TAK1–TAB1 complex. Journal of Experimental Medicine. 2013; 210: 1575–1590.

[13] Zou Q, Jin J, Hu H, Li HS, Romano S, Xiao Y, *et al*. USP15 stabilizes MDM2 to mediate cancer-cell survival and inhibit antitumor T cell responses. Nature Immunology. 2014; 15: 562–570.

[14] Chandrashekar DS, Karthikeyan SK, Korla PK, Patel H, Shovon AR, Athar M, *et al*. UALCAN: an update to the integrated cancer data analysis platform. Neoplasia. 2022; 25: 18–27.

[15] Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, *et al*. TIMER2.0 for analysis of tumor-infiltrating immune cells. Nucleic Acids Research. 2020; 48: W509–W514.

[16] Liu SH, Shen PC, Chen CY, Hsu AN, Cho YC, Lai YL, *et al*. DriverDBv3: a multi-omics database for cancer driver gene research. Nucleic Acids Research. 2020; 48: D863–d870.

[17] Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, *et al*. Inferring tumour purity and stromal and immune cell admixture from expression data. Nature Communications. 2013; 4: 2612.

[18] Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, *et al*. TIMER: a Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells. Cancer Research. 2017; 77: e108–e110.

[19] Li X, Yuan J, Song C, Lei Y, Xu J, Zhang G, *et al*. Deubiquitinase USP39 and E3 ligase TRIM26 balance the level of ZEB1 ubiquitination and thereby determine the progression of hepatocellular carcinoma. Cell Death & Differentiation. 2021; 28: 2315–2332.

[20] Dong L, Yu L, Li H, Shi L, Luo Z, Zhao H, *et al*. An NAD+-

Dependent Deacetylase SIRT7 Promotes HCC Development through Deacetylation of USP39. IScience. 2020; 23: 101351.
[21] Dong X, Liu Z, Zhang E, Zhang P, Wang Y, Hang J, *et al*. USP39 promotes tumorigenesis by stabilizing and deubiquitinating SP1 protein in hepatocellular carcinoma. Cellular Signalling. 2021; 85: 110068.
[22] Deng Q, Wu M, Deng J. USP36 promotes tumor growth of non-small cell lung cancer via increasing KHK-a expression by regulating c-MYC–hnRNPH1/H2 axis. Human Cell. 2022; 35: 694–704.
[23] Li J, Olson LM, Zhang Z, Li L, Bidder M, Nguyen L, *et al*. Differential display identifies overexpression of the USP36 gene, encoding a deubiquitinating enzyme, in ovarian cancer. International Journal of Medical Sciences. 2008; 5: 133–142.
[24] Sun X, He X, Yin L, Komada M, Sears RC, Dai M. The nucleolar ubiquitin-specific protease USP36 deubiquitinates and stabilizes c-Myc. Proceedings of the National Academy of Sciences. 2015; 112: 3734–3739.
[25] Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, *et al*. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. Nature. 2004; 431: 1112–1117.
[26] Hu H, Sun S. Ubiquitin signaling in immune responses. Cell Research. 2016; 26: 457–483.
[27] Antao AM, Tyagi A, Kim KS, Ramakrishna S. Advances in Deubiquitinating Enzyme Inhibition and Applications in Cancer Therapeutics. Cancers. 2020; 12: 1579.
[28] Park HB, Kim JW, Baek KH. Regulation of Wnt Signaling through Ubiquitination and Deubiquitination in Cancers. International Journal of Molecular Sciences. 2020; 21: 3904.
[29] Glickman MH, Ciechanover A. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. Physiological Reviews. 2002; 82: 373–428.
[30] Mansour MA. Ubiquitination: Friend and foe in cancer. the International Journal of Biochemistry & Cell Biology. 2018; 101: 80–93.
[31] Park J, Cho J, Song EJ. Ubiquitin–proteasome system (UPS) as a target for anticancer treatment. Archives of Pharmacal Research. 2020; 43: 1144–1161.
[32] Cai JB, Shi GM, Dong ZR, Ke AW, Ma HH, Gao Q, *et al*. Ubiquitin-specific protease 7 accelerates p14(ARF) degradation by deubiquitinating thyroid hormone receptor-interacting protein 12 and promotes hepatocellular carcinoma progression. Hepatology. 2015; 61: 1603–1614.
[33] Ling S, Shan Q, Zhan Q, Ye Q, Liu P, Xu S, *et al*. USP22 promotes hypoxia-induced hepatocellular carcinoma stemness by a HIF1α/USP22 positive feedback loop upon TP53 inactivation. Gut. 2020; 69: 1322–1334.
[34] Liao Y, Li L, Liu H, Song Y. High Expression of Ubiquitin-Specific Protease 39 and its Roles in Prognosis in Patients with Hepatocellular Carcinoma. Evidence-Based Complementary and Alternative Medicine. 2021; 2021: 1–11.
[35] Gao Q, Zhu H, Dong L, Shi W, Chen R, Song Z, *et al*. Integrated Proteogenomic Characterization of HBV-Related Hepatocellular Carcinoma. Cell. 2019; 179: 561–577.e22.
[36] Wang L, Yan K, He X, Zhu H, Song J, Chen S, *et al*. LRP1B or TP53 mutations are associated with higher tumor mutational burden and worse survival in hepatocellular carcinoma. Journal of Cancer. 2021; 12: 217–223.
[37] Shi L, Cao J, Lei X, Shi Y, Wu L. Multi-omics data identified TP53 and LRP1B as key regulatory gene related to immune phenotypes via EPCAM in HCC. Cancer Medicine. 2022; 11: 2145–2158.
[38] Yuan J, Luo K, Zhang L, Cheville JC, Lou Z. USP10 Regulates p53 Localization and Stability by Deubiquitinating p53. Cell. 2010; 140: 384–396.
[39] Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004; 432: 316–323.
[40] Mellman I, Yarden Y. Endocytosis and Cancer. Cold Spring Harbor Perspectives in Biology. 2013; 5: a016949–a016949.
[41] Zou T, Lin Z. The Involvement of Ubiquitination Machinery in Cell Cycle Regulation and Cancer Progression. International Journal of Molecular Sciences. 2021; 22: 5754.
[42] Dang F, Nie L, Wei W. Ubiquitin signaling in cell cycle control and tumorigenesis. Cell Death & Differentiation. 2021; 28: 427–438.
[43] Mukhopadhyay D, Riezman H. Proteasome-Independent Functions of Ubiquitin in Endocytosis and Signaling. Science. 2007; 315: 201–205.
[44] Hinshaw DC, Shevde LA. The Tumor Microenvironment Innately Modulates Cancer Progression. Cancer Research. 2019; 79: 4557–4566.
[45] Niu Y, Lin Z, Wan A, Sun L, Yan S, Liang H, *et al*. Loss-of-Function Genetic Screening Identifies Aldolase a as an Essential Driver for Liver Cancer Cell Growth under Hypoxia. Hepatology. 2021; 74: 1461–1479.
[46] Hu B, Lin J, Yang X, Sang X. Aberrant lipid metabolism in hepatocellular carcinoma cells as well as immune microenvironment: a review. Cell Proliferation. 2020; 53: e12772.
[47] Song M, He J, Pan Q, Yang J, Zhao J, Zhang Y, *et al*. Cancer-Associated Fibroblast-Mediated Cellular Crosstalk Supports Hepatocellular Carcinoma Progression. Hepatology. 2021; 73: 1717–1735.
[48] Wu Q, Zhou L, Lv D, Zhu X, Tang H. Exosome-mediated communication in the tumor microenvironment contributes to hepatocellular carcinoma development and progression. Journal of Hematology & Oncology. 2019; 12: 53.
[49] Li B, Yan C, Zhu J, Chen X, Fu Q, Zhang H, *et al*. Anti-PD-1/PD-L1 Blockade Immunotherapy Employed in Treating Hepatitis B Virus Infection-Related Advanced Hepatocellular Carcinoma: A Literature Review. Frontiers in Immunology. 2020; 11: 1037.
[50] Chen P, Chen Y, Chu T, Ma T, Wu M, Lin H, *et al*. Nucleolar control by a non-apoptotic p53-caspases-deubiquitinylase axis promotes resistance to bacterial infection. The FASEB Journal. 2020; 34: 1107–1121.
[51] Dou L, Shi X, He X, Gao Y. Macrophage Phenotype and Function in Liver Disorder. Frontiers in Immunology. 2019; 10: 3112.
[52] Ding T, Xu J, Wang F, Shi M, Zhang Y, Li SP, *et al*. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. Human Pathology. 2009; 40: 381–389.