Ferroptosis-related genes coordinated with PD-L2 shapes matrix stiffness-driven tumor environment and predict hepatocellular carcinoma development

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Research Article

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Abstract

Background: Ferroptosis have been implicated in tumorigenesis, tumor progression, and immuno-therapy in cirrhotic hepatocellular carcinoma (HCC), indicating its association with extracellular matrix stiffness and immune checkpoint inhibitor. Here, we postulated that increased matrix stiffness reduces ferroptosis and impairs tumor immunity by regulating the expression of ferroptosis- and immune-related genes in HCC, which might be a robust predictor of therapeutic efficacy.

Methods: Using publicly available tissue microarray datasets, liver cancer rat model, and clinical specimen, ferroptosis-related differential genes in cirrhotic HCC and its mechanical heterogeneous pattern of expression were screened and identified. Further investigation on the underlying mechanism of matrix stiffness-regulated ferroptosis and the expression of immune mediator were performed.

Results: SLC7A11 and STEAP3 were identified as the ferroptosis-related differential genes in cirrhotic HCC, especially induced by HBV and HCV virus. Stiffer matrix increased SLC7A11 and decreased STEAP3 in the invasive front area of HCC and the liver cirrhotic tissue. Contrarily, softer matrix reduced SLC7A11 and induced STEAP3 in the central area of HCC and the normal liver tissue. Immunological correlation of SLC7A11 and STEAP3 in cirrhotic HCC showed that SLC7A11- or STEAP3-mediated immune infiltration of CD4+ T and CD8+ T cells, macrophages, neutrophils, and dendritic cells and HCC prognosis, predicting to regulate immune infiltration. Knockdown of SLC7A11 or overexpression of STEAP3 induced ferroptosis and inhibited the expression of immune mediator of PD-L2 on a stiff matrix.

Conclusions: This finding identifies matrix stiffness impairs ferroptosis and anti-tumor immunity by mediating SLC7A11, STEAP3, and PD-L2, and thus targeting it could be a potential diagnosis and treatment strategy for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth commonest cancer worldwide, and causes nearly 600,000 deaths each year. Approximately 80% of HCC frequently emerge on the background of liver cirrhosis by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [1]. Early detection and timely intervention are the best strategies to reduce morbidity in HCC patients [2]. Current multiple diagnostic methods including ultrasound, computed tomography, magnetic resonance imaging, selective hepatic angiography, and tumor biomarkers such as alpha-fetoprotein, and therapeutic methods including chemotherapy, immunotherapy, and surgery are widely used in HCC patients [3; 4]. However, HCC still has a poor prognosis and low 5-year overall survival rate, due to delayed diagnosis and limited clinical efficacy. Therefore, the clinical development of diagnostic, prognostic and predictive biomarkers is imminent.

Ferroptosis driven by accumulated iron-dependent lipid ROS is a novel form of nonapoptotic regulated cell death, which was first observed in Ras mutant tumor cells by treatment of oncogenic Ras-selective lethal small molecules, such as erastin and RSL3 [5]. Ferroptosis is mainly mediated by mevalonate (MVA) pathway, sulphur-transfer pathway, and the heat-shock factors (HSFs) -HSPB1 pathway in

mammalian cells [6]. Among them, the activation of cystine/glutamate antiporter SLC7A11 suppresses ferroptosis by improving the import of cysteine for glutathione biosynthesis and antioxidant defense [7], whereas the inactivation of STEAP3 (six-transmembrane epithelial antigen of prostate 3) inhibits ferroptosis by blocking the transport of iron [8]. Recent evidences showed that ferroptosis are associated with the pathological process of various diseases, including cirrhosis and HCC [9; 10]. For example, ferritinophagy-mediated ferroptosis of hepatic stellate cells mitigates carbon tetrachloride-induced hepatic fibrosis and reduces the development of HCC [11]. Moreover, sorafenib-induced ferroptosis significantly prolong the survival of advanced HCC patients[12], suggesting that ferroptosis induction may provide a promising new approach to the efficient triggering of cancer cell death. Altogether, a reasonable drug target and predictive biomarkers based on ferroptosis are required to select responsive HCC patients.

Tissue mechanics provide higher-order guidance for development and homeostasis, and are consistently modified in tumor progression. Excessive extracellular matrix (ECM) deposition and cross-linking lead to increased tissue stiffness in various cancer, such as HCC, breast cancers and other solid tumors [13]. Previous reports demonstrated that cells recognize and respond to matrix stiffness by exerting contractile force and sensing counter-tension [14]. Increased matrix stiffness promotes invasion, metastasis and chemoresistance capacity of tumor cells by triggering epithelial-mesenchymal transition (EMT) and activating the related signaling pathway, such as, Wnt/beta-catenin, RhoA/ROCK, TGF-beta, JNK/c-JUN and JAK/STAT [15; 16; 17]. Moreover, stiffer matrix regulates tumor immunity by activating immune infiltration of myeloid cells and the polarization of macrophage and neutrophil [18; 19]. Altogether, growing evidences have shown that matrix stiffness controls the tumorigenesis, progression and occurrence of HCC. However, the effect of matrix stiffness on ferroptosis and its immunity response in cirrhotic HCC remains unclear.

In addition, the tumor microenvironment (TME) contains a variety of cells (e.g., immune cells, stromal cells, endothelial cells, and cancer-associated fibroblasts), which facilitate tumor progression. Among them, infiltrating immune cell interactions with other components by immunoactivators and immunosuppressors in TME can impact tumor fate [20]. New therapeutic strategies target immune cells within the TME, including checkpoint blockade and chimeric antigen receptor (CAR) T cell therapies, have proven to be a promising treatment for various cancers. Antibodies targeting other immune checkpoints, such as programmed cell death-1 (PD-1) and its ligand (PD-L1), now have multiple approvals in patients with NSCLC, CRC, and other cancers [21; 22]. Recent studies reported that immune-related prognostic biomarker (e.g., PD-L1, PD-L2, SFRP4, CPXM1, and COL5A1) might bring some potential implications in the precision treatment of various cancers [23; 24; 25]. Therefore, exploring tumor immune microenvironment associated with tumor tissue mechanics and ferroptosis contributes to improved outcomes in patients with advanced HCC.

Given CD8⁺ T cells induced ferroptosis to suppress tumor growth by IFN- γ , the effect of immunomediator on ferroptosis and its predictive value emergently need to be explored [26]. Here, we investigated whether matrix stiffness mediates ferroptosis- and immune-related genes expression in cirrhotic HCC, which

participates in the regulation of ferroptosis and tumor immune environment, and affects patient prognosis. We first screened out differentially ferroptosis- and immune-related genes SLC7A11, STEAP3, and PD-L2, and verified mechanical heterogeneity of these gene expressions in HCC. Next, the effect of matrix stiffness on ferroptosis and the expression of PD-L2, and the underlying molecular mechanism were determined in vitro. We further explored the clinical value of matrix stiffness-dependent ferroptosis- and immune-related gene SLC7A11, STEAP3 and PD-L2 as predictor for prognosis. Collectively, these findings demonstrated that matrix mechanics mediated the ferroptosis of HCC cells and tumor immune environments through SLC7A11, STEAP3, and PD-L2.

Materials And Methods

Patients

In March 2021, HCC tissues were obtained from the Department of Hepatobiliary Surgery, Chongqing University Three Gorges Hospital (CUTGH), and collected during surgery and fixed in 4% paraformaldehyde. Clinical data were available to obtain from hospital records. This research was supported by the Independent Ethics Committee (IEC) of the CUTGH and all patients were well informed of storing and upcoming use of their resected specimens for further research purposes.

Sequencing datasets

Four independent microarray data (accession number: GSE45050, GSE17548) were downloaded from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), and the quality of the data was assessed by RMAExpress software (<http://rmaexpress.bmbolstad.com>). Quality evolution of the Affymetrix datasets was carried out by affyPLM (<http://www.bioconductor.org>). Class comparison analysis were performed by BRB-Array tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>; Version 4.2.0) after normalization with RMAExpress software. RNA sequencing and clinical data of 160 normal people and 369 HCC patients were obtained from the TCGA data portal (<http://cancergenome.nih.gov/>). The GSE45050 dataset included 3 normal samples and 6 HCC tissue samples. Expression data from the GSE17548 dataset included 14 cirrhosis samples with HBV and HCV positive and 13 HCC samples.

Identification of differential expression genes and clinical prognosis analysis

The identification of differentially expressed genes (DEGs) in normal control (NC) liver cirrhotic (LC) and HCC tissues from four independent microarray data (accession number: GSE45050, GSE17548) were detected by the empirical Bayes method [27]. A false discovery rate (FDR) adjusted *P-value* < 0.01 and |log fold change| ≥ 1.0 was set as the threshold for differentially expressed genes. Functional enrichment analysis of the DEGs with GO and KEGG pathways was carried out using G: Profiler (<https://biit.cs.ut.ee/gprofiler/gost>).

Meanwhile, the expression analysis of DEGs was performed by using the free online web platform at <http://gepia.cancer-pku.cn/index.html>. Briefly, the expression of each indicated gene was evaluated in

LIHC, data set matched with 'TCGA normal and GTEx data' set (normal tissue), with a $|Log_2FC|$ Cutoff of 1, a p -value Cutoff of 0.01, and results showed using a $log_2(TPM + 1)$ log scale. The overall survival curves were generated by using the following parameters: Group Cutoff = Median, with Cutoff-High (%) = 50 and Cutoff-Low (%) = 50; Hazards Ratio (HR) = Yes.

Correlation analysis of SLC7A11 and STEAP3 with immune cells infiltration

The abundance of immune infiltrations was estimated by TIMER, which is a comprehensive resource for systematic analysis of clinical impact across diverse cancer types (<https://cistrome.shinyapps.io/timer/>), including 10,897 samples across 32 cancer types from The Cancer Genome Atlas (TCGA). Simply, the abundance of six immune cell types: B cell, CD4⁺ T cell, CD8⁺ T cell, neutrophil, macrophage and dendritic cell in the tumor microenvironment is estimated. The correlations between SLC7A11 or STEAP3 and infiltrating levels of various immune cells in HCC were checked. The expression level (displayed with log_2 RSEM) and copy number of SLC7A11 or STEAP3 were shown on X axis, and the infiltration level is shown on Y-axis. An integrated repository portal for tumor-immune system interactions (TISIDB, <http://cis.hku.hk/TISIDB/index.php>) was utilized to examine tumor and immune system interactions in 28 types of tumor infiltrating lymphocytes (TILs) across human cancers. Then, the correlations between prognostic genes and TILs were measured by Spearman's test. Some other databases used are listed in **Table S1**.

Animal models

All animal experiments were approved by the Institutional Animal Care and Use Committees and performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines (<http://www.aaalac.org>). 5-week-old male sprague dawley (SD) rats were given a single injection of diethylnitrosamine (DEN) at a dose of 100 mg/kg body weight as an initiation of liver carcinogenesis. Rats were administered 40 ppm N-nitrosomorpholine (NMOR) in drinking water for 14, 20 and 24 weeks. An interim sacrifice was performed at week 14, 20 and 24. After liver tumors were excised, plane sections of the livers were prepared for each rat, processed for production of paraffin sections, and stained with hematoxylin and eosin (HE).

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC). HepG2 and HCCLM3 cells lines were cultured in high-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin.

Preparation of matrix with different stiffness

Matrix with various stiffness was prepared by adjusting the chemical composition of polyvinylalcohol (PVA) solution according to Table S1 [28]. Briefly, 8 g PVA powder (Aladdin, Los Angeles, CA, USA) was dissolved in 100 mL water and stirred for 2 h at 95 °C to obtain PVA solution. 37% HCl (catalyst) and 25% Glutaraldehyde (Ourchem, Shanghai, China) with the desired composition (**Table S2**) were mixed to 8% PVA hydrogel of 10 mL. The hydrogels were soaked in 75% ethanol and then incubated with *N*-hydroxyethylpiperazine-*N*-ethanesulfonic acid (HEPES, pH7.2-7.4, H3375, Sigma–Aldrich, St. Louis, MO, USA) for 45 min, and were washed by sterilized HEPES three times. Next, Collagen-I (10 µg/mL, Corning, China) of rat tail was cross-linked to the PVA gel surface at 4 °C for 12 h by the addition of Sulfo-SANPAH (1 mg/ml in HEPES, Thermo, USA) to facilitate the cell adhesion. Finally, the gels were washed twice with phosphate buffer solution (PBS) to remove unreacted fibronectin before cell culture.

Quantitative PCR analysis

Total cellular RNA was extracted from cells with the RNeasy Mini Kit (TIANGEN, DP419, China) according to the manufacturer's guidelines. Then, 1 µg of total RNA was reversed transcript to cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Scientific, Waltham, MA). Quantitative PCR (qPCR) was performed using iQSYBR Green Master Mix (Bio-rad) on the CFX96 Touch Quantitative PCR Detection System. Gene expression was quantified using the primers from **Table S3**.

Western blotting

Cells were dissolved in RIPA buffer (10 mM Tris-HCl pH 7.5, 1% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 5 mM EDTA) plus a protease inhibitor). Protein concentrations were quantified using a BCA Protein Assay Kit (Beyotime Biotechnology). The same amount of protein (40 µg) was separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene fluoride (PVDF) membranes (Immobilon -P^{SO}). After transfer, the membranes were blocked with 5% skim milk powder in TBST buffer (50 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20, pH7.4) at RT for 1 h and were incubated with the following antibodies at the indicated concentrations overnight at 4°C. The antibodies included anti-GAPDH (loading control); anti-SLC7A11; anti-STEAP3; anti-SLC3A2; anti-GPX4; anti-Ferritin. Proteins were visualized using ECL plus fluorescence detection reagent (Thermo Scientific, Waltham, MA).

Immunohistochemical staining

IHC staining was performed on representative tissue sections from formalin-fixed (4%) and paraffin-embedded tissue blocks from mice hepatic tumor tissue. Simply, sections were first deparaffinized in xylene, hydrated in gradient alcohol followed by antigen retrieval in citric acid buffer (pH 6.0). After washing with PBS (3 × 5 min), sections were incubated with 3% H₂O₂ for 10 min and then blocked with blocking reagent (QuickBlock™ Blocking Buffer, Beyotime, Beijing, China) for 30 min at 37°C. Thereafter,

the sections were covered overnight at 4°C using the following antibodies at the indicated concentrations: anti-SLC7A11 (Proteintech) 1:50; anti-STEAP3 (Proteintech) 1:20; anti-GPX4 (Proteintech) 1:50. Relevant second antibody IgG HRP (Horse-Radish Peroxidase) were incubated for 30 min at 37 °C. Subsequently, samples were developed by incubation in diaminobenzidine (DAB) Detection Kit (PV9000, ZSGB-BIO, China). The stained slides were mounted for observation and all images were taken using bright-field microscopy (Leica, Germany).

Immunofluorescence staining

Liver cancer cells with different matrix stiffness were fixed in 4% (v/v) paraformaldehyde (Solarbio, Beijing) solution at room temperature for 15 min, permeabilized with 0.2% (v/v) Triton X-100 (Solarbio, Tongzhou, BJ) for 15 min, washed with PBS three times, and blocked with 2% (w/v) BSA (Solarbio, Beijing) at 37 °C for 30 min. Then, cells were incubated with primary antibodies against SLC7A11 (Proteintech, 1:100), STEAP3 (Proteintech, 1:100), PD-L1 (Proteintech, 1:100), PD-L2 (Thermo, 1:100) and at 4 °C overnight, followed by an incubation with goat anti-rabbit IgG (H&L) TRITC secondary antibodies (ZEN BIO, Chengdu) and donkey anti-rabbit Alexa Fluor 555 secondary antibodies (Beyotime, Jiangsu) at 37 °C for 30 min. Nuclei were stained with DAPI (Cell Signaling, Danvers, MA). Images were obtained by using a laser confocal fluorescent microscope (Leica, Germany).

Young's modulus analysis

The Young's modulus of tumor center and invasive frontier area of HCC tissue was measured by atomic force microscopy (AFM, JPK Instruments, NanoWizard II, Germany). The silicon nitride spherical probe used in the experiment was purchased from Nanosensors (Neuchatel, Switzerland) with a diameter of 4 µm, a spring constant of 0.2 N/m, and a poisson's ratio of 0.5. The selected tapping mode was used to detect Young's modulus. Finally, the Young's modulus of liver tumor tissue was calculated by Force-displacement curves.

Statistical analysis

Statistical analysis was performed by GraphPad Prism (version 8, San Diego California USA). All experiments were analyzed with a minimum of three independent repeats. Statistical tests applied were One-Way analysis of variance (ANOVA) by Tukey-test. Data are presented as mean ± standard error of mean (SEM). * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Differential expression pattern of SLC7A11 and STEAP3 in cirrhotic HCC

To identify the expression pattern of ferroptosis-related genes in HCC, mRNA datasets (GSE45050) and 33 ferroptosis-related genes were screened. The screening results showed that there were 1119 DEGs and 2 ferroptosis-related DEGs (SLC7A11 and STEAP3) in HCC vs. normal liver tissue (Fig. 1A, B and **Figure S1A**). To validate the expression difference of SLC7A11 and STEAP3 in HCC, the mRNA level was analyzed. Compared with the normal liver tissues, SLC7A11 was significantly upregulated and STEAP3 was downregulated in HCC (Fig. 1C). Previous research demonstrated that HCC cases mainly developed in a background of HBV- and HCV- induced cirrhosis. To further confirm these DEGs in cirrhotic HCC caused by hepatitis B and C, mRNA datasets (GSE17458) and 33 ferroptosis-related genes were screened. The results showed that there were 674 DEGs (Fig. 1D, S1B) and 1 ferroptosis-related DEGs (SLC7A11) in HBV-infected cirrhotic HCC (Fig. 1E). The mRNA expression of SLC7A11 was significantly upregulated in HBV-infected cirrhotic HCC compared with the normal liver tissues, but STEAP3 was no significant (Fig. 1F). Correspondingly, 590 DEGs (Fig. 1G, S1B) and 1 ferroptosis-related DEGs (STEAP3) were observed in HCV-infected cirrhotic HCC (Fig. 1H). The mRNA level of STEAP3 was significantly reduced, and SLC7A11 was no significant in HCV-infected cirrhotic HCC (Fig. 1I). These results suggested that there was a significant difference in the expression of ferroptosis-related genes SLC7A11 and STEAP3 in cirrhotic HCC.

To investigate the possible mechanism underlying the differential expression of SLC7A11 and STEAP3, mRNA dataset (GSE17458) from HBV- and HCV-infected cirrhotic patients was screened. The results showed that there were 254 DEGs in HBV- and HCV-infected cirrhosis (Fig. 2A). Subsequently, to identify these DEGs function, DAVID database analysis was performed. 452 biological process (BP) terms, 69 cellular component (CC) terms, 134 molecular function (MF) terms, and 67 pathways were obtained (Fig. 2B, C). These signaling pathways included extracellular matrix structural constituent and organization, collagen fibril organization and collagen-containing extracellular matrix, indicating the expression of SLC7A11 and STEAP3 are association with matrix stiffness.

Mechanical heterogeneity of SLC7A11 and STEAP3 expression in cirrhotic HCC

Increasing evidences showed that extracellular matrix stiffness from tumor central (TC) to invasion frontier (IF) area is gradually harden in cirrhotic HCC progression, which is named as mechanical heterogeneity of tumor tissue [29]. To investigate whether matrix stiffness involves in the regulation of SLC7A11 and STEAP3, we firstly observed its expression in specimens of cirrhotic human HCC. Representative picture of HE staining displayed obvious tumor characteristics in cirrhotic HCC specimen (**Figure S2B**). Compared with the TC region, immunofluorescent staining images showed that SLC7A11 expression was significantly increased, while STEAP3 was decreased in the IF region of HCC tissues (Fig. 1J-L), indicating that matrix stiffness predisposes the expression of SLC7A11 and STEAP3. Subsequently, to further validate the relation between matrix stiffness and the expression of SLC7A11 and STEAP3, a DEN + NMOR-induced rat model of cirrhosis and HCC was used. HE staining and masson images showed the lesions of cirrhosis and HCC, especially (**Figure S2A**). AFM results showed that there was a higher young modulus in liver cirrhotic (LC) tissues than in the normal liver tissues (Fig. 3A).

Accompanied with the normal liver tissues, SLC7A11 expression was significantly increased in cirrhotic tissues, while STEAP3 was decreased (Fig. 3B). Correspondingly, increased young's modulus from TC to IF region in rat HCC tissues was observed (Fig. 3C). Immunohistochemically staining images showed that SLC7A11 expression was significantly increased, while STEAP3 was decreased in the IF region, compared with the TC region of rat HCC tissues (Fig. 3D). Meanwhile, the expression of glutathione peroxidase 4 (GPX4), a key inhibitory enzymes of ferroptosis, was higher expressed in IF region than that in TC region (**Figure S2C**).

To further verify whether the expression of SLC7A11 and STEAP3 are mediated by matrix stiffness, two human HCC cell lines, HCCLM3 and HepG2, were cultured and checked on tunable PVA hydrogel with different stiffness (**Table S2**). The mRNA and protein level of SLC7A11 on stiff matrix gels (40kPa) were significantly higher than those on soft (2kPa), but STEAP3 were lower (Fig. 3E, F). Subsequently, immunofluorescent staining images also showed the consistent results (Fig. 3G). These results demonstrated that matrix stiffness directs heterogeneity of SLC7A11 and STEAP3 expression, indicating that it might mediate ferroptosis of HCC cells.

Matrix stiffness regulates ferroptosis in HCC cells via SLC7A11 and STEAP3

To explore the role of SLC7A11 and STEAP3 in cirrhotic HCC, gene functional enrichment analysis of the top 100 correlated genes was performed. The results showed that ferroptosis-related lipid metabolism (BP) and iron ion binding (MF) were enriched in both SLC7A11- and STEAP3- correlated gene sets (Fig. 4A, B), indicating its association with ferroptosis of HCC cells. To investigate ferroptosis of HCC cells, HCCLM3 and HepG2 cells were treated by 0, 1, 5, and 10 μ M RSL3 (ferroptosis activators) for 24h. The CCK-8 assay results showed that cell activity was significantly decreased with the increased concentration of RSL3 in HCCLM3 and HepG2 cells (**Figure S3A**). The mRNA and protein level of SLC7A11 were increasingly reduced, and STEPA3 were increasingly induced by RSL3 in a dose-dependent manner (**Figure S3 B–D**), indicating that 10 μ M and 1 μ M RSL3 is available for the induction of ferroptosis of HCCLM3 and HepG2 cells. To verify whether matrix stiffness regulates ferroptosis of HCC cells, HCCLM3 and HepG2 cultured on the soft (2KPa) and stiff (40KPa) PVA hydrogel were treated by 10 μ M RSL3 for 24 h. The CCK-8 assay results showed that stiff matrix significantly impaired RSL3-reduced cell activity in HCCLM3 and HepG2 cells (Fig. 5A). Meanwhile, stiff substrate significantly increased the mRNA and protein expression of SLC3A2 and GPX4 (Fig. 5B, C), and reduced ROS production, a ferroptosis-related downstream maker, in HCCLM3 and HepG2 cells (Fig. 5D). These results identified matrix stiffness as the key mediator of ferroptosis in HCC cells.

To further clarify whether matrix stiffness regulates ferroptosis via SLC7A11 and STEAP3 in HCC cells, knockdown of SLC7A11 or overexpression of STEAP3 in HCCLM3 and HepG2 cells were performed. The results of qRT-PCR and western blot showed that knockdown of SLC7A11 significantly reduced the expression of GPX4 (Fig. 5E and **Figure S3E**), and overexpression of STEAP3 significantly inhibited the

expression of GPX4 and ferritin on a stiff matrix (Fig. 5F **and Figure S3F**). MDA assay showed that knockdown of SLC7A11 promoted RSL3-induced ferroptotic cell death in both HCCLM3 and HepG2 cells (Fig. 5G). To evaluate STEAP3 functions as a ferrireductase, the level of ROS production was quantified by DCFH-DA in HCCLM3 and HepG2 cells. The results showed that the level of ROS was significantly up-regulated in STEAP3-overexpressed HCCLM3 and HepG2 cells (Fig. 5H). Subsequently, the CCK-8 assay results showed that knockdown of SLC7A11 or overexpression of STEAP3 significantly enhanced RSL3-reduced cell activity in HCCLM3 and HepG2 cells (Fig. 5I, J). Collectively, these results suggested that matrix stiffness suppressed ferroptosis in HCC cells by upregulating SLC7A11 and downregulating STEAP3.

Prognostic significance of SLC7A11 and STEAP3 in HCC patients

To investigate the prognostic significance of SLC7A11 and STEAP3 in HCC, gene expression profile, mutation spectrum, and methylation were comprehensively analyzed (Fig. 6). The heatmap showed that the expression of ferroptosis-related genes was mainly affected by copy number (Fig. 6B). Compared with the normal tissues ($n = 160$), the expression of SLC7A11 was upregulated in HCC tissues ($n = 369$) (Fig. 7A). By contrast, the expression of STEAP3 was downregulated (Fig. 7B). An approximately upward trend with SLC7A11, and downward trend with STEAP3 in different tumor stages (TS) and tumor grades (TG) of HCC patients were observed, respectively (Fig. 7C–F). To further evaluate the role of SLC7A11 and STEAP3 expression on the prognosis of HCC patients, mRNA data and overall survival (OS) time from TCGA databases were evaluated. Survival analysis showed that HCC patients with high expression of SLC7A11, or with low expression of STEAP3 had a short OS time (Fig. 7G, H). These results suggested that SLC7A11 and STEAP3 were the potential biomarkers of the prognosis in HCC patients.

Given mutation implicated in the survival and recurrence of cancer patients [30], the mutation landscape of 33 ferroptosis-related genes in HCC samples was analyzed. It was found that the mutation rates of SLC7A11 and STEAP3 in HCC were 4% and 6%, respectively (Fig. 6A). To explore the effect of SLC7A11 and STEAP3 mutation on mRNA expression and DNA methylation, cBioPortal databases were used. The results showed that the mRNA expression was significantly up-regulated ($P < 0.0001$) after SLC7A11 or STEAP3 mutation, but the methylation level was significantly down-regulated in HCC ($P < 0.0001$) (Fig. 8A–D). The OS analysis of SLC7A11 mutation showed that there was significantly shorter survival in the altered SLC7A11 groups ($P = 0.038$), compared with the unaltered groups (Fig. 8E). The altered STEAP3 was no significant effect ($P = 0.431$) on the survival and prognosis of patients (Fig. 8F). These results indicated that SLC7A11 mutation contributed to worse overall survival.

Given DNA methylation, most frequently occurred at CpG dinucleotides, correlated to mRNA expression and prognosis of cancer patients [31], methylation analysis with Mexpress databases was performed. The methylation heatmap showed that the expression of ferroptosis-related genes was affected by methylation, and its methylation degree was significantly varied (Fig. 6C). Accordingly, a total of 13 types' methylations of SLC7A11 and 3 types' methylations of STEAP3 were obtained. Among them,

cg06206831 and cg26888222 predicted a worse prognosis (Fig. 8G). To further predict the sensitive and specific of survival, the area under the curves (AUC) was assessed by receiver operating characteristic (ROC) curve analysis. The results showed that the AUC of SLC7A11 and STEAP3 were 0.979 and 0.846, respectively (Fig. 8H, I). All the above, these results suggested that SLC7A11 and STEAP3 were associated with overall survival of HCC patients.

SLC7A11 and STEAP3 are correlated with immune infiltration in HCC

Previous studies demonstrated that adaptive immunity implicated in the pathogenesis, development and treatment of cancer [32]. To better understand the effect of SLC7A11 and STEAP3 on immune contexture of HCC, the proportion of immune cell infiltration, immune cell recruitment and signature genes expression in different stages of cirrhosis and HCC samples were investigated. The heatmap showed that the immune score of each patient sample was specificity. Among them, Treg cells, Th cells and pDC cells with different proportion were involved (Fig. 9A). Observingly, the degree of immune cell infiltration in HBV-induced liver cirrhosis was significantly higher than that in HCC at different stages of cancer immunity, indicating that HBV-induced HCC may be immunosuppression. Unexpectedly, due to the small number of HCV-induced cirrhosis and HCC samples, no significantly difference was observed (Fig. 9A and Fig. 10A). Contrarily, the signature genes expression analysis showed the expression of immune-related genes in HCV-induced HCC was higher than that in cirrhosis. Nevertheless, no significantly difference was observed in HBV-induced cirrhosis and HCC (Fig. 9B). These results indicated that immune cell infiltration might orchestrate the development of HCC.

To further explore whether SLC7A11 and STEAP3 mediate immune infiltration, the correlation analysis was performed. The results showed that there was no significant relationship between immune infiltration of B cell, CD4 + T cell, CD8 + T cell, macrophage, dendritic cell, and neutrophil, and HCC patient survival (Fig. 10B). The mRNA level of SLC7A11 were negatively correlated with tumor purity, but positively correlated with the infiltration of B cell, CD8 + T cell, CD4 + T cell, macrophage, neutrophil and dendritic cell. Similarly, STEAP3 was negatively correlated with tumor purity and the infiltration of CD8 + T cell, macrophage and dendritic cell, but positively correlated with CD4 + T cell and neutrophil (Fig. 10C). Among them, copy number (deep deletion) of SLC7A11 had a significant effect on the immune infiltration level of CD8 + T cell ($P < 0.001$) (Fig. 10D, E). Subsequently, tumor immunologically quiet, TGF- β dominant and molecular subtypes of HCC patients were analyzed. The results showed that the expression of STEAP3 was significantly correlated with immune and molecular subtypes, but no significant for SLC7A11 (Fig. 10F, G). Taken together, these results demonstrated that SLC7A11 and STEAP3 directed immune microenvironment through immune infiltration in cirrhotic HCC, indicating its association with tumor immunity.

SLC7A11 and STEAP3 mediate immunogulator PD-L2 expression in HCC

Previous studies have proved immune checkpoints as a therapeutic target in malignancies [33]. To investigate whether SLC7A11 and STEAP3 direct tumor immunity, gene expression profile analysis of all immune checkpoints in HCC patients was firstly checked. Compared with the normal liver tissues, there was a significantly increased expression of PD-L2, CTLA4, HAVCR2, PDCD1, TIGIT and Siglec-15 in HCC patients, (Fig. 11A). Subsequently, the analysis results of q-PCR and western blot showed that stiff substrate significantly increased the gene expression of PD-L2 in HCCLM3 and HepG2 cells *in vitro* (Fig. 11B). To further evaluate the relation between matrix stiffness and PD-L2, a liver cirrhotic rat model and human cirrhotic HCC specimens were used. Accompanied with the normal liver tissues and TC region, PD-L2 expression was significantly increased in cirrhotic tissues (Fig. 11C) and in the IF region of HCC tissues, respectively (Fig. 11D, E-2). Unexpectedly, there was no significant differences for PD-L1. However, accompanied with the TC region, PD-L1 expression was significantly decreased in the IF region of HCC tissues (Fig. 11D, E-1). To further identify its correlation with SLC7A11 and STEAP3, the analysis of correlation coefficients was performed. The results showed that PD-L1 was significant correlation with STEAP3, but no significant correlation with SLC7A11. No significant correlation between PD-L2 and SLC7A11 or STEAP3 was observed (data no show). Knockdown of SLC7A11 or overexpression of STEAP3 significantly suppressed the expression of PD-L2 in HCCLM3 and HepG2 cells cultured on a stiff matrix (Fig. 11H, I). Similarly, there were no significant differences for PD-L1 (Fig. 11F, G). Generally, these results suggested SLC7A11 and STEAP3 could affect tumor immune microenvironment via regulating matrix stiffness-dependent immune checkpoint receptor PD-L2 in HCC cells.

In addition, gene expression profile analysis of all immune stimulators and chemokines in HCC patients was performed. Compared with the normal liver tissue, the expression of immunogulator CCL14 and CXCL2 was significantly decreased in HCC patients. Subsequently, the analysis results of correlation coefficients showed that CCL14 was significantly negative correlation with SLC7A11. CCL-14 and CXCL2 were significantly positive correlation with STEAP3. Knockdown of SLC7A11 or overexpression of STEAP3 significantly increased the expression of CCL14, and decreased the expression of CXCL2 in HCCLM3 and HepG2 cells. Unexpectedly, the analysis results of q-PCR showed that CCL14 expression was no significant differences under stiff substrate conditions, indicating that SLC7A11 and STEAP3 could mediate the tumor immune microenvironment partly by matrix stiffness-independent way in HCC cells (data no show).

Discussion

Previous evidences proposed that mechanical cues (eg, matrix stiffness) of the environment orchestrates the pathogenesis and development in various cancers, including HCC, and breast cancers as well as other solid cancers [16; 34]. Ferroptosis contribute to tumorigenesis and tumor progression in HCC caused mainly liver cirrhosis, which has frequently been a potential target of tumor therapy [35]. However, the role of matrix stiffness on the regulation of ferroptosis and tumor immunity in HCC, and its underlying mechanism remains elusive. Here, we identified that matrix stiffness suppressed ferroptosis of HCC cells through regulating the expression of SLC7A11 and STEAP3, which in turn shaped matrix mechanic-driven tumor immunity environment in HCC partily via PD-L2.

Ferroptosis is a novel non-apoptotic form of cell death through inhibiting SLC7A11-dependent glutathione biosynthesis or activating STEAP3-mediated lipid peroxidation by iron-dependent lipid reactive oxygen species (ROS) [5]. Here, we verified that matrix stiffness induces SLC7A11 expression and reduces STEAP3 expression to suppress ferroptosis of HCC cells, indicating matrix stiffness promotes HCC growth. This results are consistent with previous reports of SLC7A11 and STEAP3. SLC7A11 (also commonly known as xCT) functions as a cystine/glutamate antiporter to import cystine for glutathione biosynthesis and antioxidant defense, which is frequently overexpressed in multiple human cancers [7]. The overexpression of SLC7A11 promotes HCC growth through suppressing ferroptosis. Correspondingly, STEAP3 is a major ferric reductase, which suppresses ferroptosis through deoxidizing Fe^{3+} to Fe^{2+} [36]. The reduction of STEAP3 impairs ferroptosis of tumor cells and promotes HCC progression [37]. In addition, we also demonstrated that matrix stiffness induces the expression of GPX4, a central regulator of ferroptosis-triggering mechanisms, and impairs the accumulation of ROS in HCC cells. Given the inhibition of glutathione biosynthesis and iron-dependent accumulation of lipid peroxidation kills cells undergoing ferroptosis, both glutathione biosynthesis and iron metabolism are two critical processes involved in the mechanism of ferroptosis. Therefore, our results verified that tissue mechanical cues direct ferroptosis of HCC cells in these two aspects.

Mechanical features of liver tissues play a vital role not only in tuning of physiological liver functions but also in its pathological progression. Accordingly, mechanical factors of liver tissues have been provoked as diagnostic markers and potential therapeutic targets [38; 39]. In HCC tissues, matrix stiffness from tumor central (TC) to invasion frontier (IF) area is gradually harden [29]. Consisted with this studies, our results showed an increased matrix stiffness in liver tumor tissue of DEN-induced rats. The stiffness of normal human liver tissue was shown to be approximately less than 2.5KPa in physiological conditions, increasing to 2.5–12.5 kPa in fibrotic liver tissues, and 12.5–75 kPa in cirrhotic HCC tissues [14]. Consisted with previous studies, our results showed an increased matrix stiffness in fribrosis tissue of DEN-induced rats. Recently, polydimethylsiloxane (PDMS) substrate or other matrix gel have been designed to represent the stiffness of healthy (1-2.5kPa) and diseased livers (10–55 kPa), respectively. Indeed, we here observed that the 40 kPa (TC) PVA substrates induces SLC7A11 expression and reduces STEAP3 expression in human HCC cells in vitro, compared with 2 kPa (IF). Similarly, we demonstrated that matrix stiffness induced the expression of immune checkpoint receptor PD-L2, not PD-L1, indicating PD-L2-dependent less anti-tumor immunity in IF area. Previous studies have demonstrated that SLC3A2 (CD98hc), ubiquitously expressed heavy subunit of heteromeric amino acid transporters that bind to an SLC7 family member (e.g., SLC7A11), was required for ECM to fully exert its structural and contributed to carcinogene [40; 41]. Overexpression of CD98hc occurs widely in HCC cells, and is associated with poor prognosis clinically [42]. Here, we observed that the expression of CD98hc was promoted by stiffer matrix. Therefore, we suspected that matrix stiffness might mediate ferroptosis to promote HCC development partly via CD98hc. In addition, many positive and negative regulators (e.g., p53, ATF3/4, Nrf2, STAT3 and BAP1) have been identified in regulating the expression and activity of SLC7A11 and STEAP3 [43; 44; 45; 46].

Additionally, cancer immuno-therapy and prediction based on immune checkpoint inhibitors (ICIs) has achieved considerable success in the clinic [47]. However, the clinical application of ICIs was significantly limited by the fact that less than one-third of patients with most types of cancer respond to these agents. As most tumors harbor innate resistance to apoptosis, the induction of ferroptosis has gradually emerged as a novel cancer treatment strategy. Previous studies reported that CD8 + T cells induced ferroptosis by suppressing the expression of SLC7A11 and SLC3A2 in ovarian tumor cells [26]. Immune checkpoint inhibitor PD-L1 blocked treatment enhanced the accumulation of lipid ROS in macrophage [48]. SLC7A11 regulated the proliferation of immune cells, and systemic inhibition of xCT may compromise antitumor immunity [7]. Accordingly, STEAP3 have been implicated in the regulation of immune response in various cell types [36]. Consistent with previous studies, our results showed that the gene feature (expression level and copy number) of SLC7A11 and STEAP3 were closely correlated with the numbers of tumor-infiltrated immune cells (e.g., CD8 + T cells) in HCC. Knockdown of SLC7A11 or overexpression STEAP3 impaired the expression of PD-L2 on stiffer matrix in vitro. This results indicated that SLC7A11/STEAP3 and PD-L2 may play a synergistic role in HCC immune microenvironment.

We and others have shown that matrix stiffness is a elegant physical environment cues constantly being challenged in physiology and disease [49]. Athouth the underlying mechanism remains unclear, the occurrence of ferroptosis and anti-tumor immunity inhibition associated with matrix stiffness is likely to be a common phenomenon. In normal tissues, environment factors would trigger cellular ferroptosis and adaptive immunity, contributing to developmental programs and tissue homeostasis, or even facilitating in tissue fibrosis and tumorigenesis. In contrast, in tumor tissues, in which the proliferative arrest and anti-tumor immunity has been lost, matrix stiffness-inhibited ferroptosis and immune evasion in the IF area of tumor would lead to an aberrant tumor growth, and metastasis as well as drug resistance.

Conclusions

Our findings identify that SLC7A11 and STEAP3 characterize matrix mechanical heterogeneity and immune environment of tumor, and possess prognosis and predictive value of clinical outcome in cirrhotic HCC. Particularly, matrix stiffness impairs ferroptosis by upregulating SLC7A11 and downregulating STEAP3 in cirrhotic HCC, which in turn direct the infiltration of tumor immune cells, and exacerbate anti-tumor immunity partly via PD-L2. This finding provides a novel insights and clinical application on ferroptosis, illustrating SLC7A11 and STEAP3 supplemented with PD-L2 as the potential therapeutic targets and predictive biomarker for cirrhotic HCC.

Abbreviations

HCC: hepatocellular carcinoma; LC: liver cirrhotic; NC: normal group; HBV: hepatitis B virus; HCV: hepatitis C virus; DEGs: differentially expressed genes; GEO: Gene Expression Omnibus; ECM: extracellular matrix; TS: tumor stages; TG: tumor grades; OS: overall survival; TCGA: the Cancer Genome Atlas; PVA: polyvinylalcohol; DEN: diethylnitrosamine; NMORN-nitrosomorpholine; IHC: Immunohistochemical; ROC:

Receiver operating characteristic; AUC: area under the curve; GPX4: glutathione peroxidase 4; SLC3A2: solute carrier family 3 member 2.

Declarations

Author' contributions

LWQ, WSX and CL conceived the study. WSX performed the experiments and drafted the manuscript. CL supported the bioinformatics technique. LWQ provided the financial support. LWQ, WSX and CL check all the typing in manuscript, as well as the responses to reviewers. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no conflict of interest with regard to this manuscript.

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Consent for publication

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Ethics approval and consent to participate

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Figures

Figure 1

Expression profile of ferroptosis-related DEGs in normal liver tissue vs. HCC and HBV- and HCV- induced cirrhosis vs. HCC from GEO datasets. (A, B) The volcano and venn diagrams of the expression of differential gene SLC7A11 and STEAP3 in cirrhotic HCC tissues. **(C)** The RT-PCR results showing the expression level of SLC7A11 and STEAP3 in normal liver tissues and HCC tissues. **(D, E)** The volcano and venn diagrams of the expression of differential gene SLC7A11 and STEAP3 in HBV-induced cirrhotic HCC tissues. **(F)** The RT-PCR results showing the expression level of SLC7A11 and STEAP3 in HBV-induced cirrhotic HCC tissues. **(G, H)** The volcano and venn diagrams of the expression of differential gene SLC7A11 and STEAP3 in HCV-induced cirrhotic HCC. **(I)** The RT-PCR results showing the expression level of SLC7A11 and STEAP3 in HCV-induced cirrhotic HCC tissues. **(J, K)** Representative immunofluorescence images of SLC7A11 and STEAP3 in TC and IF region of cirrhotic HCC specimen. **(L)** Fluorescence statistical results showing the expression of SLC7A11 and STEAP3 in IF and TC area. All data were presented as mean $\pm$ SEM, $N \geq 3$. *, $p < 0.05$; **, $p < 0.01$.

Figure 2

Enrichment analysis of the differentially expressed genes in HBV- and HCV- induced cirrhosis. (A) The heat map showing the differential expression genes of HBV- and HCV-induced cirrhosis. **(B, C)** The enriched BP, CC, MF and pathway items of DEGs.

Figure 3

Matrix stiffness mediates the expression of SLC7A11 and STEAP3 in cirrhotic HCC. (A) Analytic results of AFM showing young modulus of cirrhotic tissue and normal liver tissue. (B) Representative immunofluorescence images showing the expression of SLC7A11 and STEAP3 in normal liver tissue and cirrhosis tissue of SD rat. (C) Analytic results of AFM showing young modulus of the IF and TC area in HCC tissues of SD rat. (D) Representative immunohistochemical staining images showing the expression of SLC7A11 and STEAP3 in TC and IF region from HCC tissue of SD rat. (E, F) The effects of matrix stiffness on mRNA and protein expression of SLC7A11 and STEAP3 in HCCLM3 and HepG2 cells cultured on PVA gels (2 and 40 kPa). (G) Representative immunofluorescence images of HCCLM3 and HepG2 cells cultured on different stiffness for 24 h, stained for SLC7A11 (red) STEAP3 (green), and nuclei (blue), and then merged. Image were obtained using a confocal microscopy with a 10 × objective. Bar: 25μM. All data were presented as mean ± SEM, $N \geq 3$. *, $p < 0.05$; **, $p < 0.01$.

Figure 4

Enrichment analysis of SLC7A11/STEAP3-related genes in HCC. (A) The top 100 related genes of SLC7A11 in HCC, and positive correlation on the left and a negative correlation on the right. Enrichment pathways (BP/CC/MF/KEGG) of the top 100 related genes of SLC7A11 in HCC. (B) The top 100 related genes of STEAP3 in HCC, and positive correlation on the left and a negative correlation on the right. Enrichment pathways (BP/CC/MF/KEGG) of the top 100 related genes of STEAP3 in HCC. The Abscissa represents the normalized enrichment score, and the ordinate represents the enrichment pathway. Red indicates that the enrichment score is positive and green represents negative.

Figure 5

Matrix stiffness regulates ferroptosis in HCC cells via SLC7A11 and STEAP3. (A) CCK-8 assay results showing the effect of matrix stiffness on RSL3-induced cell viability in HCCLM3 and HepG2 cells, respectively. Indicated cells were treated with RSL3 (10 μM) for 24 h. (B, C) The mRNA and protein expression level of SLC3A2 and GPX4 in HCCLM3 and HepG2 cells cultured on different stiff PVA gels (2 and 40 kPa). (D) The level of RSL3-induced lipid ROS in HCCLM3 and HepG2 cells cultured on the different stiff PVA gels (2 and 40 kPa). Indicated HCCLM3 and HepG2 cells were treated with 10 μM and 1 μM RSL3 for 24 h. (E, F) The protein expression level of GPX4 and ferritin in SLC7A11-knockdown and STEAP3-overexpressed HCCLM3 and HepG2 cells on a stiff matrix gel (40 kPa). (G) The level of MDA production in SLC7A11-knockdown HCCLM3 and HepG2 cells on a stiff matrix gel (40 kPa). Indicated cells were treated with RSL3 (10 μM) for 24 h. (H) The level of lipid ROS in STEAP3-overexpressed

HCCLM3 and HepG2 cells on a stiff matrix gel (40 kPa). Indicated cells were treated with RSL3 (10 μ M) for 24 h. **(I, J)** CCK-8 assay results showing the effect of knockdown of SLC7A11 or overexpression of STEAP3 on RSL3-induced cell viability in HCCLM3 and HepG2 cells, respectively. All data were presented as mean $\pm$ SEM, $N \geq 3$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 6

The landscape of ferroptosis-related genes in HCC. (A) Integrated plot of clinical data and ferroptosis-related genes mutation in 442 HCC samples. From top to bottom panels indicate: American joint committee on cancer tumor stage code, mutation spectrum, international classification of disease for oncology, sex, diagnosis age, overall survival, mutation count. The key to the color-coding is at the bottom. **(B, C)** The heat maps showing the mRNA expression and methylation of ferroptosis-related genes, respectively.

Figure 7

Prognostic significance of SLC7A11 and STEAP3 in HCC patients. (A, B) The box plots showing the gene expression of SLC7A11 and STEAP3 in HCC (369 samples) vs. normal liver tissues (160 Samples). **(C–F)** Boxplots for SLC7A11 and STEAP3 gene expression in different tumor stages (TS) and tumor grades (TG) in HCC patients. **(G, H)** Analysis of SLC7A11 and STEAP3 gene expression with survival prognosis in HCC patients.

Figure 8

Alteration and methylation analysis of ferroptosis-related DEGs and its correlations with survival prognosis in HCC. (A–D) Analysis of the expression and methylation in HCC patients with or without SLC7A11 and STEAP3 alterations. **(E, F)** Overall survival (OS) in HCC patients with or without SLC7A11 and STEAP3 alterations. **(G)** The heat map showing the information of 13 type's methylations of SLC7A11 and 3 type's methylations of STEAP3 in HCC. **(H, I)** The ROC curve for prediction survival prognosis of HCC.

Figure 9

Immune characteristic assay of HBV- and HCV- induced cirrhosis and HCC patients. (A) Immune cell recruitment of HBV- and HCV-induced cirrhosis, and HCC samples. **(B)** Immune signature genes

expression of HBV- and HCV-induced cirrhosis, and HCC samples.

Figure 10

SLC7A11 and STEAP3 are correlated with immune infiltration in HCC. (A) Proportion of immune cell (B cell, CD4+T cell, CD8+T cell, macrophage, dendritic cell, monocytes and natural killer cell) infiltration in cirrhosis and cirrhotic HCC samples. (B) Visual survival differences of various immune cells (B cell, CD8+T cell, CD4+T cell, macrophage, neutrophil, and dendritic cell) in HCC. The red line represents high level and the blue line represent low level. (C-E) The correlation of SLC7A11/STEAP3 expression level vs. immune infiltration and copy number vs. immune infiltration using two-sided wilcoxon rank sum test. (F, G) The correlation between SLC7A11/STEAP3 expression level and immune subtype or molecular subtype.

Figure 11

Matrix stiffness-dependent SLC7A11 and STEAP3 mediate immunogulator PD-L2 expression in HCC. (A) The expression profiles of differential immune checkpoint genes in HCC and normal liver tissues. (B) The mRNA expression level of PD-L2, CTLA4, HAVCR2, PDCD1, TIGIT, Sigleci-15 and protein expression level of PD-L2 in HCCLM3 and HepG2 cells on different stiff PVA gels (2 and 40 kPa). (C) Representative immunofluorescence images showing the expression level of PD-L1 and PD-L2 in normal liver tissue and cirrhosis tissue of SD rat. (D) Representative immunofluorescence images showing the expression of PD-L1 and PD-L2 in TC and IF region of cirrhotic HCC specimens. (E) Fluorescence statistical results showing the expression level of PD-L1 and PD-L2 in IF and TC region. (F, G) The mRNA expression level of PD-L1 in SLC7A11-knockdown or STEAP3-overexpressed HCCLM3 and HepG2 cells on a stiff matrix gel (40 kPa). (H, I) The mRNA expression level of PD-L2 in SLC7A11-knockdown or STEAP3-overexpressed HCCLM3 and HepG2 cells on a stiff matrix gel (40 kPa). All data were presented as mean $\pm$ SEM, $N \geq 3$. *, $p < 0.05$; **, $p < 0.01$.

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