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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Clinical Significance of Signal Regulatory Protein Alpha (SIRPα) Expression in Hepatocellular Carcinoma

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ABSTRACT

Background. Signal regulatory protein alpha (SIRPα), expressed in the macrophage membrane, inhibits phagocytosis of tumor cells via CD47/SIRPα interaction, which acts as an immune checkpoint factor in cancers. This study aimed to clarify the clinical significance of SIRPα expression in hepatocellular carcinoma (HCC).

Methods. This study analyzed SIRPα expression using RNA sequencing data of 372 HCC tissues from The Cancer Genome Atlas (TCGA) and immunohistochemical staining of our 189 HCC patient cohort. The correlation between SIRPα expression and clinicopathologic factors, patient survival, and intratumor infiltration of immune cells was investigated.

Results. Overall survival (OS) was significantly poorer with high SIRPα expression than with low expression in both TCGA and our cohort. High SIRPα expression correlated with lower recurrence-free survival (RFS) in our cohort. High SIRPα expression was associated with higher rates of microvascular invasion and lower serum albumin levels and correlated with greater intratumor infiltration of CD68-positive macrophages and myeloid-derived suppressor cells (MDSCs). Multivariate analysis showed that SIRPα expression and high infiltration of CD8-positive T cells and MDSCs were predictive factors for both RFS and OS. Patients with high SIRPα expression and infiltration of CD8-positive T cells and MDSCs had significantly lower RFS and OS rates. In spatial transcriptomics sequencing, SIRPα expression was significantly correlated with CD163 expression.

Conclusions. High SIRPα expression in HCC indicates poor prognosis, possibly by inhibiting macrophage phagocytosis of tumor cells, promoting MDSC infiltration and inducing antitumor immunity. Treatment alternatives using SIRPα blockage should be considered in HCC as inhibiting macrophage antitumor immunity and MDSCs.

Primary liver cancer is the fourth most common type of cancer in the world. Hepatocellular carcinoma (HCC) is caused by liver cirrhosis, hepatitis B/C virus infection, and alcoholic or nonalcoholic steatohepatitis. Although liver resection is an effective and safe treatment for patients with HCC, the possibility of recurrence after the procedure remains high.[1,2]

Cancer immunotherapy, such as immune checkpoint inhibition, has emerged as an essential therapeutic option for various cancer types. Tumors have a variety of immune

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escape mechanisms,[3–5] and the activation of T cells in HCC is insufficient. Therefore, combination therapy targeting both innate and adaptive responses is essential and has shown remarkable clinical efficacy in HCC.[6]

Signal regulatory protein alpha (SIRPα) is a transmembrane protein, activating Src-homology-2 domain-containing protein tyrosine phosphatase 1/2 (SHP-1/2) through its cytoplasmic region via ligand interaction (CD47).[7,8] Signal regulatory protein alpha is especially abundant in hematopoietic cells such as macrophages, neutrophils, and dendritic cells.[9,10] In contrast, CD47 is ubiquitously expressed on the surface of various cells, including HCC cells.[11,12] The interaction between SIRPα and CD47 serves as a suppressive signal for phagocytosis. Therefore, this signaling is termed the "don't-eat-me" or antiphagocytic signal.[13,14]

Tumor cells evade antitumor immunity through immune checkpoint signaling. Therefore, therapies targeting this pathway have been considered as immunologic approaches to cancer treatment. Indeed, preclinical research showed that blocking the SIRPα-CD47 pathway promoted macrophage-mediated phagocytosis *in vivo* and exhibited robust synergistic antitumor efficacy in a mouse model.[15,16]

This study aimed to examine the clinical significance of SIRPα expression in HCC tumors using RNA sequencing (RNA-seq) data from a public database and immunohistochemical data from a public database of patients who underwent surgical resection for HCC. We clarified the association between SIRPα expression and clinicopathologic factors and determined the prognostic value of SIRPα expression in patients with HCC. We also investigated the relationship between SIRPα expression and tumor-infiltrating immune cells in HCC tissues. Finally, we examined the correlation between SIRPα expression and tumor-infiltrating immune cells as a prognostic marker.

METHODS

Patients

The study enrolled 189 patients with HCC who underwent initial liver resection at the Department of Surgery and Science of Kyushu University Hospital between July 2004 and December 2015. The surgical technique details and patient selection criteria for liver resection in HCC have been previously reported.[17] Outpatient follow-up visits were carried out for 1–3 months after discharge. Dynamic computed tomography was performed upon a suspected recurrence. Clinical information and follow-up data were obtained from medical records. This study was approved by the ethics committee of Kyushu University (approval code 2021-467) and conducted in accordance with the Declaration of Helsinki of 1996.

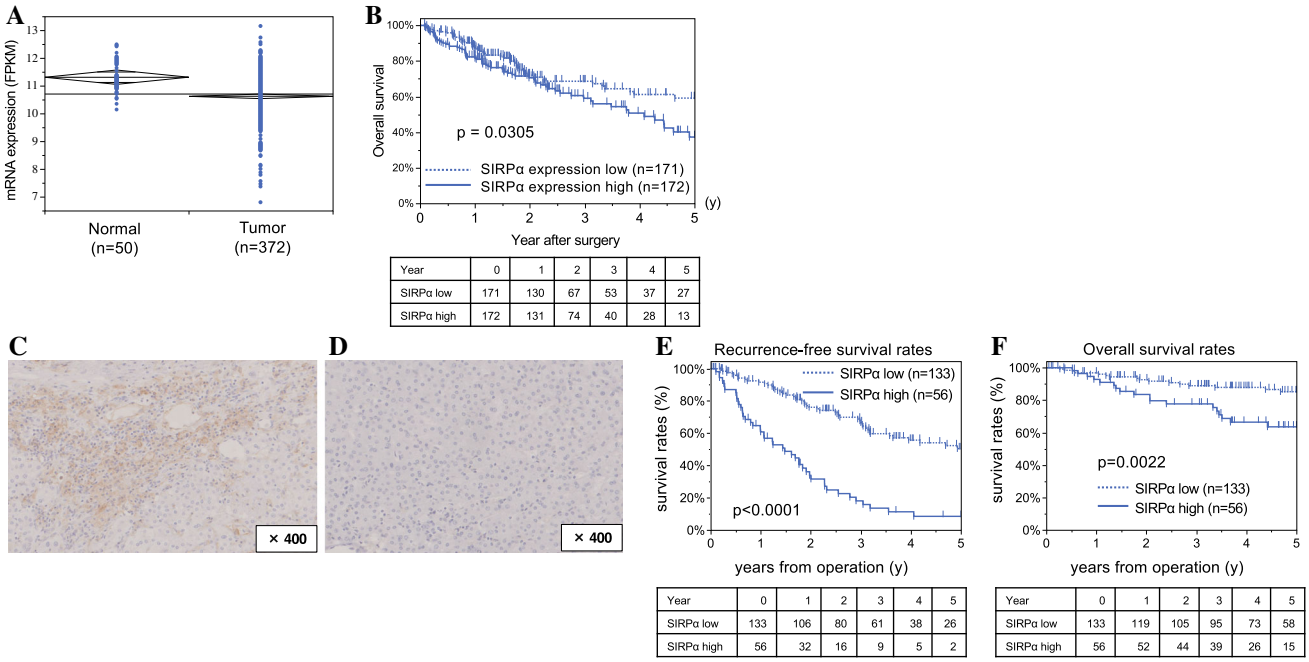
Immunohistochemical Staining

Immunohistochemical staining for CD8,[5] CD33,[4] and CD68[18] was performed as previously described. For SIRPα, immunohistochemical staining was performed on 4-μm formalin-fixed and paraffin-embedded sections. The sections were first deparaffinized. After inhibition of endogenous peroxidase activity for 30 min with 10% hydrogen peroxidase in methanol, the sections were pretreated with Target Retrieval Solution (Dako, Glostrup, Denmark) in a decloaking chamber at 121 °C for 15 min, then incubated with monoclonal antibodies at 4 °C overnight. Immune complexes were detected using an EnVision Detection System (Dako, Carpinteria CA, USA). Finally, the sections were incubated in 3,3'-diaminobenzidine, counterstained with hematoxylin, and mounted.

The primary antibodies used were SIRPα (clone D613M, 1:200 dilution; Cell Signaling Technology, Beverly, MA, USA) and a CD33 mouse antibody (PA0555, no dilution; Leica Biosystems, Wetzlar, Germany). The stained slides were scanned using a NanoZoomer digital slide scanner (Hamamatsu Photonics K.K., Shizuoka, Japan). Cells exhibited plasma membrane staining for SIRPα. Spleens were used as positive controls for SIRPα. Immunohistochemical data for SIRPα, CD33, CD68, and CD8 staining were evaluated by three experienced researchers (T.T., K.K., and S.I.) who were blinded to the clinical status of the patients. The final assessments were achieved by consensus.

Gene Analysis with a Visium Platform

Gene analysis with a Visium platform (10x Genomics, Pleasanton, CA, USA) was performed as previously reported. Briefly, a single HCC sample was embedded in optimal slicing temperature compound (TissueTek Sakura) in a 10-mm × 10-mm cryomold at − 80 °C and sectioned at a thickness of 10 μm. Tissue was permeabilized for 3 min, which was defined as the optimal time in tissue optimization time course experiments. Visium Spatial Gene Expression Libraries were prepared according to the Visium Spatial Gene Expression Reagent Kits User Guide. Libraries were sequenced on the NovaSeq 6000 System (Illumina, Tokyo, Japan) in a paired-end mode at sufficient sequence depth. Raw FASTQ files and histology images were processed using Space Ranger Software v1.1.0 (https://support.10xgenomics.com/spatial-gene-expression/software/pipelines/latest/© nstallation).To visualize spatial expression using histologic images, the raw Visium files for each sample were read into Loupe Browser software v4.0.0 (https://support.10xgenomics.com/spatial-gene-expression/software/downloads/latest). We obtained mean sequence read counts of 288,702 and identified median genes of 3300 per spot. The median-normalized average of



Year	0	1	2	3	4	5
SIRPα low	171	130	67	53	37	27
SIRPα high	172	131	74	40	28	13

Year	0	1	2	3	4	5
SIRPα low	133	106	80	61	38	26
SIRPα high	56	32	16	9	5	2

Year	0	1	2	3	4	5
SIRPα low	133	119	105	95	73	58
SIRPα high	56	52	44	39	26	15

FIG. 1 Overall survival rates in the signal regulatory protein alpha (SIRPα) high-expression group were worse than in the low-expression group using a public dataset of liver hepatocellular carcinoma (LIHC). **A** The mRNA expression level of signal regulatory protein alpha (SIRPα) in tumor tissue compared with normal tissue using the Cancer Genome Atlas. The median mRNA expressions (Log_2FPKM) were 10.7 (range, 6.80–13.2) in tumors and 11.2 (range, 10.1–12.5) in normal tissues ($p < 0.0001$). Overall survival rates for high-expression ($n = 172$), and low-expression ($n = 171$) groups of SIRPα were analyzed by the Kaplan-Meier method. Statistical analysis was performed by the log-rank test. Signal regulatory protein alpha (SIRPα) expression was the indicator of poor prognosis of hepatocellular carcinoma (HCC) in the current cohort. **B** Immunohistochemical staining of SIRPα expression in patients with HCC. **C** Image of a high SIRPα expression pattern. **D** Image of a low SIRPα expression pattern. **E** Recurrence-free survival and **F** overall survival of high ($n = 56$), and low ($n = 133$) expression of SIRPα were analyzed by the Kaplan–Meier method. Statis analysis was undertaken by the log-rank test

gene in cluster is calculated as the mean of observed unique molecular identifier (UMI) counts normalized by the size factor for each cell in the representative cluster.

Statistical Analysis

Standard statistical analyses were used to evaluate descriptive statistics such as medians, frequencies, and percentages. Continuous variables without a normal distribution were compared using the Mann–Whitney *U* test. Logistic regression analysis was performed to identify variables associated with SIRPα expression. Categorical variables were compared using the chi-square test or Fisher's exact test. Survival data were used to establish a univariate Cox proportional hazard model. Covariates significant at a *p* value lower than 0.05 were included in the multivariate Cox proportional hazards model. The cumulative overall survival (OS) and recurrence-free survival (RFS) rates were calculated using the Kaplan–Meier method, and differences between curves were evaluated using the log-rank test. Differences were considered statistically significant at a *p* value lower than 0.05. All statistical analyses were performed using the JMP15 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Signal Regulatory Protein Alpha Expression was a Prognostic Factor of HCC in the TCGA Dataset

We investigated whether SIRPα affects the prognosis of patients with liver hepatocellular carcinoma (LIHC). The association between SIRPα expression and HCC patient prognosis was examined using a public dataset.

First, we estimated the mRNA expression level of SIRPα in 372 patients with LIHC using The Cancer Genome Atlas (TCGA) dataset. Unlike other cancer types, the expression of SIRPα mRNA in cancer tissue was significantly lower than in noncancerous tissue (respective median values: 10.7 [range, 6.80–13.2] vs 11.2 [range, 10.5–12.5]; $p < 0.0001$; Fig. 1A). However, diverse patterns of SIRPα mRNA expression have been observed in cancer tissues compared with that in noncancerous tissues. We divided 343 patients into high-expression ($n = 172$) and low-expression ($n = 171$) groups according to their SIRPα mRNA expression levels. The OS in the high-expression group was significantly lower than in the low-

TABLE 1 Association between patients' background characteristics and intra-tumoral signal regulatory protein alpha (SIRPα) expression

Variable	Low SIRPα (n = 133) n (%)	High SIRPα (n = 56) n (%)	p Value
Median age: years (range)	68.0 (31–87)	69.5 (42–87)	0.9292
Sex (male)	95 (71.4)	40 (71.4)	1.0000
Median BMI, (kg/m^2 (range)	22.9 (16.0–32.6)	22.4 (15.7–28.8)	0.1402
HBs-Ag positivity	17 (12.8)	10 (17.9)	0.3626
HCV-Ab positivity	82 (61.7)	36 (64.3)	0.7325
Non-virus disease	38 (28.6)	11 (19.6)	0.2752
Child-Pugh classification, grade B	2 (1.5)	2 (3.6)	0.3672
Median albumin: g/dL (range)	4.0 (2.9-4.8)	3.8 (1.8-4.7)	**0.0024**
Median DCP: mAU/ml (range)	51 (9–250,400)	77 (12–75,000)	0.4033
Median AFP: ng/ml (range)	10.5 (1.2–44,137)	19.3 (0.5–50,649)	0.2237
Median tumor size: cm (range)	2.8 (0.9–15)	3.0 (1.1–20)	0.3268
Multiple tumors	27 (20.3)	12 (21.4)	0.8611
BCLC staging, B or C	20 (15.0)	8 (14.3)	0.8943
Without Milan criteria	32 (24.1)	17 (30.4)	0.3670
Gross classification, single nodular type	87 (66.4)	34 (60.7)	0.4552
Poor differentiation	33 (24.8)	19 (33.9)	0.2000
Microscopic vascular invasion	30 (22.6)	23 (41.1)	**0.0097**
Microscopic intrahepatic metastasis	18 (13.5)	9 (16.1)	0.6489
F3 or F4	60 (45.1)	33 (58.9)	0.0828

BMI, body mass index; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; DCP, des-gamma-carboxy prothrombin; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer

P values indicate the statistical significance are described in bold

expression group (respectively, 37.3% vs 59.2%; $p = 0.0305$, log-rank test; Fig. 1B).

Signal Regulatory Protein Alpha Expression and Clinicopathologic Factors

Our cohort of 189 patients with HCC had 135 (71.4%) male patients. The median age of the patients was 68 years (range, 61–76 years; 25–75% quantile). Among the 189 patients, 27 (14.2%) and 118 (62.4%) were positive for hepatitis B surface antigen and hepatitis C virus antibody expression, respectively. The median observation period was 4.11 years (range, 2.28–6.32 years; 25–75% quantile).

Figure 1C, D show representative immunohistochemical staining of SIRPα in HCC tissues. The staining exhibited SIRPα in the intratumor cytoplasm and plasma membrane of mononuclear cells. The median protein expression level of SIRPα was 15% (range, 25–75%; quantile, 3–35%). The patients were divided into high-expression (n = 56) and low-expression (n = 133) groups according to the SIRPα staining.

The associations between SIRPα protein expression and clinicopathologic factors are shown in Table 1. The serum albumin level was lower in the SIRPα high-expression group than in the low-expression group ($p = 0.0004$). Tumors with microvascular invasion were more frequent in the SIRPα high-expression group than in the low-expression group ($p = 0.0097$). The primary disease of the patients did not differ significantly.

Survival analysis according to SIRPα expression using the Kaplan-Meier method in our patient cohort showed that both RFS and OS were significantly poorer in the SIRPα high-expression group than in the low-expression group ($p < 0.0001$ and $p = 0.0022$, respectively; Fig. 1E, F).

SIRPα High Expression was Associated with Tumor-Infiltrating Immune Cells

We examined whether SIRPα is involved in the regulation of antitumor immunity in HCC. We used immunohistochemistry (IHC) analysis to evaluate the association between SIRPα expression and the number of tumor-infiltrating immune cells such as cytotoxic T cells, macrophages, and myeloid-derived suppressor cells (MDSCs). Studies show that CD8, CD68, and CD33 are used as cell surface markers for cytotoxic T cells, macrophages, and MDSCs, respectively.[4,5,19] Representative images of CD8-positive T cells, CD68-positive macrophages, and MDSC staining are shown in Fig. 2A–C.

Although the number of tumor-infiltrating CD8-positive T cells did not differ significantly between the SIRPα high- and low-expression groups (Fig. 2D; $p = 0.7115$), the

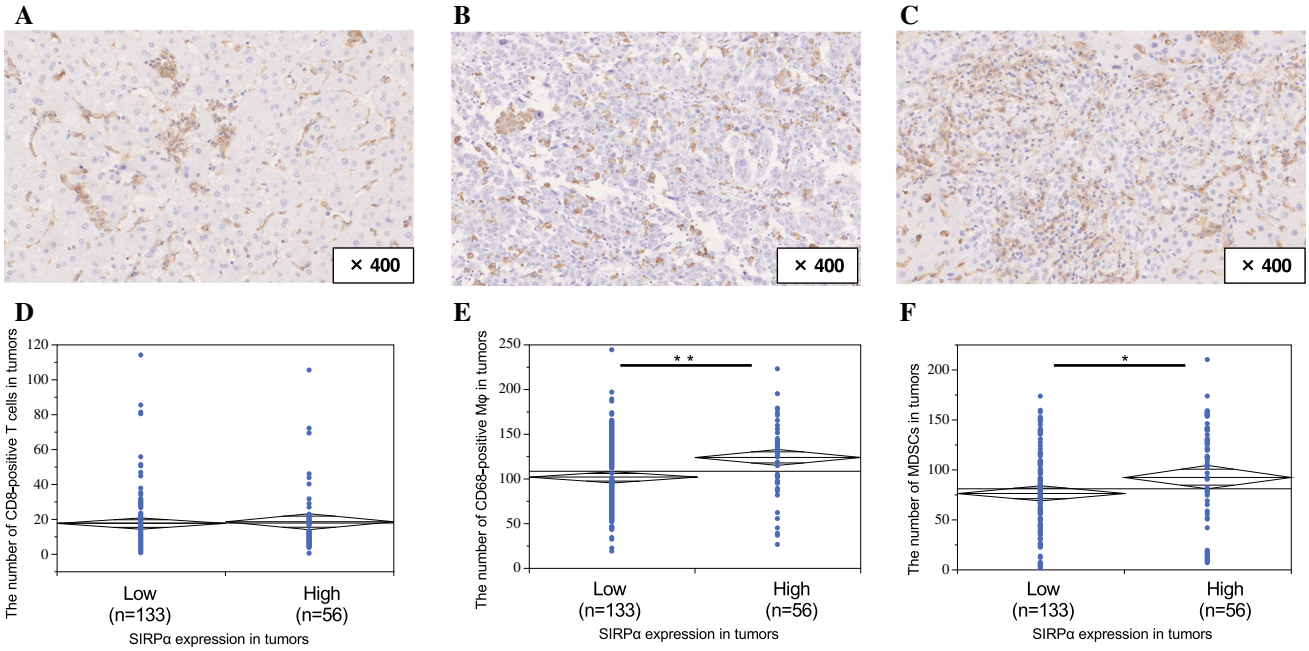


FIG. 2 Signal regulatory protein alpha (SIRPα) expression was correlated with tumor infiltration of macrophage and myeloid-derived suppressor cells (MDSCs). Representative immunohistochemical staining of **A** CD8-positive T cells, **B** CD68-positive macrophages, and **C** myeloid-derived suppressor cells (MDSCs) in patients with hepatocellular carcinoma (HCC) is shown. **D** The median number of intratumor CD8-positive T cells was 12.3 (range, 0.667–114) in the high-SIRPα group and 13.0 (range, 0.333–105) in the low-SIRPα group ($p = 0.7115$). **E** The median number of intratumor CD68-positive macrophages was 131 (range, 26.3–223) in the high-SIRPα group and 95.0 (range, 19.0–244) in the low-SIRPα group ($p = 0.0007$). **F** The median number of intratumor MDSCs was 99.3 (range, 6.67–210) in the high-SIRPα group and 74.7 (range, 1.33–2173) in the low-SIRPα group ($p = 0.0266$). $^{*}p < 0.05$. $^{**}p < 0.001$

SIRPα high-expression group had a significantly higher number of tumor-infiltrating macrophages and MDSCs than the low-expression group (CD68-positive macrophages [$p = 0.0007$], MDSCs [$p = 0.0266$]; Fig. 2E, F). Multivariate analysis showed that high SIRPα expression was significantly associated with the number of tumor-infiltrating CD68-positive macrophages (odds ratio [OR], 3.81; 95% confidence interval [CI] 1.65–8.84; $p = 0.0018$) (Table S1).

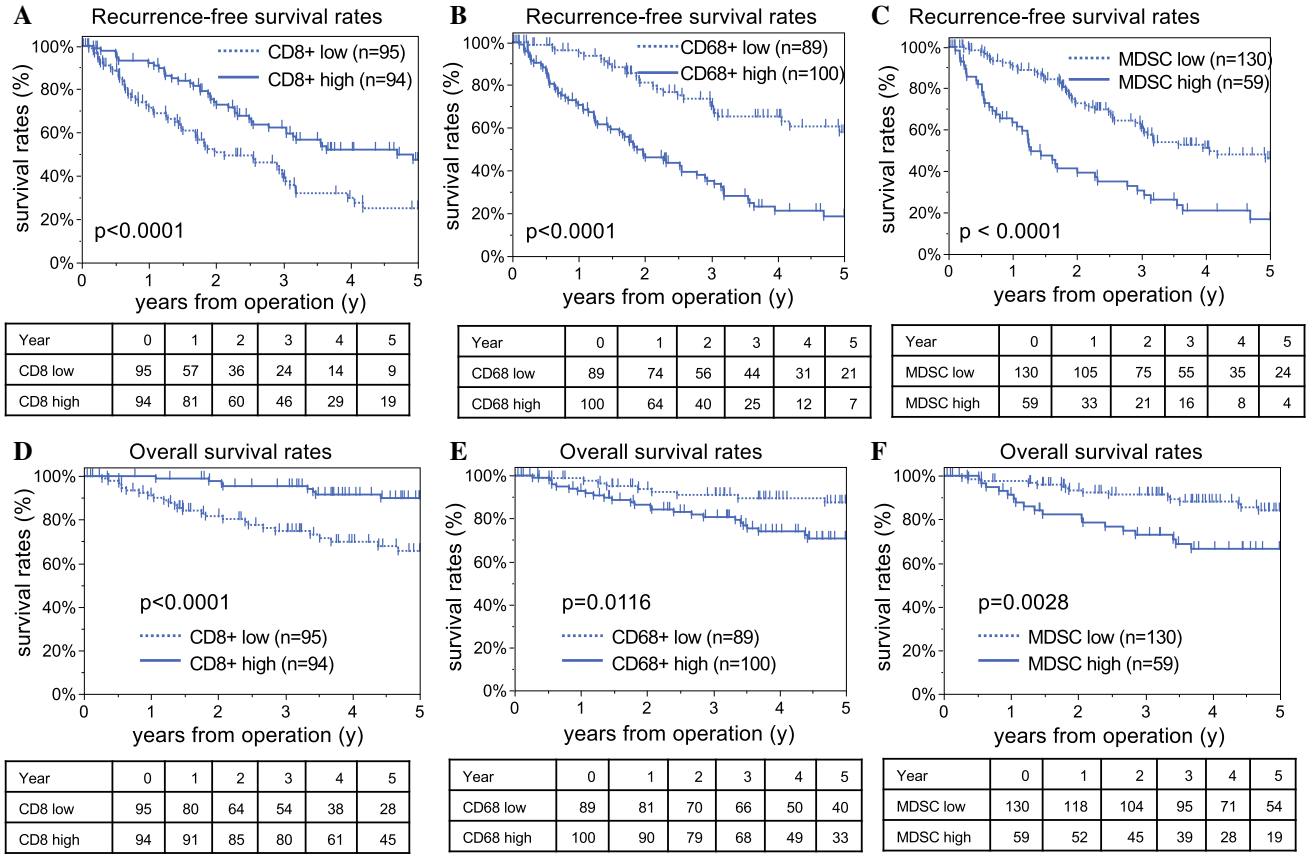
We analyzed the contribution of CD8-positive T cells, CD68-positive macrophages, and MDSCs to patient survival. All were significant prognostic factors for RFS and OS with HCC (RFS: CD8-positive T cells [$p < 0.0001$], CD68-positive macrophages [$p < 0.0001$], MDSCs [$p < 0.0001$]; OS: CD8-positive T cells [$p < 0.0001$], CD68-positive macrophages [$p = 0.0116$], MDSCs [$p = 0.00028$]; Fig. 3A–F).

Uni- and Multivariate Analysis of Prognostic Factors for RFS and OS

Tables 2 and 3 lists the uni- and multivariate analyses results associated with RFS and OS for the patients with surgically resected HCC. In the multivariate analysis, Cox proportional hazard regression models showed that high expression of SIRPα in tumors was associated with significantly worse RFS and OS (RFS: hazard ratio [HR], 2.78 [95% CI 1.78–4.33, $p < 0.0001$]; OS: HR, 2.45 [95% CI 1.16–5.20, $p = 0.0194$]). Low intratumor CD8-positive T cell infiltration in tumors was associated with significantly worse RFS (HR, 2.02; 95% CI 1.27–3.19; $p = 0.0028$) and OS (HR, 4.08; 95% CI 1.72–9.67; $p = 0.0014$). High intratumor MDSC infiltration also was associated with significantly worse RFS (HR, 2.42; 95% CI 1.41–4.17; $p = 0.0014$) and OS (HR, 2.52; 95% CI 1.00–6.35; $p = 0.0493$). In addition, the prognostic factors for RFS were liver fibrosis (HR, 1.72; 95% CI 1.03–2.89; $p = 0.0338$) and intrahepatic metastasis (HR, 1.82; 95% CI 10.1–3.26; $p = 0.0449$). In this analysis, CD68-positive macrophages were not significantly associated with RFS or OS.

Examination of SIRPα Expression in M2 Macrophage

We evaluated the distribution of SIRPα expression using the Visium platform. Figure 4A, B show the distributions of SIRPα and CD163, respectively. The distributions of SIRPα and CD163 were similar. Compared with CD163-



Year	0	1	2	3	4	5
CD8 low	95	57	36	24	14	9
CD8 high	94	81	60	46	29	19

Year	0	1	2	3	4	5
CD68 low	89	74	56	44	31	21
CD68 high	100	64	40	25	12	7

Year	0	1	2	3	4	5
MDSC low	130	105	75	55	35	24
MDSC high	59	33	21	16	8	4

Year	0	1	2	3	4	5
CD8 low	95	80	64	54	38	28
CD8 high	94	91	85	80	61	45

Year	0	1	2	3	4	5
CD68 low	89	81	70	66	50	40
CD68 high	100	90	79	68	49	33

Year	0	1	2	3	4	5
MDSC low	130	118	104	95	71	54
MDSC high	59	52	45	39	28	19

FIG. 3 Kaplan–Meier curves showing the survival of patients with hepatocellular carcinoma (HCC) according to the number of tumor-infiltrating CD8-positive T cells, CD68-positive macrophages, or myeloid derived suppressor cells (MDSCs). Recurrence-free survival rates for all the patients according to **A** high and low intratumor CD8-positive T cell infiltration, **B** CD68-positive macrophage infiltration, and **C** MDSC infiltration are shown. Overall survival rates for all the patients according to **D** high and low CD8-positive T cell infiltration, **E** CD68-positive macrophage infiltration, and **F** MDSCs infiltration are shown

negative cells, a significantly higher percentage of CD163-positive cells exhibited SIRPα expression (Fig. 4C).

Stratification of SIRPα Expression and MDSC and CD8-Positive T Cell Infiltration in HCC

Next, we evaluated the significance of SIRPα expression and MDSC and CD8+ T cell infiltration in predicting OS and RFS. High SIRPα expression, low CD8 infiltration, and high MDSC infiltration each was assigned a prognostic risk score of 1 point, and the patients were divided into four groups (0–3 points). The four groups were found to differ significantly in both RFS ($p < 0.0001$, log-rank test) and OS ($p < 0.0001$, log-rank test) (Fig. 5A, B). The differences in clinicopathologic characteristics between the patients with the highest risk scores and the others are shown in Table S2. Poor differentiation and smaller tumor size were observed in the group with the highest risk factor score compared with the others ($p = 0.0109$ and 0.0352, respectively). In addition, the serum albumin level was lower in the group with the highest risk score than in the other groups ($p = 0.0007$).

DISCUSSION

The current study analyzed SIRPα expression in the TCGA and our HCC cohorts. In both cohorts, we identified SIRPα expression as an indicator of poor prognosis in HCC patients compared with the conventional markers such as alpha fetoprotein and des-gamma-carboxy pro-thrombin. Although SIRPα was associated with tumor-infiltrating macrophages and MDSCs, the correlation coefficient was small, with r^2 below 0.1 for both the number of intratumor CD68-positive macrophages and SIRPα expression ($r^2 = 0.065$ for the number of intratumor MDSCs and $r^2 = 0.038$ for SIRPα expression). Therefore, the relationship was weak as a confounding factor, and multivariate analysis was used to examine these factors.

TABLE 2 Results of uni- and multivariate analyses of recurrence-free survival

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age (years)						
< 75 (*n* = 134)	1.00	(Referent)				
≥ 75 (*n* = 55)	1.18	0.76–1.85	0.4585			
Sex						
Male (*n* = 135)	1.05	0.66–1.66				
Female (*n* = 54)	1.00	(Referent)	0.8317			
hBs-Ag						
Positivity (*n* = 27)	0.87	0.49–1.53				
Negativity (*n* = 162)	1.00	(Referent)	0.6213			
HCV-Ab						
Positivity (*n* = 118)	1.03	0.68–1.57				
Negativity (*n* = 71)	1.00	(Referent)	0.882			
Alb (g/dL)						
< 3.5 (*n* = 18)	1.13	0.55–2.35				
≥ 3.5 (*n* = 171)	1.00	(Referent)	0.7345			
DCP (mAU/ml)						
< 300 (*n* = 139)	1.00			1.00	(Referent)	
≥ 300 (*n* = 50)	1.79	1.17–2.75	**0.0079**	1.56	0.86–2.83	0.1460
AFP (ng/ml)						
< 500 (*n* = 166)	1.00	(Referent)				
≥ 500 (*n* = 22)	1.05	0.57–1.93	0.8692			
Tumor size (cm)						
≤ 5.0 (*n* = 147)	1.00	(Referent)		1.00	(Referent)	
> 5.0 (*n* = 42)	2.09	1.35–3.25	**0.0018**	1.64	0.62–4.34	0.3228
Macroscopic tumor numbers						
Multiple (*n* = 39)	2.57	1.67–3.96		1.82	1.01–3.26	
Solitary (*n* = 150)	1.00	(Referent)	< **0.0001**	1.00	(Referent)	**0.0449**
BCLC staging, B or C						
0 or A (*n* = 161)	1.00	(Referent)		1.00	(Referent)	
B or C (*n* = 28)	2.10	1.29–3.42	**0.0029**	0.43	0.18–1.02	0.0547
Milan criteria						
Within Milan criteria (*n* = 137)	1.00	(Referent)		1.00	(Referent)	
Without Milan criteria (*n* = 52)	2.07	1.36–3.14	**0.0007**	1.22	0.45–3.25	0.6969
Poorly differentiated						
Present (*n* = 52)	1.31	0.83–2.05				
Absent (*n* = 137)	1.00	(Referent)	0.2419			
Tumor types						
Boundary type (*n* = 121)	1.00	(Referent)				
Nonboundary type (*n* = 66)	1.24	0.82–1.89	0.312			
Microscopic vascular invasion						
Present (*n* = 53)	2.26	1.48–3.45		1.26	0.74 –2.12	
Absent (*n* = 136)	1.00	(Referent)	**0.0003**	1.00	(Referent)	0.3949
Microscopic intrahepatic metastasis						
Present (*n* = 27)	2.95	1.80–4.83		1.72	0.93–3.16	
Absent (*n* = 162)	1.00	(Referent)	< **0.0001**	1.00	(Referent)	0.0882

TABLE 2 continued

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Fibrosis						
F0–2 (*n* = 96)	1.00	(Referent)		1.00	(Referent)	
F3, 4 (*n* = 93)	1.90	1.17–3.10	**0.0095**	1.72	1.03–2.89	**0.0388**
SIRPα expression						
High (*n* = 56)	4.04	2.66–6.13		2.78	1.78–4.33	
Low (*n* = 133)	1.00	(Referent)	< **0.0001**	1.00	(Referent)	< **0.0001**
CD8-positive T cell infiltration						
High (*n* = 94)	1.00	(Referent)		1.00	(Referent)	
Low (*n* = 95)	2.01	1.33–3.03	**0.0009**	2.02	1.27–3.19	**0.0028**
CD68-positive cell infiltration						
High (*n* = 100)	3.32	2.11–5.21		1.73	0.96–3.12	
Low (*n* = 89)	1.00	(Referent)	< **0.0001**	1.00	(Referent)	0.0705
MDSC infiltration						
High (*n* = 59)	2.75	1.82–4.15		2.42	1.41–4.17	
Low (*n* = 130)	1.00	(Referent)	< **0.0001**	1.00	(Referent)	**0.0014**

HR, hazard ratio; CI confidence interval; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; Alb, albumin; DCP, des-gamma-carboxy prothrombin; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; SIRPα, signal regulatory protein alpha; MDSC, myeloid-derived suppressor cells

A previous study reported that SIRPα expression was an effective prognostic marker in esophageal and intrahepatic cholangiocarcinomas.[10,20] In HCC, CD47, the ligand of SIRPα, was positive in 21.7% of the patients and associated with a shorter RFS for the patients who underwent surgical resection.[12] However, the clinical significance of SIRPα expression in HCC remained unclear. Therefore, the current study is the first to determine the effect of SIRPα expression on HCC prognosis.

In our cohort, SIRPα expression was associated with microvascular invasion and poor prognosis, indicating that SIRPα may partially contribute to patient survival by enhancing tumor invasiveness.

Suppression of SIRPα expression in dendritic cells also enhanced T cell activation through antigen presentation and suppressed tumor growth in a mouse model.[21]

Several preclinical studies have shown that blocking the SIRPα-CD47 pathway using anti-SIRPα antibody, SIRPα-Fc fusion proteins, or anti-CD47 antibody, which act as effective decoy receptors, has an antitumor effect against several solid organ or hematopoietic cancers.[22–27] Several clinical trials for blocking the SIRPα-CD47 pathway have been performed.[28,29] Our results provide a basis for further investigations of the therapeutic effects of SIRPα-CD47 inhibition in HCC.

The results of the current study showed that SIRPα expression was significantly correlated with the infiltration of CD68-positive macrophages, indicating that these might have high SIRPα expression. In HCC, the number of tumor-infiltrating CD68-positive macrophages is reported to be a prognostic marker for OS.[30] In the multivariate analysis, SIRPα expression was found to be a prognostic factor for both RFS and OS, whereas the number of CD68-positive macrophages was not. This result suggested that macrophage polarization may be more important than the number of tumor-infiltrating macrophages.

Macrophages in malignant tumors are classified into two subtypes. On the one hand, M1 macrophages are involved in efficient antigen presentation and pathogen killing, secreting large amounts of pro-inflammatory molecules and promoting type 1 T helper cells and antitumor immunity. On the other hand, M2 macrophages have high phagocytic activity but secrete small amounts of pro-inflammatory molecules, which are involved in type 2 T helper responses and suppress the antitumor immune response.[31,32] In HCC, tumor cells promote M2 polarization in a paracrine manner, and tumor-associated macrophages (TAMs) predominantly consist of M2 macrophages.[33,34] Moreover, M2 macrophages promote neovascularization, cancer spreading, and suppression of innate and adaptive immune responses.[32] Expression of SIRPα correlates with M2 macrophage differentiation and regulates anti-tumor immunity.[10] Actually, in the current study, SIRPα expression was significantly upregulated in CD163-positive cells compared with CD163-negative cells.

Our IHC data also showed that SIRPα expression was correlated with MDSC infiltration. We previously reported that high intratumor MDSC infiltration was correlated with poor RFS and OS for patients with HCC,[4] with MDSCs enhancing tumor proliferation and resistance to immunotherapy.[35] In addition, MDSCs express SIRPα, and

TABLE 3 Results of uni- and multivariate analyses on overall survival

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Age (years)						
< 75 (*n* = 134)	1.00	(Referent)				
≥ 75 (*n* = 55)	1.60	0.80–3.21	0.1788			
Sex						
Male (*n* = 135)	1.33	0.60–2.93				
Female (*n* = 54)	1.00	(Referent)	0.4855			
hBs-Ag						
Positivity (*n* = 27)	1.26	0.52–3.04				
Negativity (*n* = 162)	1.00	(Referent)	0.6086			
HCV-Ab						
Positivity (*n* = 118)	0.82	0.42–1.63				
Negativity (*n* = 71)	1.00	(Referent)	0.5809			
Alb (g/dL)						
< 3.5 (*n* = 18)	1.57	0.22–1.82				
≥ 3.5 (*n* = 171)	1.00	(Referent)	0.4009			
DCP (mAU/ml)						
< 300 (*n* = 139)	1.00	(Referent)		1.00	(Referent)	
≥ 300 (*n* = 50)	2.01	1.01–3.97	**0.046**	1.33	0.54–3.24	0.5357
AFP (ng/ml)						
< 500 (*n* = 166)	1.00	(Referent)				
≥ 500 (*n* = 22)	0.89	0.31–2.53	0.8289			
Tumor size (cm)						
≤ 5.0 (*n* = 147)	1.00	(Referent)		1.00	(Referent)	
> 5.0 (*n* = 42)	2.79	1.41–5.52	**0.0033**	2.57	0.54–12.9	0.2383
Macroscopic tumor numbers						
Multiple (*n* = 39)	1.82	0.89–3.75				
Solitary (*n* = 150)	1.00	(Referent)	0.1020			
BCLC staging, B or C						
0 or A (*n* = 161)	1.00	(Referent)				
B or C (*n* = 28)	2.13	0.99–4.57	0.0520			
Milan criteria						
Within Milan criteria (*n* = 137)	1.00	(Referent)		1.00	(Referent)	
Without Milan criteria (*n* = 52)	2.67	1.36–5.22	**0.0044**	0.53	0.09–3.01	0.4733
Poorly differentiated						
Present (*n* = 52)	2.66	1.35–5.22		1.73	0.84–3.57	
Absent (*n* = 137)	1.00	(Referent)	**0.0057**	1.00	(Referent)	0.1394
Tumor types						
Boundary type (*n* = 121)	1.00	(Referent)				
Nonboundary type (*n* = 66)	1.27	0.63–2.52	0.5045			
Microscopic vascular invasion						
Present (*n* = 53)	2.72	1.39–5.35		1.15	0.50–2.63	
Absent (*n* = 136)	1.00	(Referent)	**0.0036**	1.00	(Referent)	0.6620
Microscopic intrahepatic metastasis						
Present (*n* = 27)	4.03	1.99–8.17		1.86	0.73–4.74	
Absent (*n* = 162)	1.00	(Referent)	**0.0001**	1.00	(Referent)	0.1906

TABLE 3 continued

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Fibrosis						
F0–2 (*n* = 96)	1.00	(Referent)				
F3, 4 (*n* = 93)	1.01	0.51–1.98	0.9844			
SIRPα expression						
High (*n* = 56)	2.74	1.40–5.38		2.45	1.16–5.20	
Low (*n* = 133)	1.00	(Referent)	**0.0034**	1.00	(Referent)	**0.0194**
CD8-positive T cell infiltration						
High (*n* = 94)	1.00	(Referent)		1.00	(Referent)	
Low (*n* = 95)	4.27	1.93–9.44	**0.0003**	4.08	1.72–9.67	**0.0014**
CD68-positive cell infiltration						
High (*n* = 100)	2.58	1.20–5.52		1.10	0.37–3.30	
Low (*n* = 89)	1.00	(Referent)	**0.015**	1.00	(Referent)	0.8582
MDSC infiltration						
High (*n* = 59)	2.68	1.37–5.26		2.52	1.00–6.35	
Low (*n* = 130)	1.00	(Referent)	**0.0041**	1.00	(Referent)	**0.0493**

HR, hazard ratio; CI confidence interval; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; Alb, albumin; DCP, des-gamma-carboxy prothrombin; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; SIRPα, signal regulatory protein alpha; MDSC, myeloid-derived suppressor cells

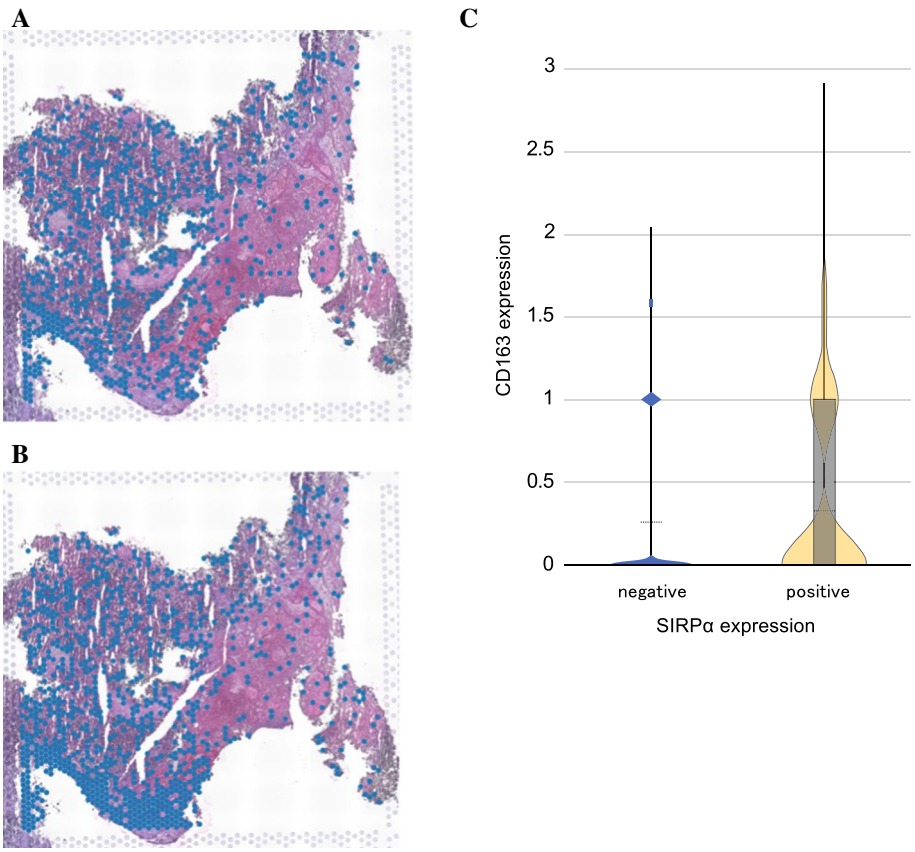
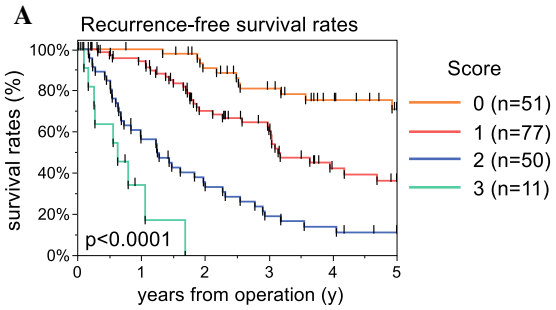
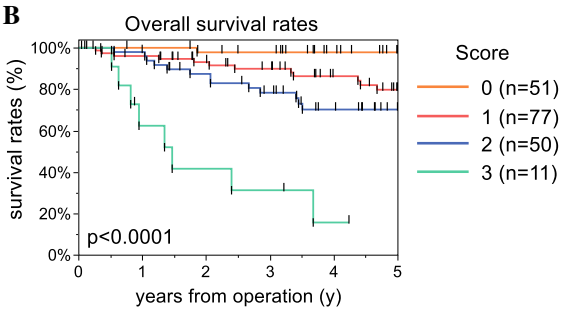


FIG. 4 Distribution of signal regulatory protein alpha (SIRPα)- and CD163-positive cells. The distributions of **A** SIRPα-positive cells and **B** CD163-positive cells are shown. **C** The violin plot of CD163 expression in SIRPα-positive or SIRPα-negative cells is shown



Year	0	1	2	3	4	5
Score 0	51	47	40	32	23	15
Score 1	77	64	41	30	15	11
Score 2	50	26	16	9	6	2
Score 3	11	3	0	0	0	0



Year	0	1	2	3	4	5
Score 0	51	48	45	43	34	26
Score 1	77	70	61	55	41	32
Score 2	50	48	40	34	24	15
Score 3	11	7	5	4	1	

FIG. 5 Kaplan–Meier curves classified with the numbers of risk factors, including signal regulatory protein alpha (SIRPα), myeloid-derived suppressor cells (MDSCs), and CD8-positive T cells. Kaplan–Meier curves for **A** recurrence-free survival (RFS) and **B** overall survival (OS) for the patients with hepatocellular carcinoma (HCC) according to the numbers of risk factors are shown

SIRPα blockage reduces the number of MDSCs in a mouse model. Hence, SIRPα blockage may be useful in suppressing MDSCs.

Recently, a clinical trial (983P phase 1 dose-escalation study) of BI 765063, a selective SIRPα inhibitor, was performed in patients with advanced solid tumors and in patients with HCC, indicating a growing interest in therapies that inhibit this signaling. Several clinical trials also are currently evaluating the efficacy of CD47 blockage (Hu5F9-G4, TTI-621) for solid tumor,[36] although no clinical trials are dealing primary with HCC. In addition, humanized anti-CD47 antibodies, evaluated in a current phase 1 clinical trial, are reported to be well-tolerated for patients with advanced solid and hematologic malignancies when used in initial and maintenance doses.[37]

Interest in anti-CD47 antibodies also is growing, and further validation of their efficacy is needed. Therefore, we believe that examination of the SIRPα immune status in patients with HCC is essential to the development of more effective cancer therapies. To the best of our knowledge, no report has described a method to evaluate SIRPα expression by liquid biopsy in solid tumors. However, it will be an important subject of future research to find a method to evaluate SIRPα expression using a peripheral blood-based platform to determine the therapeutic effect of immunotherapy before treatment. In addition, programmed death 1 (PD-1) blockage suppressed intratumor SIRPα and CD47 expression and deteriorated intratumor MDSC invasion in mouse model experiments of head and neck squamous cell carcinoma.[38] In our study, we were unable to examine the correlation with SIRPα expression and immunotherapy because our cases did not include patients treated with preoperative immunotherapy. In HCC patients, the relationship between PD-1 and CTLA-4 antibody therapy and SIRPα expression should be verified in the future.

One limitation of the current study was its retrospective single-center design. Further analyses from multiple institutions are required. Additionally, several types of cells in the tumor microenvironment of HCC, such as macrophages and MDSCs, express SIRPα. It is unknown whether these cells suppress the immune reaction via SIRPα. Therefore, further experiments are required to confirm this hypothesis. In conclusion, SIRPα is associated with a poor prognosis in HCC through M2 macrophage and MDSC accumulation.

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