Important Role of Hematopoietic Proteoglycan Serglycin in Liver Hepatocellular Carcinoma Associated with Tumor Microenvironment

Zengcheng Zou[1], Heping Xie[1], Wenhai Guo[1], Yue Li[1], Jiongshan Zhang[1], Yongwei Li[1*]*

[1] *Department of Traditional Chinese Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China*

[*] *Corresponding author: Dr. Yongwei Li, E-Mail: liyongw@mail.sysu.edu.cn, Jiongshan Zhang [1☆], E-Mail:zhjshan2@mail.sysu.edu.cn*

Zengcheng Zou, Heping Xie and Wenhai Guo are co-authors

600 Tianhe Road, Guangzhou, Guangdong, China. Tel: +0086 020 85253497

Funding statement: Supported by Science and Technology Planning Project of Guangdong Province, China, no. 2017A030313738, 2022A1515011689, 2023A1515011937

the construction project of inheritance studio of national famous and old traditional Chinese Medicine experts, no.140000020132

Abstract

Background Serglycin (SRGN) is a prominent hematopoietic proteoglycan that regulates tumorigenesis; however, its role in tumor immunity is unclear.

Materials and methods We investigated the expression and prognostic potential of SRGN in liver hepatocellular cancer (LIHC) in the context of pan-cancer (for showing the similarity and heterogeneity) using the PrognoScan, GEPIA, Kaplan–Meier Plotter, and TIMER bioinformatics databases. HepG2 cells were transfected with an SRGN over-expression vector, and their proliferation, invasion, sorafenib resistance, and vasculature were examined *in vitro*. A subcutaneous xenograft tumor model was created in nude mice.

Results SRGN expression was prominent in M2 macrophages in LIHC. The Kaplan–Meier Plotter indicated that SRGN RNA was a favorable prognostic factor after correcting for clinical factors. TIMER 2.0 showed that the immune infiltrates of CD8+ T cells, M1 and M2 macrophages, and endothelial cells were strongly correlated with SRGN RNA expression (r=0.552, P=5.79e^{-29}; r=0.517, P=5.84e^{-25}; r=0.696, P=3.26e^{51}; and r=0.522, P=1.67e^{-25}, respectively), and had prognostic potential in LIHC in patients with low or high levels of SRGN, in addition to resting memory CD4+ T cells, hematopoietic stem cells (HSCs), and myeloid-derived suppressor cells (MDSCs). SRGN promoted the proliferation of HepG2 cells *in vitro* and *in vivo*, and was associated with weak sorafenib resistance, invasion, and vasculature. CD206 and CD80 were up-regulated and down-regulated, respectively, in subcutaneous tumor tissues.

Conclusions The results comprehensively revealed relationships between SRGN and tumor microenvironment(TME)-infiltrating cells, especially monocyte/macrophage subsets. These may constitute an important TME because the pro-tumorigenicity of SRGN in liver cancer.

Key words: Serglycin; Liver hepatocellular carcinoma; Prognosis; Pro-tumorigenicity

Introduction

It is estimated that 296 million people are infected with hepatitis B virus (HBV) worldwide [1]. HBV has a broad spectrum of clinical manifestations ranging from a healthy carrier state to acute and chronic hepatitis (chronic hepatitis B), liver cirrhosis, primary liver cancer (PLC, mainly liver hepatocellular carcinoma, LIHC), and end-stage liver disease. Most patients recover from acute HBV infection, but 1%–5% of patients develop LIHC [2]. HBV-specific B-cell antibodies and HBV-specific T cells combat HBV infection [3]. The HBV infection-based microenvironment consists of immunosuppressive and inflammatory cytokines and immune cells, including macrophages, neutrophils, and lymphocytes [4-6], which can complicate the LIHC tumor microenvironment (TME). Immunotherapy with PD-1/PD-L1 blockade has been regarded as a hot topic and promising therapy in LIHC [4]. However, there is a need to clarify the mechanisms linking immunity and malignancy to investigate new treatments for LIHC. Serglycin (SRGN, gene *SRGN*) is a prominent hematopoietic proteoglycan with inflammatory and malignant properties, which interacts with proteases, chemokines, and cytokines and is required for the formation of secretory granules [7-9].

The HepG2.2.15 cell line is derived from HepG2 human hepatoma cells chronically infected with HBV [10]. We previously analyzed the Human Genome U133 Plus 2.0 Array (Platform GPL570; Affymetrix, Santa Clara, CA) and revealed extensive changes in global gene transcription in HepG2.215 compared with HepG2 cells, with SRGN being the most significantly differentially expressed gene [11]. Levels of SRGN detected by flow

cytometry were increased in peripheral monocytes, neutrophils, and lymphocytes in HBV-infected LIHC patients compared with healthy adults, and hematopoietic SRGN was associated with an unfavorable prognosis in terms of overall survival (OS) [12]. Based on these results, we comprehensively evaluated the prognostic potential of SRGN in pan-cancer using bioinformatics databases and further investigated its role in LIHC. Other PLC such as cholangio carcinoma (CHOL) is rarely mentioned in these databases.

Materials and methods

SRGN expression, prognosis analysis and correlation analysis

We examined the differential expression and prognosis of *SRGN* RNA in pan-cancer using Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html, http://gepia2021.cancer-pku.cn/)[13], Kaplan–Meier plotter databases (http://kmplot.com/analysis/ (registration-freeKM-plotter))[14], the Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/ timer/, http://timer.comp-genomics.org/)[15], and PrognoScan database (http://www.abren.net/ PrognoScan/) [16], respectively. SRGN protein expression was analyzed by Clinical Proteomic Tumor Analysis Consortium (CPTAC) analysis. The expression of SRGN in immune cells was analyzed using GEPIA, TIMER and The Human Protein Atlas (HPA).

The correlation module was used to investigate correlations between SRGN and TME cells or gene markers using Spearman's correlation analysis via TIMER. Spearman's correlation was determined using the following criteria: 0.1–0.3: weak correlation, 0.3–0.5: moderate correlation, and 0.5–1.0: strong correlation [17]. The results were represented as a

heatmap for pan-cancer and scatter plots for LIHC, respectively. SRGN expression in liver cell types was analyzed using HPA, SRGN–protein interaction was analyzed by STRING, and SRGN-associated genes in LIHC were analyzed using the University of Alabama at Birmingham Cancer data (UALCAN).

Over-expression of SRGN in HepG2 cells

Details of the methods, including construction of an over-expression vector, lentiviral transduction, quantitative polymerase chain reaction, western blotting, Cell Counting Kit-8 cell viability assay, Transwell invasion assay, angiogenesis, vasculogenic mimicry, immunofluorescence, and hematoxylin and eosin staining are provided in the Supplementary Methods.

Animal experiments

Male BALB/c nude mice (5-weeks-old, weight 18-20g) were purchased from Guangzhou University of Chinese Medicine Laboratory Animal Center (Guangzhou, China). Tumor cells (5×10^6/0.1 mL phosphate-buffered saline) were injected randomly into the right axillary region and the mice were monitored twice a week for 4 weeks for tumor formation, then, sacrificed by carbon dioxide asphyxiation. After animal experiments, the carcasses were returned to the Laboratory Animal Center for harmless treatment. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). The animal experiments were approved by Institutional Animal Care and Use Committee of Rulge Biotechnology (ethical approval number, 20230201002).

Statistical analysis

Measured data were compared by *t*-tests or ANOVA using SPSS 22.0 (IBM Corp. Armonk, NY, USA) and counted data were compared with χ^2 tests. Differences between means for data with a skewed distribution and variance were analyzed by rank sum tests. $P < 0.05$ was considered statistically significant.

Results

Expression of SRGN in pan-cancer and cell types

The differential expression of SRGN RNA between pan-cancer and adjacent normal tissues was analyzed using TIMER (Figure 1). SRGN levels were significantly higher in head and neck cancer (HNSC) and kidney renal clear cell carcinoma compared with adjacent normal tissues. SRGN expression was also higher in human papilloma virus (HPV)-positive compared with HPV-negative HNSC. SRGN levels were significantly lower in bladder urothelial carcinoma, breast invasive carcinoma (BRCA), colon adenocarcinoma, kidney chromophobe, kidney renal papillary cell carcinoma, LIHC, lung squamous cell carcinoma, lung adenocarcinoma (LUAD), prostate adenocarcinoma, rectum adenocarcinoma, and uterine corpus endometrial carcinoma than in adjacent normal tissues, and was lower in skin cutaneous melanoma (SKCM) tumor than in SKCM metastasis. (Figure 1A). KM-plotter analysis showed that SRGN mRNA levels differed significantly between tumor and adjacent normal tissues in 19 cancers, except for adrenal, liver, and renal_PA cancers (Supplementary Figure 1). However, KM-plotter analysis of GeneChip data showed that SRGN levels were significantly higher in adjacent normal tissues than in LIHC tumor tissues (non-paired tumor vs. adjacent normal tissues, $P = 5.85e^{-11}$; paired

tumor *vs*. adjacent normal tissues, P = $9.29e^{-10}$), which was consistent with the comparison among LIHC tumor, adjacent normal, and metastasis tissues (P = $7.92e^{-11}$; Figure 2A). There were no differences in SRGN levels based on RNA-Seq data between LIHC tissues and adjacent normal tissues as in GEPIA (Figure 2B, Supplementary Figure 2). The results for SRGN protein levels in tumor and normal samples were consistent with the RNA levels for breast cancer, lung cancer, and HNSC by CPTAC (Figure 1B), but there were no data for SRGN protein in liver cancer. Among all these cancers, SRGN levels were highest in patients with acute myeloid leukemia (AML) according to the TIMER, GEPIA, and KM-plotter databases.

HPA analysis showed that SRGN RNA was expressed in immune cell types in the order neutrophils > eosinophils > basophils > total peripheral blood mononuclear cells > classical monocytes, and in liver cell types in the order neutrophils > vascular endothelial cells (ECs) > Kupffer cells > plasma cells > cholangiocytes. SRGN was prominently expressed in T cells, Kupffer cells, B cells, hepatic stellate cells, and ECs, but was rarely expressed in hepatocytes (Figure 2C, D). However, the proportion and sub-expression analysis via GEPIA showed that the proportion of M2 macrophages and sub-expression of SRGN in M2 were highest in LIHC, adjacent normal, and liver tissues, respectively (Figure 2E-H). .

Prognostic potential of SRGN in pan-cancer according to PrognoScan, GEPIA, Kaplan–Meier Plotter Databases, and TIMER

We analyzed the relationship between SRGN expression and prognosis in pan-cancer (Supplementary Figures 3–6, Table 1). PrognoScan analysis revealed that SRGN expression had a significant impact on prognosis in five cancer types: AML, diffuse large B-cell

lymphoma (DLBCL), BRCA, lung cancer, and ovarian cancer (OV). High SRGN expression was associated with a poor prognosis in AML and DLBCL, but showed favorable prognostic potential in a small sample size of three solid tumors, BRCA, lung cancer and OV. According to GEPIA, SRGN only had prognostic value in testicular germ cell tumors, glioblastoma multiforme, and SKCM. According to the TIMER gene_outcome module, SRGN was a significant prognostic factor for OS in patients with sarcoma, SKCM, and SKCM metastasis after correcting for age, race, sex, and tumor purity (Supplementary Figure 5, Table 1).

We examined the prognostic potential of SRGN mRNA in pan-cancer based on GeneChip and RNA-seq using KM-plotter. High SRGN levels were associated with poor OS, PFS, and post-progression survival (PPS) in OV (Supplementary Figure 6A). In BRCA, SRGN was a risk factor for PPS and RFS, and a protective factor for OS (Supplementary Figure 6B). In gastric cancer, high SRGN expression was associated with a favorable prognosis in terms of OS, first progression, and PPS (Supplementary Figure 6C). In lung cancer, high SRGN levels were significantly associated with a favorable or poor prognosis for OS (201859_at and 201858_s_at, respectively), and a favorable prognosis in terms of first progression (Supplementary Figure 6D).

SRGN was a favorable prognostic factor in liver cancer according to the Kaplan–Meier Plotter Database.

We investigated the relationship between SRGN expression and survival in patients with liver cancer using KM-plotter. High SRGN expression was associated with better OS, RFS, PFS, and DSS after correcting for stage, grade, AJCC_T stage, vascular invasion, sex, race,

sorafenib treatment, hepatitis virus, and alcohol consumption (Supplementary Figure 6E; RNA_SeqID 5552; PFS HR = 0.67, 95% CI = 0.5–0.91, P = 0.0095, median survival 16.73 *vs.* 30.4 months; OS HR = 0.68, 95% CI = 0.47–0.97, P = 0.034, median survival 52 *vs.* 70.5 months; RFS HR = 0.65, 95% CI = 0.46–0.92, P = 0.015, median survival 21.87 *vs.* 40.97 months; and DSS HR = 0.58, 95% CI = 0.35–0.97, P = 0.034). However, the median DSS times were 104.17 *vs.* 84.4 months in the low *vs*. high expression cohorts. The prognostic potential of SRGN for survival may change over time. This was reflected by UALCAN, with SRGN indicating a poor prognosis in high-expression cohorts (P = 0.5).

We further analyzed SRGN in relation to clinicopathological factors in liver cancer (Table 2). High SRGN mRNA expression was correlated with favorable OS and PFS in patients with stage 2–3+4, RFS in stages 2–3, and DSS in stages 2+3–3+4, with better OS, PFS, and DSS in AJCC_T 2 and 3, and with favorable RFS in AJCC_T 2. High SRGN expression was associated with better OS and DSS in patients with grade 2 cancer, better RFS in grades 2–3, and better PFS in grades 1–3. High SRGN expression was also associated with favorable OS, RFS, PFS, and DSS in men, Asians, and in patients with a history of alcohol consumption, and with favorable OS in women and Caucasian patients. Over-expression of SRGN in patients with hepatitis virus infection was associated with favorable prognoses in terms of RFS and PFS, and with favorable OS and DSS in patients without virus infection.

Prognostic potential of SRGN expression associated with immune cells in pan-cancer

We analyzed SRGN expression in relation to six immune infiltrates using TIMER 2.0. Correcting for six immune infiltrates and clinical factors (age, sex, stage, and tumor purity),

high SRGN expression was a risk factor for OS in cholangiocarcinoma (CHOL), LUAD, and thymoma (THYM), but a favorable prognostic factor in LIHC and SKCM metastasis (Supplementary Table 7). Only SKCM metastasis was significantly different between the groups with low and high SRGN levels (log-rank test, $P < 0.001$; Supplementary Figure 8).

Correlation and prognostic potential of SRGN with immune cells in LIHC

SRGN expression was significantly positively correlated with immune infiltrations of CD4+ T cells, B cells, macrophages, CD8+ T cells, neutrophils, and dendritic cells (Supplementary Figure 9). The correlations were moderate to strong (r = 0.34, 0.483, 0.579, 0.616, 0.631, and 0.714, respectively; $P = 0.40e^{-11}$, $1.73e^{-21}$, $7.02e^{-32}$, $4.16e^{-37}$, $9.47e^{-40}$, and $2.94e^{54}$, respectively). Cancer type (LIHC), clinical characteristics (age, stage, tumor purity, sex, and race), six immune infiltrates, and gene symbols (*SRGN*) were analyzed using a multivariable Cox proportional hazard model. Of 305 patients enrolled, 101 died. Race (black), stage 4, macrophages, and dendritic cells were risk factors for OS, whereas stage 3, CD4+ T cells, CD8+ T cells, and SRGN were favorable factors (Supplementary Table 7). However, there were no significant survival differences for the six types of immune cells and SRGN in the low- *vs*. high-expression cohorts according to log-rank test (Supplementary Figure 8).

Correlations between SRGN and TME cells and gene markers in LIHC

We analyzed the correlations and outcomes for each immune cell subset and other TME cell types using TIMER 2.0. SRGN expression was significantly negatively correlated with tumor purity (Rho = −0.53, $P = 1.67e^{-26}$). Eleven proportions of cell subsets were negatively and 32 were positively correlated with SRGN expression (Table 3, Supplementary Figure

10). Immune cells included 10 CD4+ T cell subsets (CD4+ and CD4+ naïve T cells (by two algorithms CIBERSORT and XCELL), central memory, effector memory, activated memory, resting memory, and memory CD4+ T cells, CD4+ (non-regulatory), and Th1 and Th2 CD4+ T cells); two CD8+ T cell subsets (central memory and effector memory CD8+ t cells); three B cell subsets (naïve, plasma_(CIBERSORT-ABS and XCELL), and memory B cells); regulatory and follicular helper T cells; monocytes, four macrophage subsets (macrophages (EPIC and TIMER), and M0, M1, and M2 macrophages (CIBERSORT-ABS and TIDE); common lymphoid progenitors; neutrophils; three natural killer (NK) cell subsets (NK cells and resting and activated NK cells); three myeloid dendritic cell subsets (myeloid dendritic cells and activated and resting myeloid dendritic cells); and NK T cells. Naïve CD4+ T cells, plasma B cells, macrophages, and M2 macrophages were negatively or positively correlated with SRGN expression according to different algorithms: *e.g.*, M2 macrophages (CIBERSORT-ABS: $r = 0.696$, $P = 3.26e^{51}$; TIDE: $r = -0.516$, $P = 0.66e^{-25}$). CD8+ T cells, M1 macrophages, myeloid dendritic cells, activated NK cells, and ECs showed strong positive correlations with SRGN expression levels ($r = 0.552$, $P = 5.79e^{-29}$; $r = 0.517$, $P = 5.84e^{-25}$; $r = 0.627$, $P = 4.97e^{-39}$; $r = 0.524$, $P = 1.11e^{-25}$; $r = 0.522$, $P = 1.67e^{-25}$, respectively). The remaining six cell types, including hematopoietic stem cells (HSCs) and myeloid-derived suppressor cells (MDSCs), showed no correlation with SRGN expression.

We further analyzed the correlations between SRGN expression and biomarkers of CD8+ T cells, M1 and M2 macrophages, tumor-associated macrophages, and monocytes in pan-cancer, especially LIHC (Supplementary Figures 11 and 12). The gene markers have been reported previously [18]. All the markers, as well as CTLA4, PDCD1, CD274, CD80,

CD44, vascular endothelial growth factor C, and transforming growth factor-β2, were positively correlated with SRGN in LIHC. UALCAN analysis revealed SRGN-associated genes in LIHC. The monocyte marker CD86 was the top positive correlated gene (Figure 3A and B). According to HPA analysis, markers of vascular ECs, neutrophils, and Kupffer cells showed strong correlations with SRGN ($r > 0.5$, Figure 3C). STRING analysis showed SRGN–protein interactions (Figure 3D). The top three proteins were granzyme B, perforin-1, and CD44 antigen.

Prognostic potential of SRGN with TME cells in LIHC

We analyzed TME infiltrates (21 cell types), clinical characteristics (age, stage, purity, sex, and race), and SRGN in relation to OS in patients with LIHC using the Cox proportional hazards model. The cell types and subsets are shown in Table 4 and Figure 4, with SRGN as a significant prognostic factor in the Cox model. According to log-rank test, high infiltration of MDSCs, M1 macrophages, or monocyte_MCPCOUNTER was associated with unfavorable OS in the cohort with high SRGN expression (HR = 2.78, P = 0.00668; HR = 3.09, P = 0.00633; HR = 2.75, P = 0.025, respectively), whereas high infiltration of CD8+ T cells, resting memory CD4+ T cells, ECs, or HSCs was associated with a favorable prognosis for OS (HR = 0.53, P = 0.048; HR = 0.501, P = 0.0383; HR = 0.364, P = 0.00638; HR = 0.379, P = 0.00868, respectively). In the cohort with low SRGN expression, high infiltration of macrophage_TIMER, macrophage M0_CIBERSORT, and macrophage M2_CIBERSORT-ABS were unfavorable factors for OS (HR = 1.82, P = 0.0432; HR = 1.86, P = 0.0356; HR = 1.99, P = 0.0366, respectively).The three cellsubsets, together with

CD8+ T cell, showed time-dependent survival curves. The log-rank test of SRGN expression with the other six cell types (central memory and Th2 CD4+ T cells, Tregs, monocytes (XCELL), myeloid dendritic cells, and resting myeloid dendritic cells) showed no significant difference in LIHC.

In vitro and *in vivo* pro-tumorigenicity of SRGN

SRGN promoted the proliferation of SRGN-over-expressing HepG2SG cells compared with HepG2SG-NC cells (transfected with blank control vector) and HepG2, respectively ($P < 0.05$, $P < 0.01$; Figure 5A-C). HepG2SG cells showed weak sorafenib resistance (resistance index 1–2; Figure 5D). HepG2SG cells also showed increased cell invasion compared with HepG2-NC cells by Transwell assay ($P \leq 0.001$, Figure 5E). The number of tubular structures was higher in human umbilical vein ECs (HUVECs) cultured with HepG2SG supernatant compared with HepG2SG-NC supernatant ($P \leq 0.001$; Figure 5F). SRGN promoted vascular mimicry tube formation in HepG2SG cells compared with HepG2SG-NC cells ($P < 0.01$; Figure 5G). Subcutaneous xenograft tumors derived from HepG2SG cells were heavier than those from HepG2SG-NC cells ($P < 0.05$; Figure 5H). Hematoxylin and eosin staining showed that cells were distributed in clumps or strands in subcutaneous tumor tissues (Figure 5I). SRGN and CD206 were up-regulated while CD80 was down-regulated in subcutaneous tumor tissues in HepG2SG xenograft mice compared with HepG2 SG-NC mice ($P < 0.05$, $P < 0.01$; Figure 5J).

Discussion

SRGN is a hematopoietic intracellular proteoglycan expressed in secretory granules and

vesicles, and secreted into the extracellular medium [19]. The core SRGN protein consists of eight serine/glycine repeats attached by variable glycosaminoglycan bonds, depending on the cell type and status [20, 21]. SRGN exerts pro-tumorigenic effects in multiple ways [22], and several malignant cell types depend on SRGN to promote aggregation [23]. However, its role remains controversial.

TIMER, GEPIA, and KM-Plotter database analyses showed that SRGN levels were higher in AML than in other cancers, and SRGN expression was a prognostic risk factor for survival in four AML and two DLBCL groups according to PrognoScan analysis. The prognostic potential of SRGN RNA expression was consistent with that for SRGN protein, as determined by immunohistochemistry (IHC) and enzyme-linked immunosorbent assay [24]. The pro-tumorigenic effect of SRGN was recently confirmed in AML [25]. However, no consistent role has been demonstrated in solid tumors.

IHC analyses in lung, breast, prostate, and colon cancer cells showed that SRGN expression was up-regulated in advanced tumors and in the activated TME [26]. In the present study, SRGN RNA levels were lower in BRCA and lung cancer compared with normal adjacent tissues according to TIMER, KM-plotter, and GEPIA analyses, as also reported by Oncomine [27]. SRGN protein levels were decreased in the above two cancers compared with normal tissues according to CPTAC. PrognoScan analysis demonstrated that high levels of SRGN mRNA were associated with a favorable prognosis in small samples of patients with OV, lung cancer, and BRCA, but SRGN RNA expression was also a prognostic risk factor in a large sample size in three cancers analyzed via KM-plotter, and experimental and clinical studies have identified pro-tumorigenic effects of SRGN in lung

cancer and BRCA [27-33]. SRGN was correlated with drug resistance and stemness in BRCA, promoting invasion and metastasis and inducing BRCA cell aggregation [27-31]. High levels of SRGN predicted a poor prognosis in LUAD, promoting NSCLC cell migration [32, 33]. The current results for SKCM were consistent with a previous report that identified SRGN as a favorable factor in SKCM and SKCM-metastasis tissues via GEPIA and TIMER 2.0 [27], indicating that the result was reproducible by bioinformatics analysis. However, the prognostic potential and tumorigenicity of SRGN in sarcoma, glioblastoma multiforme, testicular germ cell tumors, gastric cancer, CHOL, and THYM have rarely been studied.

The controversial role of SRGN is more evident in LIHC. SRGN mRNA levels were lower in LIHC than in adjacent normal tissues via TIMER, but were higher in liver cancer *vs.* normal tissues via Oncomine [27], showed no difference via GEPIA, and were inconsistent between GeneChip and RNA-seq data via KM-plotter. IHC staining showed that SRGN levels were higher in 56.7% of HCC tissues *vs.* 3.1% of adjacent non-tumor tissues [9]. We previously showed that levels of hematopoietic SRGN examined by flow cytometry were higher in patients with chronic hepatitis B, liver cirrhosis, and LIHC than in healthy adults, while levels of SRGN mRNA were lower in HepG2.215 compared with HepG2 cells [11, 12]. HepG2 is a hepatoblastoma-derived cell line. However, it expresses the lowest SRGN level among 24 liver cancer cell lines (https://www. proteinatlas. org/ENSG00000122862-SRGN/cell+line), and HepG2 cells show poor *in vivo* tumorigenicity. In the present study, HepG2 cells over-expressing SRGN had significant proliferation *in vitro* and tumorigenicity *in vivo*, which identified pro-tumorigenicity of

SRGN in liver cancer. SRGN expression may be affected by inflammation and malignant transformation induced by HBV, thus increasing the complexity of survival analyses.

SRGN mRNA expression levels showed no prognostic potential in LIHC *via* PrognoScan, GEPIA, or TIMER 2.0 (analyzed without TME cells). According to the KM-plotter database, SRGN mRNA was a favorable prognostic factor in LIHC after adjusting for stage, grade, AJCC_T, sex, race, alcohol consumption, hepatitis virus infection, sorafenib treatment, and vascular invasion; however, SRGN protein expression was an unfavorable factor for OS and time to recurrence [9, 12]. The prognostic potential of the SRGN transcriptome and protein were thus contradictory, as in lung cancer and BRCA. As a prominent hematopoietic proteoglycan, SRGN should be analyzed together with TME cells in a multivariate Cox regression model.

The liver is the major hematopoietic organ during the fetal period. Hepatic hematopoiesis can generate lymphoid, myeloid, and erythroid lineages, and is involved in tumor surveillance and rejection [34, 35]. SRGN maintains homeostasis of the body's immune cell populations, controlling the magnitude and durability of the immune response [36]. In the present study, six prominent immune cells showed strong correlations with SRGN expression, and SRGN was a favorable prognostic factor for OS associated with macrophages, dendritic cells, and $CD4^+$ and $CD8^+$ T cells. However, there are abundant immune cell subsets, and further analyses are required.

SRGN has been studied in neutrophils, cytotoxic T lymphocytes, macrophages, CD8+ T cells, platelets, and NK cells [8, 37-41]. The negative correlation between SRGN and tumor purity suggested that SRGN may be prominently expressed in TME-infiltrating cells, and

the correlations between SRGN and TME cells was reliable. The results showed that both neutrophils and monocytes were more prevalent in normal liver tissues than in LIHC and normal tissues adjacent to LIHC. M2 macrophages acquire pro-tumorigenic properties by responding to CCL2 and interleukin-10, triggering the release of cytokines, chemokines, and growth factors to promote malignancy [42]. M2 macrophages with immunosuppressive properties were the most abundant of the five types of immune cells in healthy liver tissues, as in LIHC and adjacent normal tissues, which may reflect the fact that the liver demonstrates natural immune tolerance [4]. M1 macrophages and CD8+ T cells antagonize pro-tumorigenesis and both were second to M2 macrophages in LIHC and adjacent normal tissues. These results suggested that the differentiation and activation of monocytes/macrophages may vary in physiological and pathological liver tissues. SRGN can be induced in macrophages [37]. The glycosaminoglycan of SRGN varies according to the differentiation of monocytes to macrophages or the activation of macrophages [20]. SRGN level were higher in M2 macrophages than in neutrophils in LIHC, adjacent normal, and liver tissues by GEPIA, but SRGN in neutrophils was dominant in the liver by HPA. Spearman's correlation analysis indicated significant correlations between SRGN and 43 TME cells in LIHC. Together with the above previous studies [8, 37-41], these results suggest that SRGN may have an extensive impact on the immune system. CTLA4, PDCD1, CD274, and granzyme B, as markers of T cell exhaustion [19], were correlated with SRGN. The *in vivo* results indicated that CD206 was increased while CD80 was down-regulated in HepG2SG mice, in contrast to the positive correlation between SRGN and CD80 *via* TIMER2.0. SRGN might promote monocyte/macrophage differentiation or migration to

LIHC, as reported in myeloma [43]. The TME was immunosuppressive in HepG2SG mice, but further studies are needed to verify these bioinformatics results.

Survival analysis showed that macrophages (TIMER), M0, and pro-tumorigenic M2 macrophages were associated with low SRGN levels, while monocytes (MCPCOUNTER) and “antitumorigenic” M1 macrophages with high SRGN levels were unfavorable prognostic factors. The prior cells associated with low SRGN expression might maintain the immunosuppressive status, while the latter with high SRGN expression might promote excessive proinflammatory status into immune exhaustion or tolerance status, promoting the conversion of “antitumorigenic” M1 macrophages to pro-tumorigenic ones [44]. The prognostic potential of SRGN was closely related to monocyte / macrophage subsets. The positive correlations between SRGN and CTLA4, PD-1, and PD-L1 also suggested that SRGN was related to immune escape, and that immune therapy, e.g., PD-1 inhibitors, might have an effect in SRGN-positive LIHC. CD4+ T cell subsets had extensive, weak-to-moderate correlations with SRGN (seven positive and three negative); however, only high resting memory CD4+ and CD8+ T cells in the cohort with high SRGN expression showed protective effects against LIHC by log-rank test. It is possible that high levels of SRGN may play a role in the cytotoxicity of T cells in LIHC, as reported in virus infection [39, 41]. MDSCs have immunosuppressive activity against T-cell responses, and high SRGN expression and MDSCs indicated an unfavorable prognosis. Further studies are warranted to investigate the interaction between SRGN and these immune cells.

Vascular invasion had no effect on the prognostic potential of SRGN mRNA according to KM-plotter. However, high SRGN has been reported to up-regulate the proliferation of

HUVECs [45], and SRGN protein expression was positively related to vascular invasion in HCC patients [9]. The present study showed that ECs and vascular endothelial growth factor C were strongly correlated with SRGN, and SRGN induced the formation of tubular structures by both HUVECs and HepG2 cells. High levels of circulating HSCs and endothelial progenitor cells were associated with worse OS in HCC patients [46]; however, high SRGN RNA levels with high HSCs or ECs were associated with favorable OS by TIMER 2.0 analysis. Both high levels of SRGN and ECs might promote normalization of tumor vasculature; however, further studies are required to investigate this controversy.

The prognostic capability of SRGN may vary according to the covariates in Cox regression. Regardless of the algorithms, SRGN mRNA and protein expression patterns, in addition to clinicopathological factors, observation endpoints, different populations, and sample sizes, may result in contradictory survival results for pan-cancers, except for blood cancers. One of two SRGN transcript variants is protein-coding (GEPIA, provided by RefSeq, Jul 2010). The present study constructed an SRGN-over-expressing vector (based on the same sequence of ID5552 analyzed by KM-plotter) and identified pro-tumorigenicity and metastasis in PLC, as other cancers [27-33]. Menyhárt et al. reviewed 318 genes related to HCC survival and showed that none had equivalent prognostic value to tumor stage. They also noted that survival results according to protein expression were inconsistent with those for the transcriptome [47]. SRGN may have an important effect on TME cells according to the multivariate Cox regression model.

In addition to the above limitations, the sequencing data and microarray analysis of

tumor tissues might have systematic bias. Further studies combined with spatiotemporal single-cell RNA sequencing are needed to clarify the results. Second, variable glycosaminoglycan bonds might interfere with the function of SRGN. Although SRGN showed contradictory expression and prognostic potential in bioinformatics analysis across different databases, both *in vivo* and *in vitro* experiments were performed to identify its pro-tumorigenicity in many reports [9, 12, 27-33]. Future studies could clarify more mechanisms of SRGN in PLC.

Although SRGN was a limited prognostic factor in PLC, the results comprehensively revealed relationships between SRGN and monocyte/macrophage subsets, ECs, HSCs, MDSCs, $CD8^+$ T cells, and resting memory $CD4^+$ T cells, which may contribute to the development of new immunotherapy strategies.

Acknowledgements: We thank International Science Editing (http://www. International scienceediting.com) for editing a draft of this manuscript.

Disclosure Statement: The authors declare no potential conflicts of interest.

Availability of data and materials: Please contact the corresponding author Yongwei Li (liyongw@mail.sysu.edu.com) upon reasonable requests.

Ethics approval and consent to participate: Not Applicable for patient's ethics approval.

References

1. https://www.who.int/news-room/fact-sheets/detail/hepatitis-b

2. Gurtsevitch VE. Human oncogenic viruses: Hepatitis B and hepatitis C viruses and their role in hepatocarcinogenesis. Biochemistry (Moscow), 2008; 73(5): 504-513.

3. Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J Hepatol. 2016; 64(1 Suppl):S71-S83.

4. Li B, Yan C, Zhu J, Chen X, Fu Q, et al. Anti-PD-1/PD-L1 Blockade Immunotherapy Employed in Treating Hepatitis B Virus Infection-Related Advanced Hepatocellular Carcinoma: A Literature Review. Front Immunol. 2020; 11: 1037.

5. Guidotti LG, Inverso D, Sironi L, et al. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. Cell 2015; 161:486–500.

6. Douglas Hanahan, Robert A Weinberg. Hallmarks of cancer: The next generation. Cell. 2011; 144: 646-674.

7. Korpetinou A, Skandalis SS, Labropoulou VT, Smirlaki G, Noulas A, Karamanos NK, Theocharis AD. Serglycin: At the crossroad of inflammation and malignancy. Front Oncol. 2014; 3: 327.

8. Xin-Jian Li and Chao-Nan Qian. Serglycin in human cancers. Chin J Cancer. 2011; 30 (9):585-9.

9. He L, Zhou X, Qu C, Tang Y, Zhang Q, Hong J. Serglycin (SRGN) overexpression predicts poor prognosis in hepatocellular carcinoma patients. Med Oncol. 2013; 30: 707.

10. Li Y, Zhu M, Guo Y, Chen W, Li G. Full-length hepatitis B virus sequences from naive patients with fluctuation of viral load during ADV monotherapy. Virus Genes 2010;

40:155-162.

11. Yongwei Li, Mingfen Zhu, Gang Li. Expression of serglycin in HepG2 cells with the different HBV infection status. Journal of Gastroenterology and Hepatology. 2013; 28(Supplement 3): 913.

12. Li Y, Chen H, Lu H, Zou Z, Li Y. Prognostic significance of hematopoietic-cell serglycin for the survival of hepatocellular carcinoma: A single-center retrospective study. Comb Chem High Throughput Screen. 2021; 24(7):986-995.

13. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 2017; 45(W1):W98-W102.

14. Nagy A, Munkárcsy G, Győrffy B. Pancancer survival analysis of cancer hallmark genes, Scientific Reports, 2021; 11(1): 6047.

15. 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 Res. 2017; 77: e108-e110.

16 . Mizuno, H., Kitada, K., Nakai, K. et al. PrognoScan: a new database for meta-analysis of the prognostic value of genes. BMC Med Genomics. 2009; 2:18.

17. Cohen, J., Statistical power analysis for the behavioral sciences (2nd ed.); Hillsdale, NJ: Lawrence Erlbaum Associates. 1988:83.

18. Pan JH, Zhou H, Cooper L, Huang JL, Zhu SB, Zhao XX, et al. **LAYN** Is a Prognostic Biomarker and Correlated With Immune Infiltrates in Gastric and Colon Cancers. Front Immunol. 2019; 10: 6.

19. Dimitra Manou, Nikos K. Karamanos, Achilleas D. Theocharis. Tumorigenic functions of serglycin: Regulatory roles in epithelial to mesenchymal transition and oncogenic signaling. Seminars in Cancer Biology.2020；62:108-115.

20. Kolset SO, Kjellen L, Seljelid R, Lindah U. Changes in glycosaminoglycan biosynthesis during differentiation in vitro of human monocytes. Biochem J .1983; 210 (3):661–7.

21. Uhlin-Hansen L, Eskeland T, Kolset, S O. Modulation of the expression of chondroitin sulfate proteoglycan in stimulated human monocytes. J Biol Chem. 1989; 264(25):14916–22

22. Angeliki Korpetinou, Spyros S Skandalis, Vassiliki T Labropoulou, Gianna Smirlaki, Argyrios Noulas, Nikos K Karamanos, Achilleas D Theocharis. Serglycin: At the crossroad of inflammation and malignancy. Front Oncol. 2014; 3: 327.

23. Manou D, Bouris P, Kletsas D, Götte M, Greve B, Moustakas A, Karamanos NK, Theocharis AD. Serglycin activates pro-tumorigenic signaling and controls glioblastoma cell stemness, differentiation and invasive potential. Matrix Biol Plus. 2020; 6-7: 100033.

24. Niemann CU, Kjeldsen L, Ralfkiaer E, Jensen MK, Borregaard N. Serglycin proteoglycan in hematologic malignancies: A marker of acute myeloid leukemia. Leukemia. 2007; 21(12):2406-10.

25. Peng L, Zhang Y, Xin H. lncRNA SNHG3 facilitates acute myeloid leukemia cell growth via the regulation of miR-758-3p/SRGN axis. J Cell Biochem. 2020; 121(2): 1023-1031.

26. Korpetinou A, Papachristou DJ, Lampropoulou A, Bouris P, Labropoulou VT, Noulas A, Karamanos NK, Theocharis AD. Increased expression of serglycin in specific carcinomas and aggressive cancer cell lines. Biomed Res Int. 2015; 2015: 690721.

27. Wang X, Xiong H, Liang D, Chen Z, Li X, Zhang K. The role of SRGN in the survival and immune infiltrates of skin cutaneous melanoma (SKCM) and SKCM-metastasis patients. BMC Cancer. 2020; 20(1): 378.

28. Yang T, Fu Z, Zhang Y, Wang M, Mao C, Ge W. Serum proteomics analysis of candidate predictive biomarker panel for the diagnosis of trastuzumab-based therapy resistant breast cancer. Biomed Pharmacother. 2020; 129: 110465.

29. Zhang Z, Qiu N, Yin J, Zhang J, Liu H, Guo W, Liu M, Liu T, Chen D, Luo K, Li H, He Z, Liu J, Zheng G. SRGN crosstalk with YAP to maintain chemoresistance and stemness in breast cancer cells by modulating HDAC2 expression. Theranostics. 2020 4; 10(10):4290-4307.

30. Bouris P, Manou D, Sopaki-Valalaki A, Kolokotroni A, Moustakas A, Kapoor A, Iozzo RV, Karamanos NK, Theocharis AD. Serglycin promotes breast cancer cell aggressiveness: Induction of epithelial to mesenchymal transition, proteolytic activity and IL-8 signaling. Matrix Biol. 2018; 74: 35-51.

31. Zhang Z, Deng Y, Zheng G, Jia X, Xiong Y, Luo K, Qiu Q, Qiu N, Yin J, Lu M, Liu H, Gu Y, He Z.SRGN- TGFbeta2 regulatory loop confers invasion and metastasis in triple-negative breast cancer. Oncogenesis. 2017; 6(7):e360.

32. Guo JY, Chiu CH, Wang MJ, Li FA, Chen JY.J Proteoglycan serglycin promotes non-small cell lung cancer cell migration through the interaction of its glycosaminoglycans with CD44. Biomed Sci. 2020; 27(1):2.

33. Guo JY, Hsu HS, Tyan SW, Li FY, Shew JY, Lee WH, Chen JY. Serglycin in tumor microenvironment promotes non-small cell lung cancer aggressiveness in a CD44-dependent manner. Oncogene. 2017; 36(17): 2457-2471.

34. Kotton D. N., Fabian A. J., Mulligan R. C. A novel stem-cell population in adult liver with potent hematopoietic-reconstitution activity. Blood. 2005; 106(5):1574–1580.

35. Golden-Mason L., Curry M. P., Nolan N., et al. Differential expression of lymphoid and myeloid markers on differentiating hematopoietic stem cells in normal and tumor-bearing adult human liver. Hepatology. 2000; 31(6):1251–1256.

36. Kolset SO, Pejler G. Serglycin: a structural and functional chameleon with wide impact on immune cells. J Immunol. 2011;187(10):4927–33.

37. Zernichow L, Abrink M, Hallgren J, Grujic M, Pejler G, Kolset SO. Serglycin is the major secreted proteoglycan in macrophages and has a role in the regulation of macrophage tumor necrosis factor-alpha secretion in response to lipopolysaccharide. J Biol Chem. 2006; 281(37):26792–801.

38. Niemann CU, Abrink M, Pejler G, Fischer RL, Christensen EI, Knight SD, et al. Neutrophil elastase depends on serglycin proteoglycan for localization in granules. Blood. 2007; 109(10): 4478– 86.

39. Grujic M, Christensen JP, Sorensen MR, Abrink M, Pejler G, Thomsen AR. Delayed contraction of the CD8+ T cell response toward lymphocytic choriomeningitis virus infection in mice lacking serglycin. J Immunol. 2008; 181(2):1043–51.

40. Chanzu H, Lykins J, Wigna-Kumar S, Joshi S, Pokrovskaya I, Storrie B, Pejler G, Wood JP, Whiteheart SW. Platelet alpha-granule cargo packaging and release are affected by the luminal proteoglycan, serglycin. J Thromb Haemost. 2021; 19(4):1082-1095.

41. Sutton VR, Brennan AJ, Ellis S, Danne J, Thia K, Jenkins MR, Voskoboinik I, Pejler G, Johnstone RW, Andrews DM, Trapani JA. Serglycin determines secretory granule repertoire and regulates natural killer cell and cytotoxic T lymphocyte cytotoxicity. FEBS J. 2016 ; 283(5):947-61.

42. EvansA, CostelloE. The role of inflammatory cells in fostering pancreatic cancer cell growth and invasion. Front Physiol. 2012; 3:270.

43. Purushothaman A, Bandari SK, Chandrashekar DS, Jones RJ, Lee HC, Weber DM, Orlowski RZ.Chondroitin sulfate proteoglycan serglycin influences protein cargo loading and functions of tumor-derived exosomes. Oncotarget. 2017; 8(43):73723- 73732.

44. Kun Cheng, Ning Cai, Jinghan Zhu, Xing Yang, Huifang Liang, et al.Tumor- associated macrophages in liver cancer: From mechanisms to therapy. Cancer Commun (Lond). 2022; 42(11):1112-1140.

45. Ma Q, Gu W, Li T, Zhang K, Cui Y, Qu K, Wang N, Humphry R, Durkan C, Qiu J, Wang G. SRGN, a new identified shear-stress-responsive gene in endothelial cells. Mol Cell Biochem. 2020; 474(1-2): 15-26.

46. Zahran AM, Abdel-Rahim MH, Refaat A, Sayed M, Othman MM, Khalak LMR, Hetta HF.Circulating hematopoietic stem cells,endothelialprogenitor cells and cancer stem cells in hepatocellular carcinoma patients: contribution to diagnosis and prognosis. Acta Oncol. 2020; 59(1):33-39.

47. Menyhart O, Nagy A, Gyorffy B. Determining consistent prognostic biomarkers of overall survival and vascular invasion in hepatocellular carcinoma. R Soc Open Sci**.** 2018; 5(122):181006.

Figure legends

Figure 1 (A) Differential expression of SRGN in tumor (red column) compared with adjacent normal tissues (blue column) across all TCGA tumors by Wilcoxon's test using TIMER (Diff Exp module) (*P < 0.05, **P < 0.01, ***P < 0.001). (B) The University of ALabama at Birmingham CANcer data analysis (UALCAN) portal provides protein expression analysis using data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC). Protein expression data for liver cancer are not available.

Figure 2 (A) SRGN gene expression in tumor, adjacent normal, and metastatic liver cancer

tissues via the Kaplan–Meier plotter database. (B) Expression of SRGN RNA in LIHC and adjacent normal tissues via GEPIA. (C) SRGN RNA was expressed in immune cell types in the order neutrophils > eosinophils >basophils > total peripheral blood mononuclear cells > classical monocytes, and in liver cell types in the order neutrophils >endothelial cells > Kupffer cells > plasma cells > cholangiocytes via The Human Protein Atlas (HPA). (D) SRGN RNA in liver cells via HPA. SRGN was prominently expressed in T cells, Kupffer cells, B cells, hepatic stellate cells, and endothelial cells, and barely expressed in hepatocytes. (E, F) GEPIA proportion analysis. The proportion of each cell type shown with interactive boxplots by ANOVA test among cell types or TCGA/GTEx sub-datasets. (G, H) GEPIA sub-expression analysis. SRGN expression in each cell type shown with interactive boxplot by ANOVA test. All cell types were analyzed, but only cells with strong correlations with SRGN or prognostic potential are shown.

Figure 3 Genes positively (A) and negatively (B) correlated with SRGN in LIHC by UALCAN analysis (data from TCGA). (C) Correlations between SRGN and liver cell biomarkers via HPA. (D) Predicted functional proteins with SRGN (*Homo sapiens*) by STRING analysis (https://cn.string-db.org/ cgi/network? taskId= bHNlp6Ws7IcB& session Id=bSY6tL5m6eXy).

Figure 4 Comparisons of Kaplan–Meier survival curves between cohorts with high and low levels of SRGN expression associated with high and low levels of infiltrating cells in LIHC via TIMER2.0.

Figure 5 *In vitro* and *in vivo* experiments of pro-tumorigenicity of SRGN (A) Relative expression of SRGN in HepG2SG (over-expressing SRGN) and HepG2SG-NC (transfected

with blank control vector) examined by quantitative reverse transcription-polymerase chain reaction. (B) Expression of SRGN protein in HepG2SG and HepG2SG-NC cells examined by western blotting. (C) Proliferation rates (%) of HepG2SG and HepG2SG-NC cells by CCK8 assay (*P < 0.05, **P < 0.01). (D) Proliferation rates (%) of HepG2SG and HepG2SG-NC cells treated with sorafenib, 2> resistance index>1. (E) Invasion of HepG2SG and HepG2SG-NC cells examined by Transwell assay (***P < 0.001). (F) Angiogenesis of HUVECs cultured with supernatant from HepG2SG and HepG2SG-NC cells, respectively (***P ≤ 0.001). (G) Vasculogenic mimicry of HepG2SG and HepG2SG-NC cells (**P < 0.01). (H) Weight of HepG2SG and HepG2SG-NC subcutaneous xenograft tumors in nude mice (*P < 0.05). (I) Hematoxylin and eosin and (J) immunofluorescence staining of subcutaneous tumor tissues. Both SRGN and CD206 were up-regulated while CD80 was down-regulated in HepG2SG mice compared with HepG2SG-NC mice (*P < 0.05, **P < 0.01). Light blue: DAPI; green: fluorescein isothiocyanate-stained SRGN; red: CY3-stained CD80 or CD206.

Table1 The correlation of serglycin expression and prognosis in cancers via four bioinformatics databases

Database and cancer	**ID**	**Patients**	**Survival**	**Hazard ratio(HR)**	**p-value**
PrognoScan	ID	Sample size	Survival	HR(95%CI)	Cox P
AML	1554676_at	35/44	OS	1.35(1.03-1.76)	0.03
	201859_at	69/10	OS	2.59(1.01-6.63	0.05
	201858_s_at	9/49	OS	1.99(1.31-3.0)	0.001
	201859_at	36/22	OS	2.67(1.48-4.82)	0.001
DLBCL	201858_s_at	22/31	EFS	2.49(1.03-6.02)	0.04
	201858_s_at	22/31	OS	2.59(1.02-6.56)	0.05
Breast cancer	201859_at	86/29	DMSF	0.31(0.12-0.83)	0.02
	201858_s_at	91/195	DMSF	0.77(0.62-0.96)	0.02
Lung cancer	1554676_at	182/22	RFS	0.46(0.29-0.72)	0.001
Ovarian cancer	201859_at	87/46	OS	0.82(0.70-0.97)	0.03
GEPIA[#]	ID	Sample size	Survival	HR(high)	p-value
TGCT	NA	68/68	OS	9.1e8	0.022
GBM	NA	80/81	RFS	1.7	0.013
SKCM	NA	228/229	OS	0.51	5.2e-07
SKCM	NA	228/229	RFS	0.74	0.014
TIMER2.0	ID	Sample size	Survival	HR	p-value
SARC	NA	260	OS	0.762	0.0213
SKCM	NA	471	OS	0.746	0.000873
SKCM metastasis	NA	368	OS	0.734	0.00184
ACC	NA	79	OS	1.49	0.0659

LGG	NA	516	OS	1.19	0.0691
Kaplan-Meier plotter*	ID	Sample size	Survival	HR(95%CI)	p-value
Breast cancer	201859 _at	458	PPS	1.39(1.09-1.76)	0.0066
	201859_at	4929	RFS	1.15(1.03-1.27)	0.012
	201858_s_at	1879	OS	0.74(0.61-0.91)	0.0038
	201858_s_at	4929	RFS	1.13(1.01-1.27)	0.034
Ovarian cancer	201858_s_at	1435	PFS	1.26,(1.11-1.45)	0.00054
	201859_at	1435	PFS	1.3(1.12-1.5)	0.00038
	201859_at	1656	OS	1.15(1-1.33)	0.051
	201859_at	782	PPS	1.21(1-1.46)	0.05
Lung cancer	201859_at	982	FP	0.58(0.48- 0.71)	9.9e-08
	201859_at	1925	OS	0.78 (0.69 -0.89)	0.00022
	201858_s_at	1925	OS	1.25(1.1-1.42)	0.00081
Gastric cancer	201858_s_at	875	OS	0.55(0.46-0.66)	6.3e-12
	201858_s_at	498	PPS	0.49(0.38-0.62)	9.7e-10
	201858_s_at	640	FP	0.57(0.47-0.7)	4.8e-08
	201859_at	640	FP	0.57(0.46-0.7	1e-07
	201859_at	875	OS	0.58(0.49-0.69)	2.1e-10
	201859_at	498	PPS	0.56(0.44-0.7)	3.8e-07
Liver cancer	5552	366	PFS	0.67(0.5-0.91)	0.0095
	5552	364	OS	0.68(0.47-0.97)	0.034
	5552	313	RFS	0.65((0.46-0.92)	0.015
	5552	357	DSS	0.58(0.35-0.97)	0.034

The survival analysis of SRGN expression in pan-cancer. NA: the data was not shown; *Split patients by auto select best cutoff, other factors such as pathology, risk factors, patient, and follow up threshold, etc., were chosen all; #group cutoff:

median, cutoff -high/low: 50%. AML, acute myeloid leukemia ; DLBCL, diffuse large B-cell lymphoma ; LGG, brain lower grade glioma; ACC, adrenocortical carcinoma; SKCM, skin cutaneous melanoma; TGCT, testicular germ cell tumor; SARC, sarcoma; GBM, glioblastoma multiforme; OS, overall survival; PFS, progression free survival; RFS, relapse-free survival; PPS, post progression survival; FP, first progression; DSS, disease-specific survival; DMFS, distant metastasis-free survival; EFS, event free survival.

Table 2 Correlation of SRGN mRNA expression and prognosis in liver cancer with different clinical factors by Kaplan-Meier plotter.

Clinical characteristics*	**OS (n= 364)**			**RFS(n=313)**		
	N	**Hazard ratio**	**p-value**	**N**	**Hazard ratio**	**p-value**
Male	246	0.56(0.36-0.88)	**0.01**	208	0.56(0.36-0.88)	**0.0051**
Female	118	0.49(0.24-0.99)	**0.044**	105	0.56(0.28-1.1)	0.085
White	181	0.58(0.36-0.93)	**0.022**	147	0.75(0.48-1.18)	0.21
Asian	155	0.51(0.28-0.94)	**0.028**	143	0.55(0.33-0.92)	**0.022**
Hepatitis virus(yes)	150	0.72(0.38-1.37)	0.31	138	0.53(0.31-0.88)	**0.014**
(no)	167	0.39(0.23-0.64)	**0.00014**	142	0.65(0.38-1.12)	0.12
Alcohol consumption(yes)	115	0.35(0.18-0.7)	**0.0017**	98	0.52(0.28-0.97)	**0.037**
(no)	202	0.65(0.38-1.1)	0.1	182	0.63(0.38-1.02)	0.06
Sorafenib treatment	29	0.51(0.17-1.54)	0.23	22	0.41(0.16-1.07)	0.06
Vascular invasion(none)	203	0.65(0.38-1.12)	0.12	175	0.74(0.45-1.21)	0.23
(micro)	90	2.14(0.74-6.22)	0.15	81	0.59(0.27-1.3)	0.18
AJCC_T(1)	180	0.76(0.42-1.39)	0.37	160	1.27(0.72-2.24)	0.4
(2)	90	0.44(0.21-0.95)	**0.031**	79	0.46(0.21-1.01)	**0.046**

(3)	78	0.46(0.23-0.9)	**0.021**	65	0.56(0.27-1.15)	0.11
Grade (1)	55	0.44(0.17-1.14)	0.084	45	1.74(0.64-4.71)	0.27
2	174	0.55(0.33-0.94)	**0.026**	147	0.53(0.31-0.93)	**0.024**
3	118	1.3(0.69-2.46)	0.42	106	0.54(0.31-0.93)	**0.025**
Stage 1	170	0.73(0.39-1.38)	0.34	153	1.55(0.87-2.76)	0.14
1+2	253	0.67(0.42-1.09)	0.11	227	0.74(0.48-1.13)	0.16
2	83	0.45(0.2-1.01)	**0.046**	74	0.51(0.25-1.05)	0.063
2+3	166	0.49(0.3-0.79)	**0.0028**	142	0.52(0.33-0.81)	**0.0033**
3	83	0.41(0.21-0.8)	**0.0071**	68	0.54(0.29-1.01)	0.051
3+4	87	0.45(0.24-0.85)	**0.011**	68	0.54(0.29-1.01)	0.051
Clinical characteristics		PFS (n=366)			DSS(n=357)	
	N	Hazard ratio	p-value	N	Hazard ratio	p-value
Male	246	0.54□0.37-0.77□	**0.00073**	241	0.53□0.29-0.97□	**0.037**
Female	120	0.56□0.31-1.01□	0.051	116	0.41□0.16-1.08□	0.062
White	183	0.73□0.49-1.07□	0.11	177	0.62□0.35-1.11□	0.1
Asian	155	0.58□0.36-0.94□	**0.025**	152	0.31□0.14-0.68□	**0.002**
Hepatitis virus(yes)	152	0.55□0.34-0.88□	**0.011**	149	1.54□0.67-3.55□	0.31
(no)	167	0.64□0.39-1.03□	0.062	163	0.37□0.2-0.69□	**0.0013**
Alcohol consumption(yes)	115	0.43□0.25-0.74□	**0.002**	115	0.29□0.13-0.66□	**0.0017**
(no)	204	0.68□0.43-1.08□	0.098	197	0.63□0.31-1.29□	0.2
Sorafenib treatment	30	0.37□0.15-0.9□	**0.023**	29	0.51□0.17-1.54□	0.23
Vascular invasion(none)	204	0.84□0.54-1.3□	0.43	200	1.46□0.7-3.04□	0.31
(micro)	91	0.61□0.34-1.08□	0.084	88	4.62(0.6-35.68)	0.11
AJCC_T(1)	180	1.58□0.95-2.63□	0.073	177	3.82(0.9-16.26)	0.051

(2)	92	0.45□0.25-0.8□	**0.0049**	89	0.36(0.13-1.04)	**0.049**
(3)	78	0.46□0.24-0.88□	**0.016**	75	0.4(0.18-0.89)	**0.021**
Grade (1)	55	0.4□0.18-0.9□	**0.023**	55	0.32(0.09-1.17)	0.072
2	175	0.6□0.37-0.96□	**0.031**	169	0.48(0.24-0.95)	**0.032**
3	119	0.54□0.32-0.93□	**0.024**	116	1.31(0.61-2.83)	0.49
Stage 1	170	1.62□0.96-2.74□	0.071	167	3.18(0.74-13.74)	0.1
1+2	254	0.69□0.45-1.06□	0.088	249	0.67(0.34-1.34)	0.26
2	84	0.44□0.24-0.82□	**0.0083**	82	0.33(0.1-1.08)	0.054
2+3	167	0.49□0.33-0.74□	**0.00043**	163	0.38(0.21-0.7)	**0.0013**
3	83	0.45□0.24-0.82□	**0.0074**	81	0.36(0.17-0.78)	**0.007**
3+4	88	0.45□0.25-0.81□	**0.0066**	84	0.37(0.18-0.79)	**0.0075**

* The sample numbers with some clinical characteristics were too low for meaningful analysis, such as Black or Africa American, Grade 4, macro invasion, etc. N: sample size.

Table3. The correlation between SRGN and infiltrating cells in LIHC by TIMER2.0

TME Cells	Rho	p-value
T cell CD4+	0.368	1.76e-12
T cell CD4+ naïve_CIBERSORT)	-0.152	4.69e-03
T cell CD4+ naïve_XCELL)	0.171	1.44e-03
T cell CD4+ central memory	-0.262	7.79e-07
T cell CD4+ effector memory	0.107	4.8e-02
T cell CD4+ memory activated	0.12	2.64e-02
T cell CD4+ memory resting	0.412	1.55e-15
T cell CD4+ memory	0.305	7.2e-09

T cell CD4+(non-regulatory)	0.175	1.1e-03
T cell CD4+ Th1	-0.234	1.09e05
T cell CD4+ Th2	0.206	1.19e-04
T cell CD8+	**0.552**	**5.79e-29**
T cell CD8+ central memory	0.336	1.49e-10
T cell CD8+ effector memory	0.119	2.65e-02
B cell	0.308	5.19e-09
B cell naïve	0.145	7.06e-03
B cell plasma_CIBERSORT-ABS	0.121	2.4e-02
B cell plasma_XCELL	-0.11	4.12e-02
B cell memory	0.108	4.49e-02
T cell regulatory	0.399	1.34e-14
T cell follicular helper	0.252	2.22e-06
Monocyte	0.481	2.21e-21
Macrophage/monocyte	0.481	2.21e-21
Macrophage_EPIC	-0.382	1.9e-13
Macrophage_TIMER	0.481	2.31e-21
Macrophage M0	-0.123	2.23e-02
Macrophage M1	**0.517**	**5.84e-25**
Macrophage M2_CIBERSORT-ABS	**0.696**	**3.26e-51**
Macrophage M2_TIDE	**-0.516**	**6.66e-25**
Myloid dendritic cell	**0.627**	**4.97e-39**
Myloid dendritic cell activated	0.475	7.62e-21
Myloid dendritic cell resting	0.142	8.13e-03

Common lymphoid progenitor	0.211	8.13e-05
Neutrophil	0.426	1.11e-16
NK cell	-0.115	3.26e-02
NK cell resting	-0.164	2.18e-03
NK cell activated	**0.524**	**1.11e-25**
T cell NK	-0.147	6.11e-03
Mast cell	0.109	4.27e-02
Mast cell resting	0.192	3.23e-04
Mast cell activated	-0.166	2.03e03
Endothelial cell	**0.522**	**1.67e-25**
Cancer associated fibroblast	0.456	4.09e-19

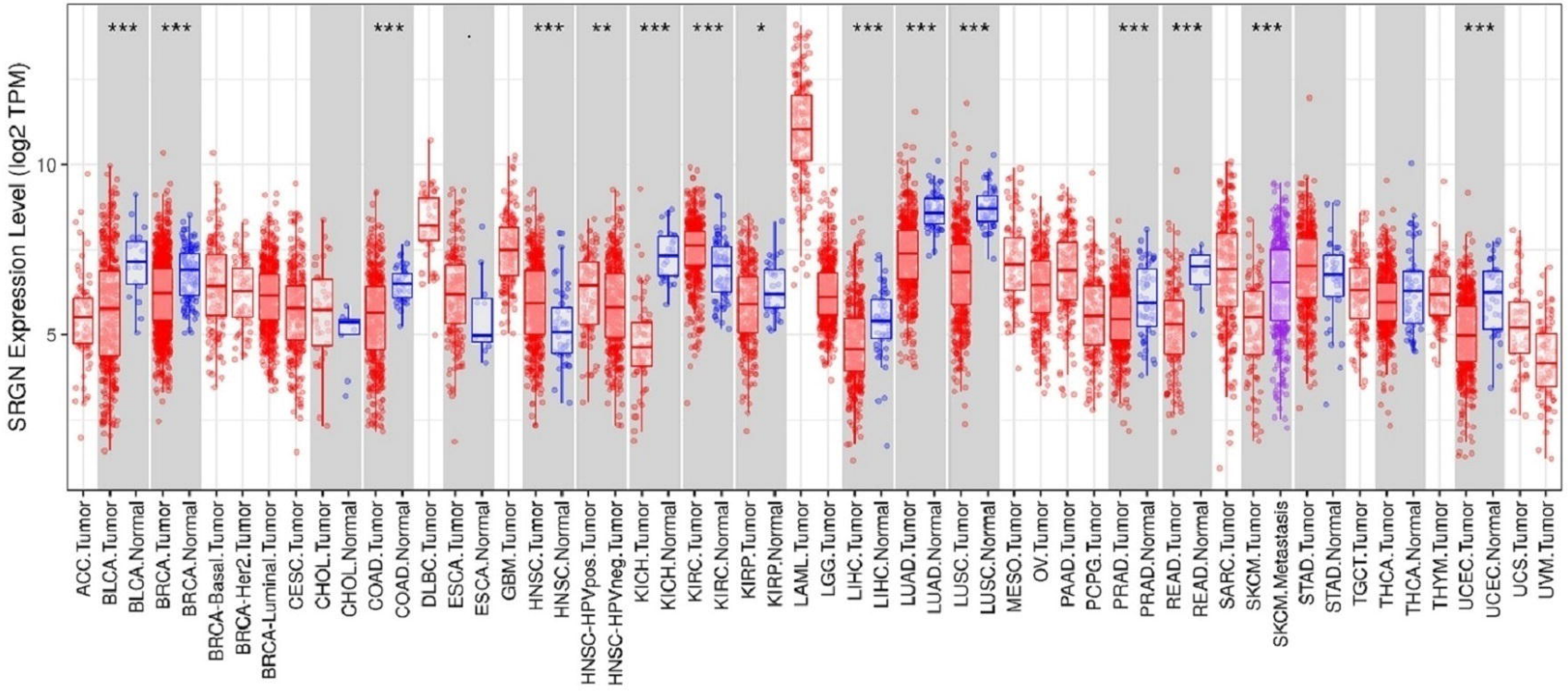
Table4. The survival analysis of SRGN expression associated with infiltrating cells in LIHC by TIMER2.0

Cells in TME	**Low SRGN expression Hazard ratio**	**p-value**	**High SRGN expression Hazard ratio**	**p-value**
T cell CD8+	0.658	0.139	0.53	**0.0448**
T cell CD4+ memory resting	0.847	0.584	0.501	**0.0383**
Hematopoietic stem cell	0.834	0.551	0.379	**0.00868**
MDSC	1.41	0.313	2.78	**0.00668**
Endothelial cell	0.565	0.0566	0.364	**0.00638**
Monocyte(MCPcounter)	1.65	0.105	2.75	**0.025**
Macrophage	1.82	**0.0432**	1.58	0.198
Macrophage M1	1.19	0.558	3.09	**0.00633**

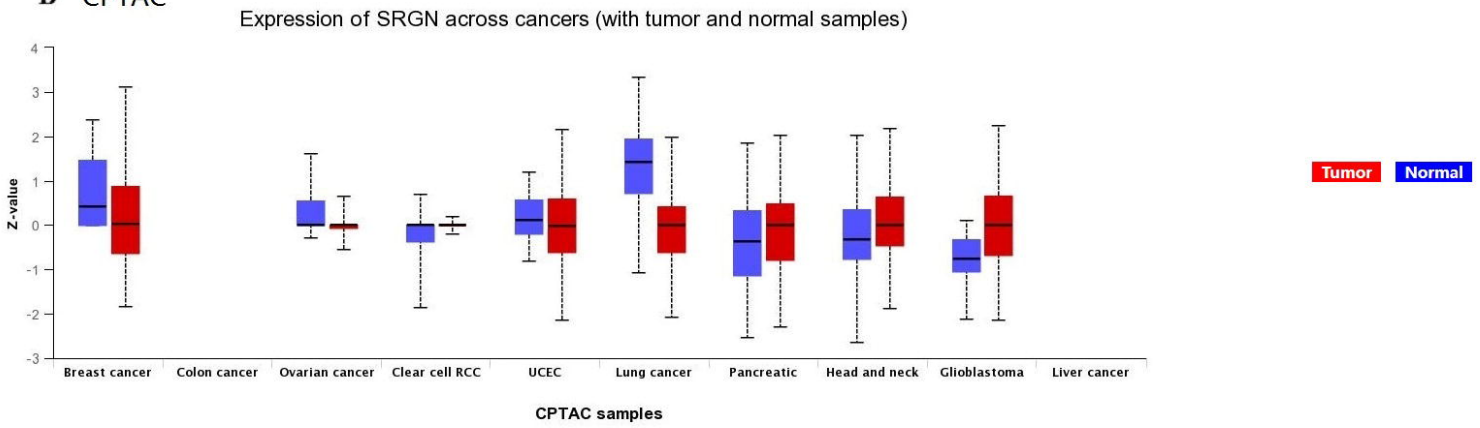
Macrophage 0	1.86	**0.0356**	1.59	0.159
Macrophage 2	1.99	**0.0366**	1.25	0.568
T cell CD4+ central memory	0.742	0.305	0.874	0.694
T cell CD4+ Th2	1.22	0.498	1.83	0.0945
Tregs	0.873	0.659	0.671	0.249
Monocyte(XCELL)	1.19	0.599	1.95	0.0811
Myloid dendrtic cell	0.924	0.796	3.28	0.0599
Myloid dendrtic cell resting	1.34	0.283	1.05	0.871

TME: tumor microenvironment

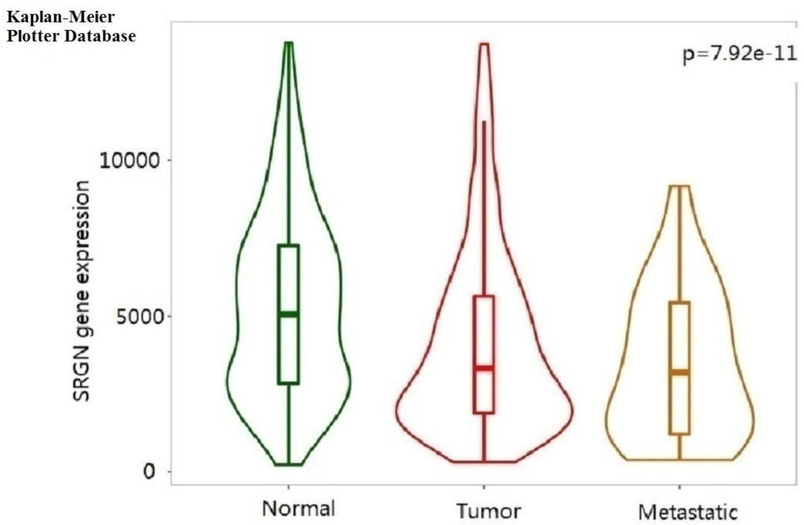
A TIMER



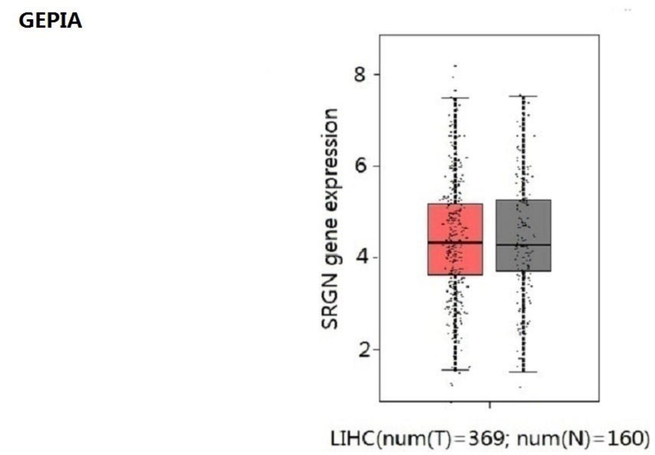
B CPTAC



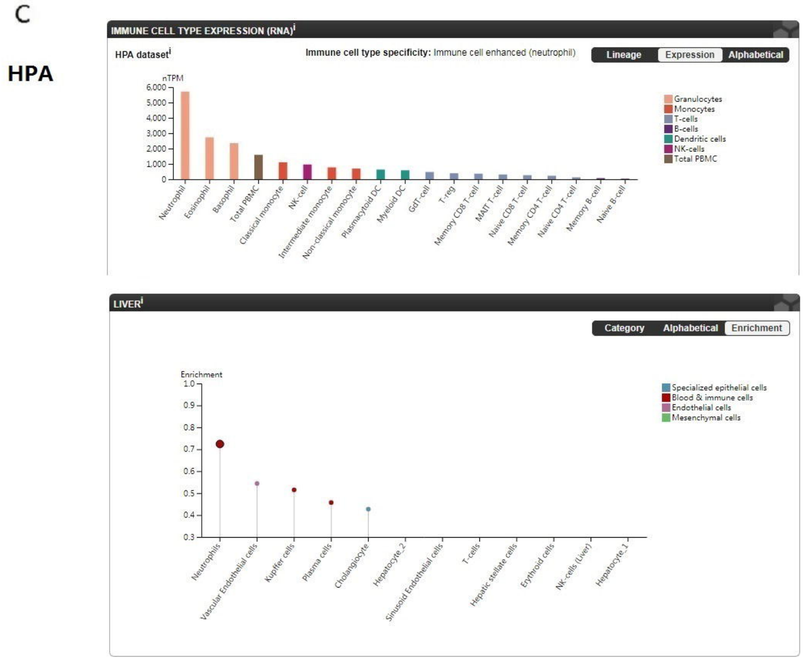
A



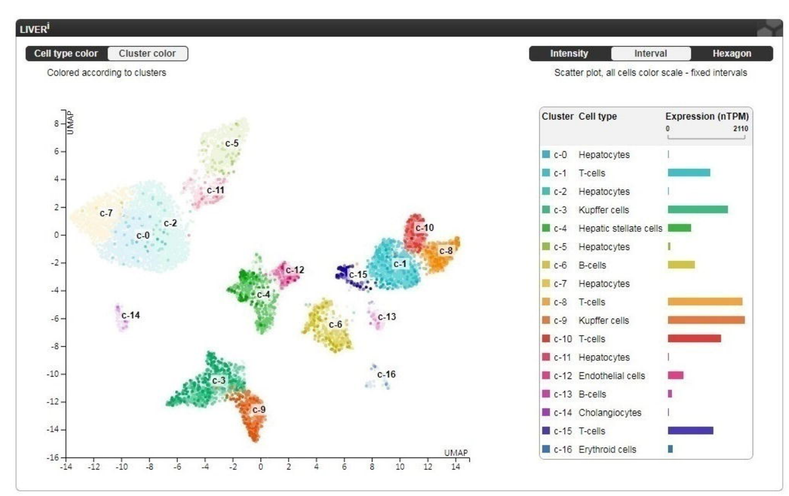
B



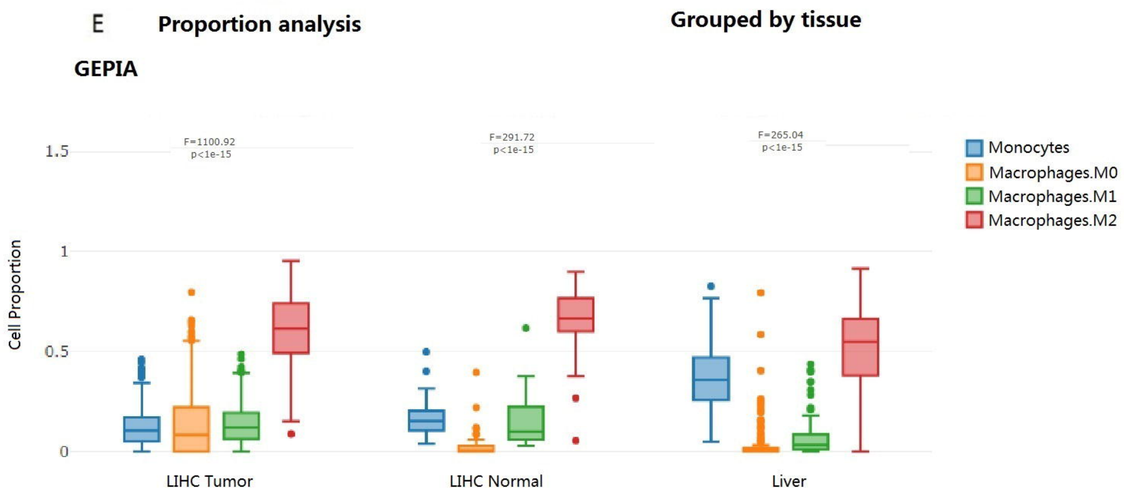
C



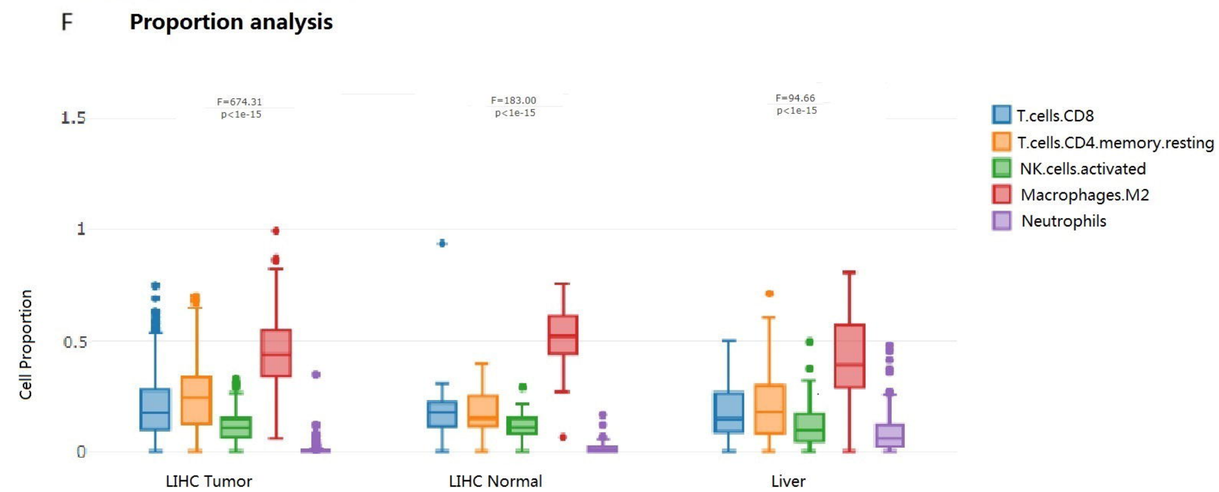
D



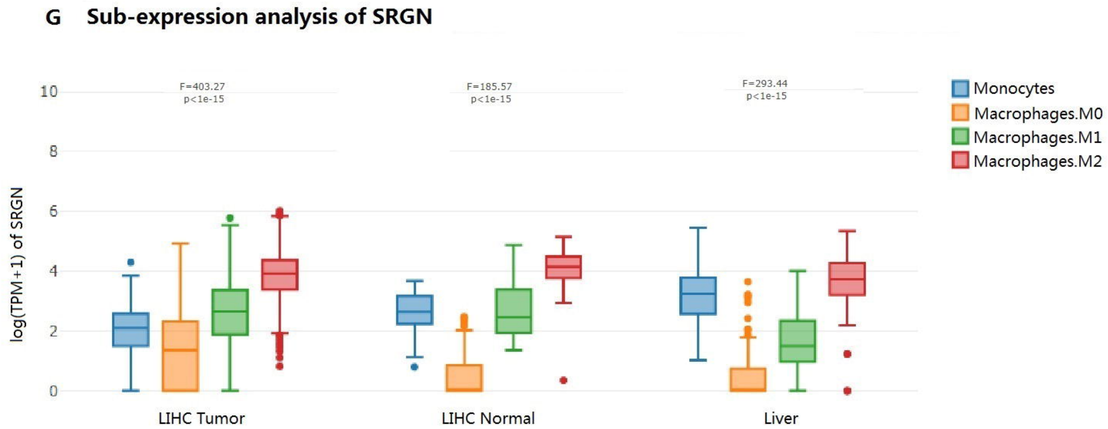
E Proportion analysis



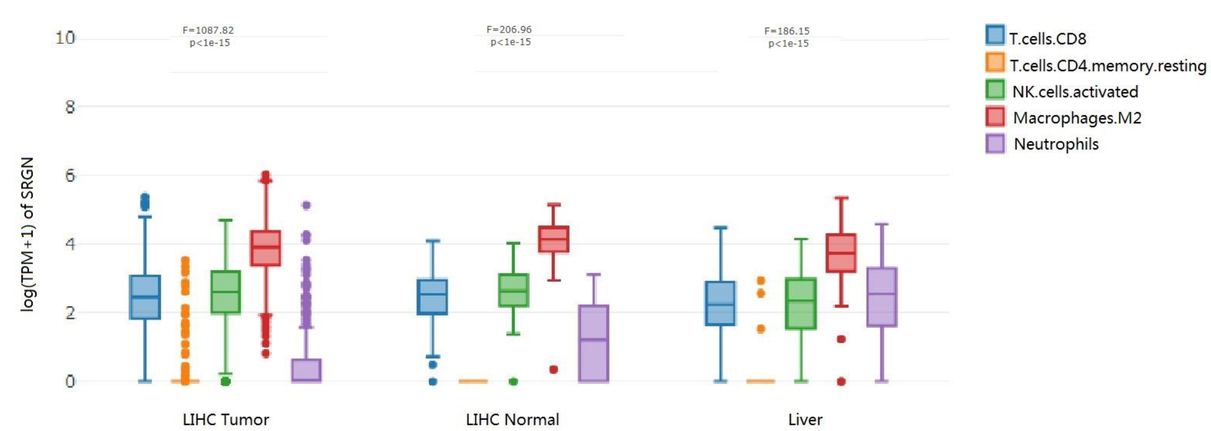
F Proportion analysis



G Sub-expression analysis of SRGN

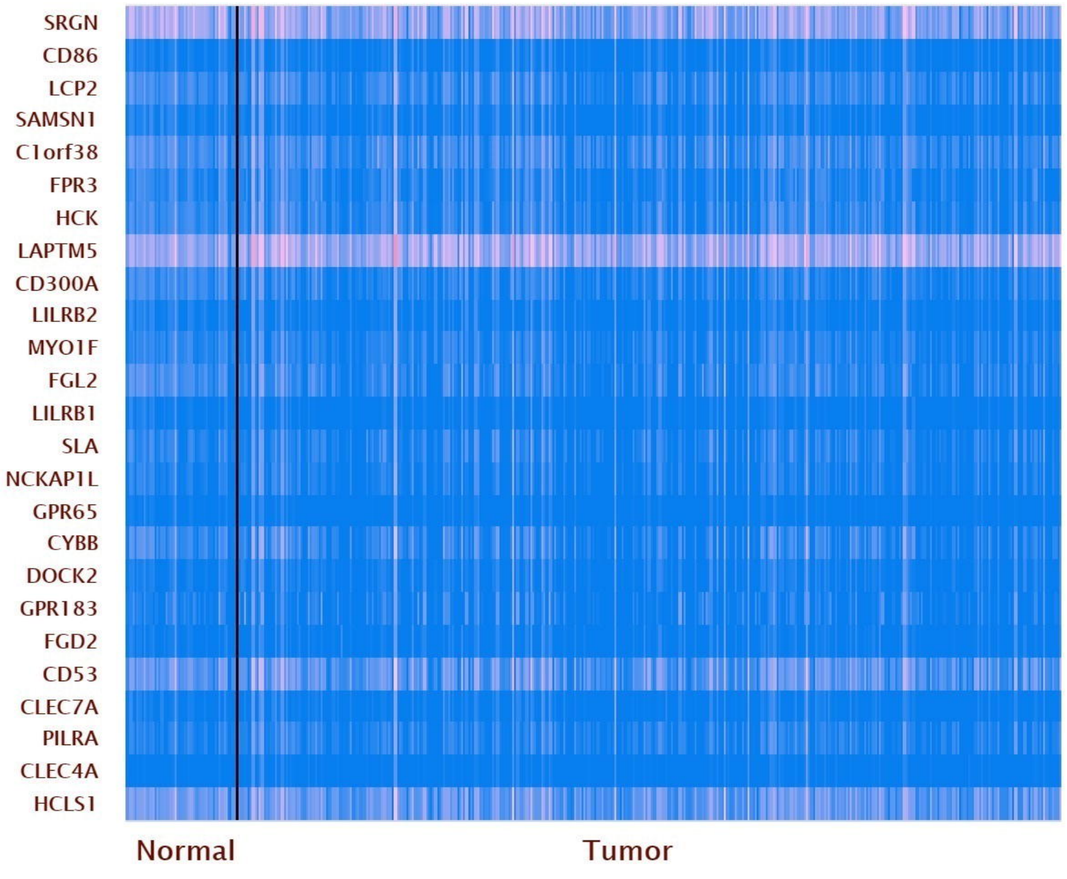


H Sub-expression analysis of SRGN

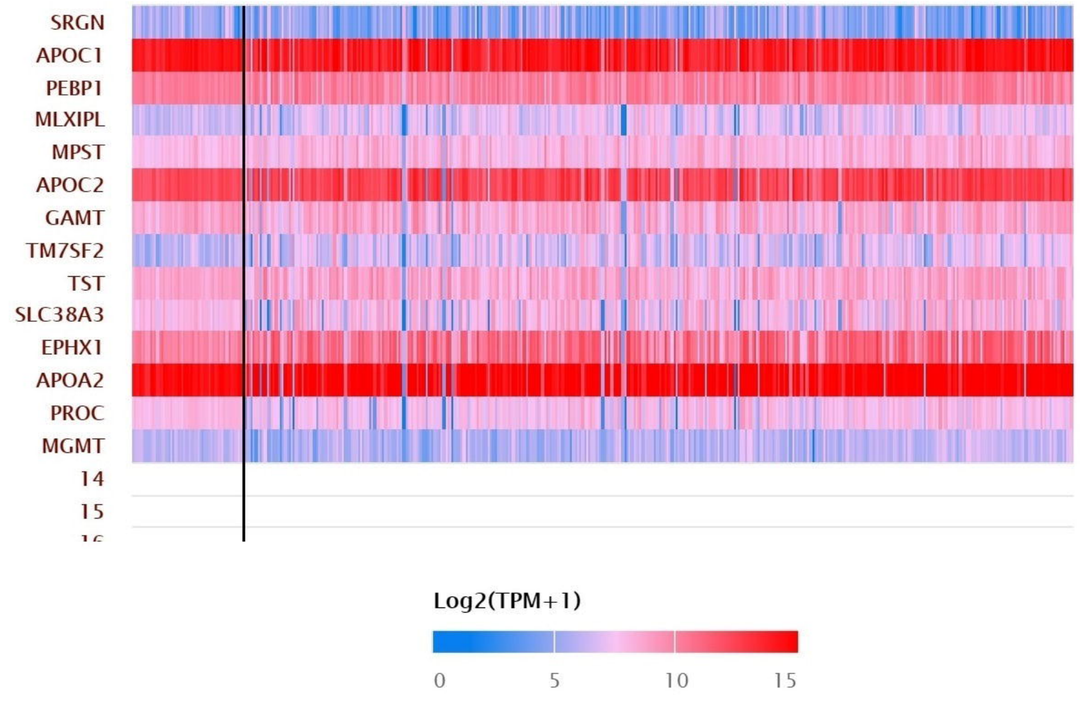


Expression pattern of input genes in Liver hepatocellular carcinoma (LIHC)

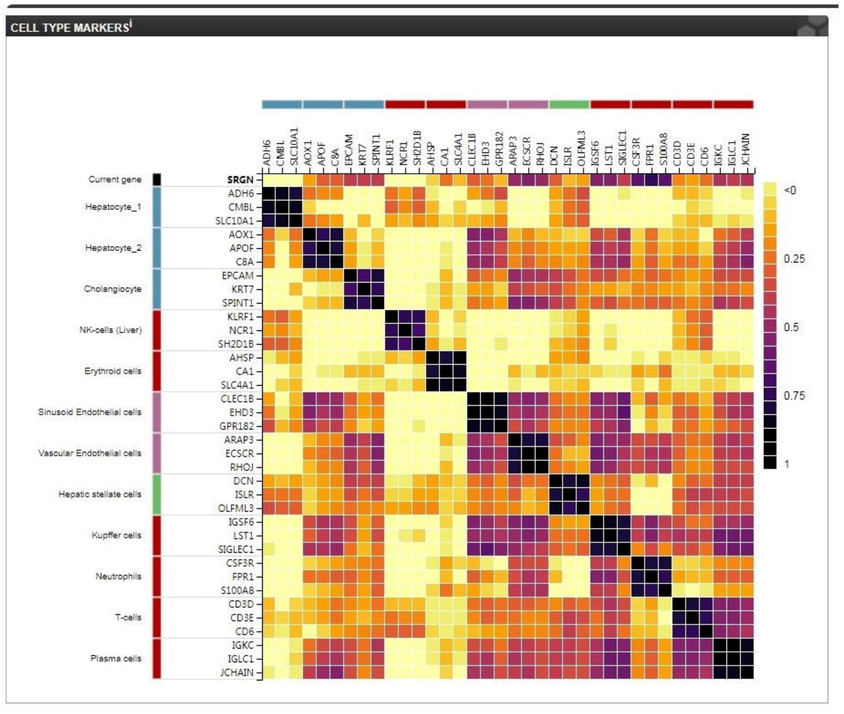
A Ualcan



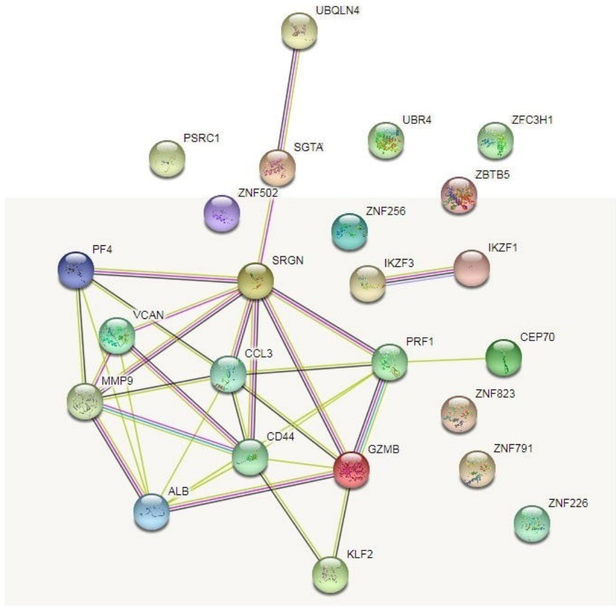
B Ualcan

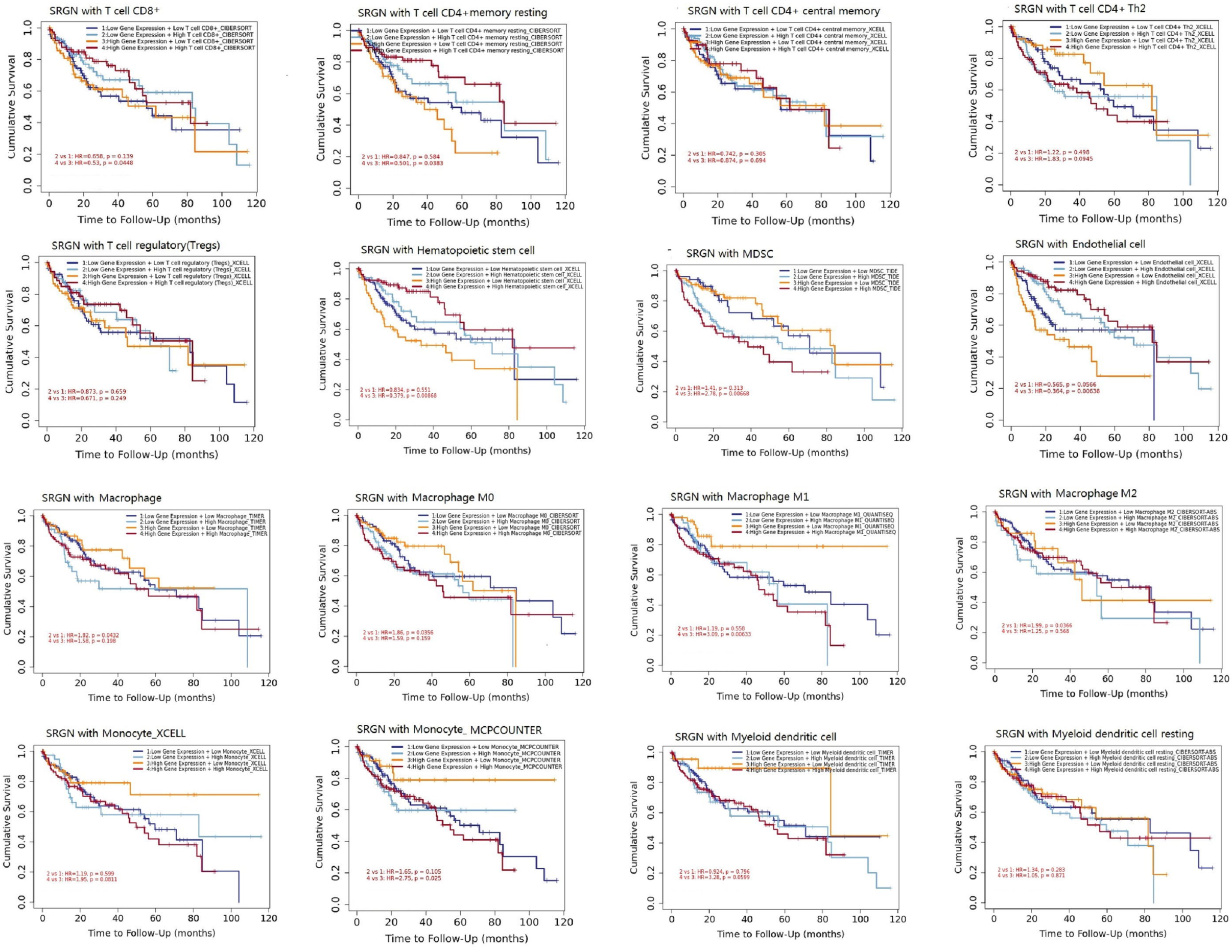


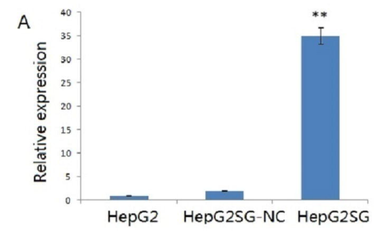
C HPA

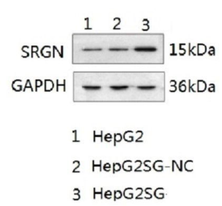


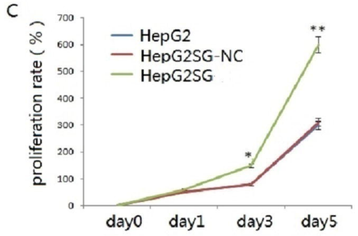
D STRING

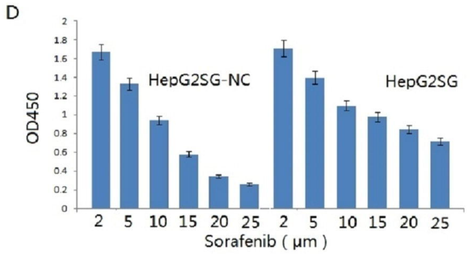



SRGN with T cell CD8+
1:Low Gene Expression + Low T cell CD8+_CIBERSORT
2:Low Gene Expression + High T cell CD8+_CIBERSORT
3:High Gene Expression + Low T cell CD8+_CIBERSORT
4:High Gene Expression + High T cell CD8+_CIBERSORT
2 vs 1: HR=0.658, p = 0.139
4 vs 3: HR=0.53, p = 0.0448
SRGN with T cell CD4+memory resting
1:Low Gene Expression + Low T cell CD4+ memory resting_CIBERSORT
2:Low Gene Expression + High T cell CD4+ memory resting_CIBERSORT
3:High Gene Expression + Low T cell CD4+ memory resting_CIBERSORT
4:High Gene Expression + High T cell CD4+ memory resting_CIBERSORT
2 vs 1: HR=0.847, p = 0.584
4 vs 3: HR=0.501, p = 0.0383
SRGN with T cell CD4+ central memory
1:Low Gene Expression + Low T cell CD4+ central memory_XCELL
2:Low Gene Expression + High T cell CD4+ central memory_XCELL
3:High Gene Expression + Low T cell CD4+ central memory_XCELL
4:High Gene Expression + High T cell CD4+ central memory_XCELL
2 vs 1: HR=0.742, p = 0.305
4 vs 3: HR=0.874, p = 0.694
SRGN with T cell CD4+ Th2
1:Low Gene Expression + Low T cell CD4+ Th2_XCELL
2:Low Gene Expression + High T cell CD4+ Th2_XCELL
3:High Gene Expression + Low T cell CD4+ Th2_XCELL
4:High Gene Expression + High T cell CD4+ Th2_XCELL
2 vs 1: HR=1.22, p = 0.498
4 vs 3: HR=1.83, p = 0.0945
SRGN with T cell regulatory(Tregs)
1:Low Gene Expression + Low T cell regulatory (Tregs)_XCELL
2:Low Gene Expression + High T cell regulatory (Tregs)_XCELL
3:High Gene Expression + Low T cell regulatory (Tregs)_XCELL
4:High Gene Expression + High T cell regulatory (Tregs)_XCELL
2 vs 1: HR=0.873, p = 0.659
4 vs 3: HR=0.671, p = 0.249
SRGN with Hematopoietic stem cell
1:Low Gene Expression + Low Hematopoietic stem cell_XCELL
2:Low Gene Expression + High Hematopoietic stem cell_XCELL
3:High Gene Expression + Low Hematopoietic stem cell_XCELL
4:High Gene Expression + High Hematopoietic stem cell_XCELL
2 vs 1: HR=0.834, p = 0.551
4 vs 3: HR=0.379, p = 0.00868
SRGN with MDSC
1:Low Gene Expression + Low MDSC_TIDE
2:Low Gene Expression + High MDSC_TIDE
3:High Gene Expression + Low MDSC_TIDE
4:High Gene Expression + High MDSC_TIDE
2 vs 1: HR=1.41, p = 0.313
4 vs 3: HR=2.78, p = 0.00668
SRGN with Endothelial cell
1:Low Gene Expression + Low Endothelial cell_XCELL
2:Low Gene Expression + High Endothelial cell_XCELL
3:High Gene Expression + Low Endothelial cell_XCELL
4:High Gene Expression + High Endothelial cell_XCELL
2 vs 1: HR=0.565, p = 0.0566
4 vs 3: HR=0.364, p = 0.00638
SRGN with Macrophage
1:Low Gene Expression + Low Macrophage_TIMER
2:Low Gene Expression + High Macrophage_TIMER
3:High Gene Expression + Low Macrophage_TIMER
4:High Gene Expression + High Macrophage_TIMER
2 vs 1: HR=1.82, p = 0.0432
4 vs 3: HR=1.58, p = 0.198
SRGN with Macrophage M0
1:Low Gene Expression + Low Macrophage M0_CIBERSORT
2:Low Gene Expression + High Macrophage M0_CIBERSORT
3:High Gene Expression + Low Macrophage M0_CIBERSORT
4:High Gene Expression + High Macrophage M0_CIBERSORT
2 vs 1: HR=1.86, p = 0.0356
4 vs 3: HR=1.59, p = 0.159
SRGN with Macrophage M1
1:Low Gene Expression + Low Macrophage M1_QUANTISEQ
2:Low Gene Expression + High Macrophage M1_QUANTISEQ
3:High Gene Expression + Low Macrophage M1_QUANTISEQ
4:High Gene Expression + High Macrophage M1_QUANTISEQ
2 vs 1: HR=1.19, p = 0.558
4 vs 3: HR=3.09, p = 0.00633
SRGN with Macrophage M2
1:Low Gene Expression + Low Macrophage M2_CIBERSORT-ABS
2:Low Gene Expression + High Macrophage M2_CIBERSORT-ABS
3:High Gene Expression + Low Macrophage M2_CIBERSORT-ABS
4:High Gene Expression + High Macrophage M2_CIBERSORT-ABS
2 vs 1: HR=1.99, p = 0.0366
4 vs 3: HR=1.25, p = 0.568
SRGN with Monocyte_XCELL
1:Low Gene Expression + Low Monocyte_XCELL
2:Low Gene Expression + High Monocyte_XCELL
3:High Gene Expression + Low Monocyte_XCELL
4:High Gene Expression + High Monocyte_XCELL
2 vs 1: HR=1.19, p = 0.599
4 vs 3: HR=1.95, p = 0.0811
SRGN with Monocyte_ MCPCOUNTER
1:Low Gene Expression + Low Monocyte_MCPCOUNTER
2:Low Gene Expression + High Monocyte_MCPCOUNTER
3:High Gene Expression + Low Monocyte_MCPCOUNTER
4:High Gene Expression + High Monocyte_MCPCOUNTER
2 vs 1: HR=1.65, p = 0.105
4 vs 3: HR=2.75, p = 0.025
SRGN with Myeloid dendritic cell
1:Low Gene Expression + Low Myeloid dendritic cell_TIMER
2:Low Gene Expression + High Myeloid dendritic cell_TIMER
3:High Gene Expression + Low Myeloid dendritic cell_TIMER
4:High Gene Expression + High Myeloid dendritic cell_TIMER
2 vs 1: HR=0.924, p = 0.796
4 vs 3: HR=3.28, p = 0.0599
SRGN with Myeloid dendritic cell resting
1:Low Gene Expression + Low Myeloid dendritic cell resting_CIBERSORT-ABS
2:Low Gene Expression + High Myeloid dendritic cell resting_CIBERSORT-ABS
3:High Gene Expression + Low Myeloid dendritic cell resting_CIBERSORT-ABS
4:High Gene Expression + High Myeloid dendritic cell resting_CIBERSORT-ABS
2 vs 1: HR=1.34, p = 0.283
4 vs 3: HR=1.05, p = 0.871
Cumulative Survival
Time to Follow-Up (months)

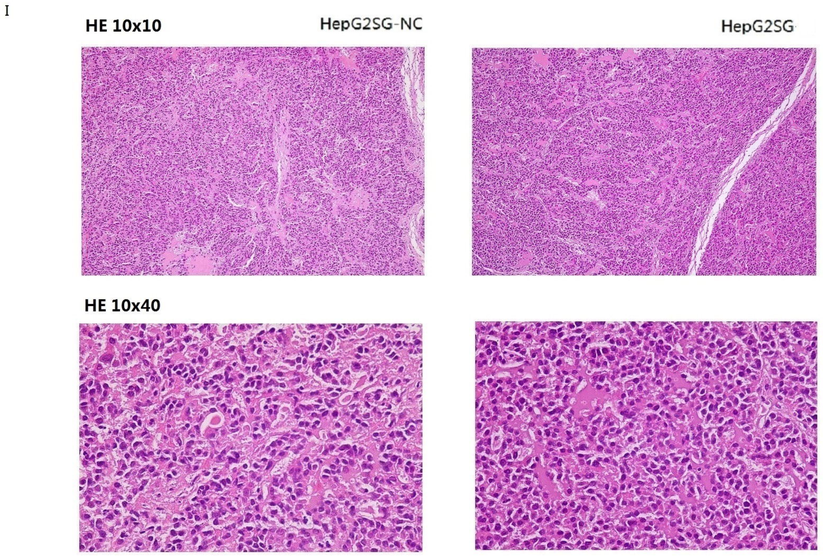

A
Relative expression
HepG2
HepG2SG-NC
HepG2SG

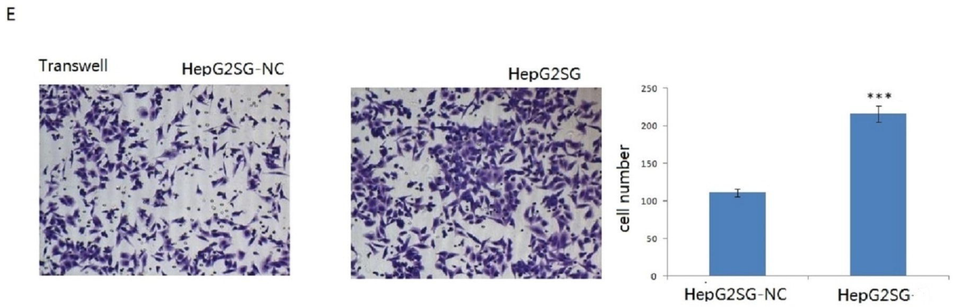

B
1 2 3
SRGN
15kDa
GAPDH
36kDa
1 HepG2
2 HepG2SG-NC
3 HepG2SG

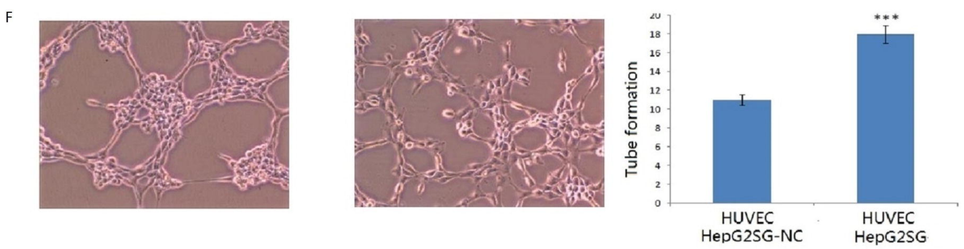

C
proliferation rate (%)
HepG2
HepG2SG-NC
HepG2SG
day0
day1
day3
day5

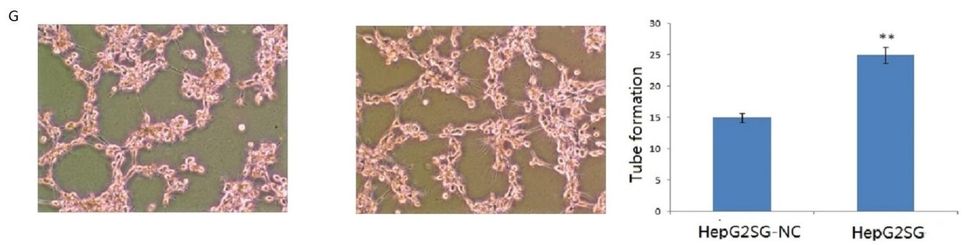

D
OD450
HepG2SG-NC
HepG2SG
Sorafenib (μm)

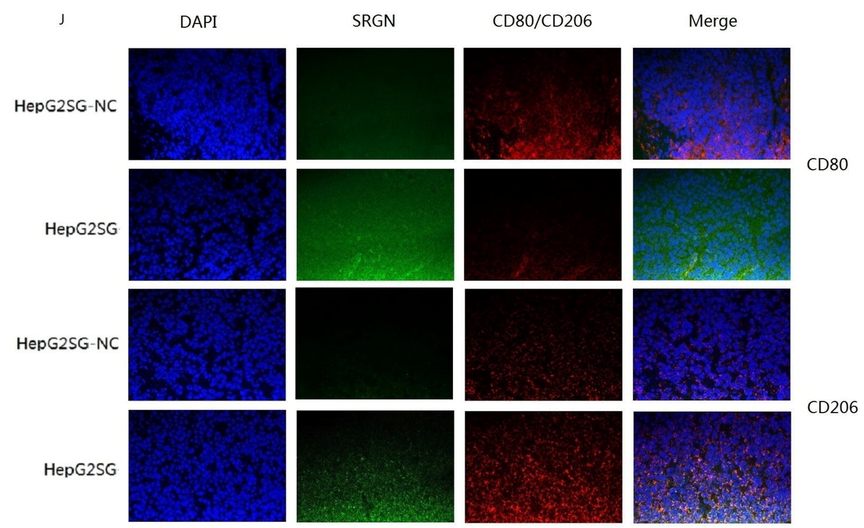
H
HepG2SG-NC
HepG2SG
weight (g)

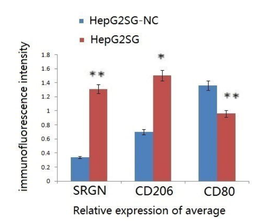

I
HE 10x10
HepG2SG-NC
HepG2SG
HE 10x40


E
Transwell
HepG2SG-NC
HepG2SG
cell number
HepG2SG-NC
HepG2SG


F
Tube formation
HUVEC
HepG2SG-NC
HUVEC
HepG2SG


G
Tube formation
HepG2SG-NC
HepG2SG


J
DAPI
SRGN
CD80/CD206
Merge
HepG2SG-NC
HepG2SG
HepG2SG-NC
HepG2SG
CD80
CD206


HepG2SG-NC
HepG2SG
immunofluorescence intensity
SRGN
CD206
CD80
Relative expression of average