

SPP1 as a risk factor for patients with acute on chronic liver failure undergoing liver transplantation

Yeping Yu^{a,b,1}, Xinyi Mao^{a,1}, Jieying Wang^{c,1}, Mo Chen^a, Fang Wang^b, Xiaoni Kong^{b,*}, Hualian Hang^{a,*}

^a Department of Liver Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

^b Central Laboratory, Department of Liver Diseases, ShuGuang Hospital Affiliated to Shanghai University of Chinese Traditional Medicine, Shanghai, China

^c Clinical Research Center, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

Background: Acute on chronic liver failure (ACLF) is characterized by systemic inflammation and significant mortality, calling for accurate assessment due to the diverse prognosis of liver transplantation (LT).

Methods: 8 patients with ACLF and 4 normal controls (NC) underwent peripheral blood mononuclear cells (PBMCs) transcriptomics, whereas 9 patients with ACLF and 3 NC had hepatic CD45⁺ T cells transcriptomics. The candidate indicator found in the transcriptomics was confirmed by a retrospective cohort (n = 137) and one prospective cohort (n = 68).

Results: Transcriptomics revealed significant differentially expression genes (DEGs) and bioprocesses related to the PBMCs and hepatic CD45⁺ T cells. Secreted phosphoprotein 1 (SPP1) was identified as a potential indicator for ACLF patients receiving LT, which was supported by evidence from the cross-sectional cohorts. As the condition of ACLF got worse, so did SPP1 levels, which were associated with liver failure and coagulation failure. SPP1 levels prior to LT were considerably greater in non-survivors of ACLF within 90 days than that in survivors. In the derivation cohort and validation cohort, ACLF patients with elevated SPP1 levels had significantly shorter cumulative survival durations than those with low SPP1 levels, P = 0.02 and P < 0.001, respectively. The SPP1-MELD and SPP1-chronic liver failure consortium (CLIF-C) ACLF scores had comparatively larger areas under the receiver operating characteristic curves (AUCs) than MELD (P = 0.0388) and CLIF-C ACLF (P = 0.045).

Conclusions: The circulating SPP1 showed promise as a predictor for ACLF patients receiving LT, which demonstrated the need for tracking the clinical outcome of LT.

1. Introduction

Common characteristics of patients with acute on chronic liver failure (ACLF) include initial hepatic decompensation, rapid deterioration of liver or extrahepatic organ system failures, and high short-term mortality without liver transplantation (LT), with rates nearing 80% at 28 days [1]. The Model for End-Stage Liver Disease (MELD) score, the

Chronic Liver Failure Consortium Organ Failure (CLIF-C OF) score, and the CLIF-C ACLF score appeared to be applied for the assessment of patients with ACLF; nevertheless, their prediction accuracy is not as fantastic as originally expected [2-4]. There is mounting evidence linking the pre-LT status of the patient to surgical outcomes, highlighting the need for precise risk categorization [5,6].

Owing to the existence of an episode of acute hepatic insult

Abbreviations: ACLF, acute on chronic liver failure; LT, liver transplantation; SPP1, secreted phosphoprotein 1; DAMP, damage-associated molecular pattern; MELD, the Model for End-Stage Liver Disease; ROC, the receiver operating characteristic; CLIF-C OF, Chronic Liver Failure Consortium Organ Failure; CLIF-C ACLF, Chronic Liver Failure Consortium ACLF; NC, normal control; Non-ACLF, AD patients without ACLF; LF, liver failure; CoF, coagulation failure; KF, kidney failure; CeF, cerebral failure.

* Corresponding authors at: Central Laboratory, Department of Liver Diseases ShuGuang Hospital Affiliated to Shanghai University of Chinese Traditional Medicine 528 Zhangheng Road, Shanghai 201203, China (Xiaoni Kong), Department of Liver Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China 160 Pujian Road, Shanghai 200127, China (Hualian Hang).

E-mail address: hanghualian@shsmu.edu.cn (H. Hang).

¹ These authors contributed equally to this study.

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associated with liver dysfunction, patients with chronic liver disease are considered to be predisposed to suffering acute decompensation (AD), a pre-ACLF condition due to the further progress to ACLF [7]. It has long been recognized that systemic inflammation and immunity can lead to tissue injury and extrahepatic organ failure as ACLF advances [8,9]. In decompensated cirrhosis, it is generally accepted that the release of damage-associated molecular patterns (DAMPs), brought on by constant

hepatocyte and tissue destruction from the underlying liver disease, is what triggers systemic inflammation [10]. In addition, prolonged exposure to an unfavorable hepatic microenvironment might contribute to excessive systemic inflammation that ultimately causes organ failure and mortality [11]. Hepatic macrophages and liver-recruited monocytes both have either an anti-inflammatory or proinflammatory function throughout this process [12,13]. However, the immunosuppressive

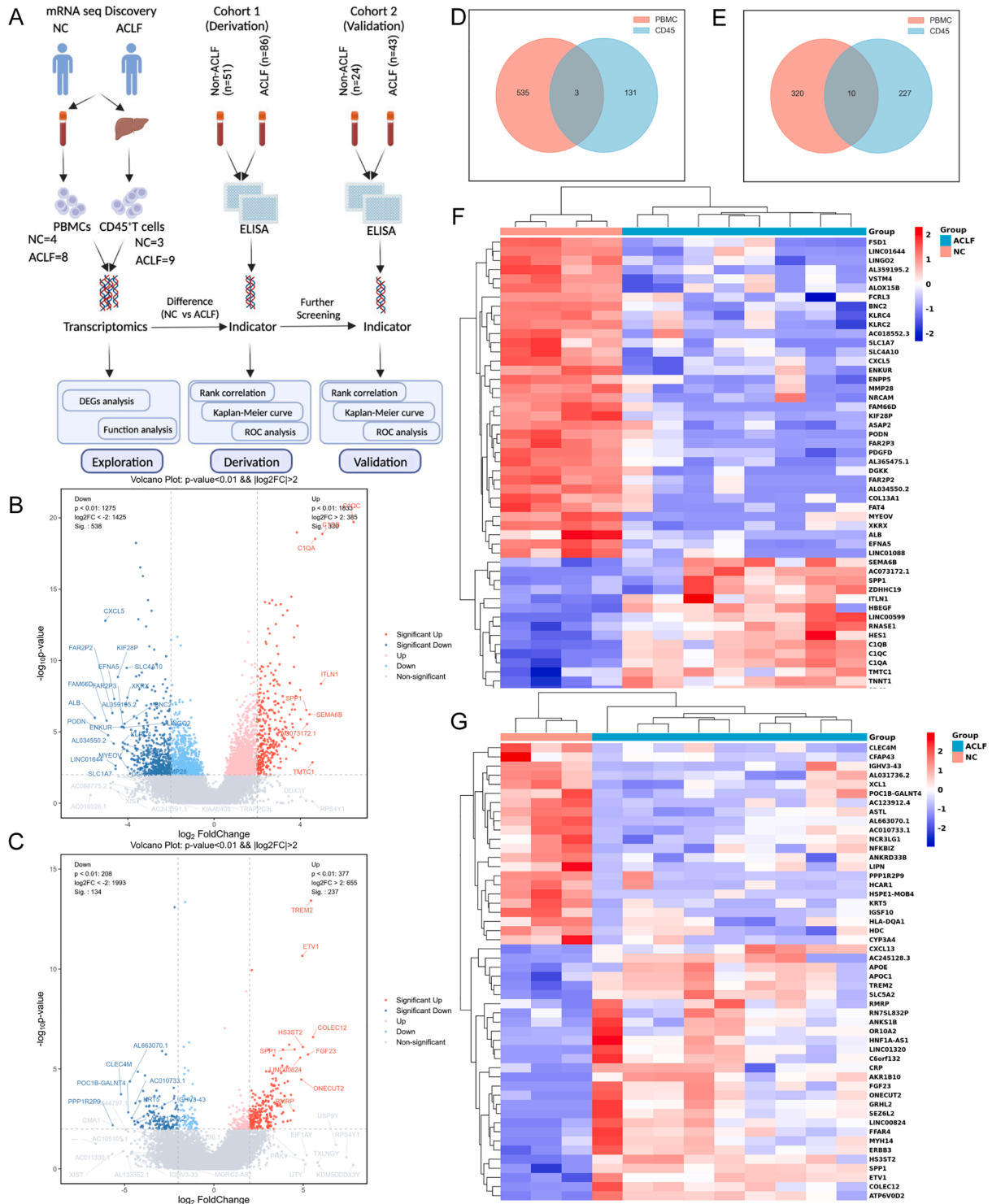


Fig. 1. Analysis for transcriptomics data. (A) Flowchart illustrating the study design. (B) Volcano plot of the DEGs in PBMCs between NC and ACLF patients. (C) Volcano plots of the DEGs in hepatic CD45⁺ T cells between NC and ACLF patients. (D) Venn diagram of upregulated DEGs in PBMCs and hepatic CD45⁺ T cells. (E) Venn diagram of downregulated DEGs in PBMCs and hepatic CD45⁺ T cells. (F) Clustering heatmap of the top 50 DEGs in PBMCs between NC and ACLF patients. (G) Clustering heatmap of the top 50 DEGs in hepatic CD45⁺ T cells between NC and ACLF patients. NC, normal controls; ACLF, acute on chronic liver failure.

phenotype of ACLF can be partially attributed to immunological tolerance, an adaptive response related to disease progression that might mitigate damage's adverse effects [14]. Thus, immunosuppression raises the risk of infection and death in patients [10].

Studies have confirmed that innate immune cells and adaptive immune cells, accordingly, played the most prominent roles in the phenotypic alterations that make a contribution to the systemic inflammation or immunosuppression of ACLF and the survival of ACLF patients undergoing LT. High-throughput investigation of phenotypic alterations in immunocytes involved in ACLF pathology, such as peripheral blood mononuclear cells (PBMCs), has been facilitated by the emergence of RNA sequencing technology [15]. However, the phenotypic modifications of immunocytes in liver tissues have gained little attention in the literature, especially for ACLF patients prior to LT.

Here, we detailed the discovery of an encouraging prognostic indicator for ACLF patients receiving LT based on immunocyte transcriptomics. First, we first analyzed the differentially expressed genes (DEGs) in PBMCs and hepatic CD45⁺ cells of ACLF patients prior to LT using mRNA sequencing and we verified a potential putative biomarker that permitted discrimination between ACLF and normal controls (NC). Then, we proved the prognostic power of secreted phosphoprotein 1 (SPP1), enhancing universal prognostic models as an auxiliary index for ACLF patients prior to LT in a retrospective cohort utilizing a series of survival analyses. This observation was finally sustained in a prospective cohort.

2. Materials and Methods

2.1. Study design and patients

As shown in Fig. 1A, hepatic CD45⁺ T cell transcriptomics was done on 9 patients with ACLF and 3 NC, while PBMCs transcriptomics was performed on 8 patients with ACLF and 4 NC. Then, a retrospective cohort of subjects composed of 51 Non-ACLF and 86 ACLF patients getting LT utilizing donation after circulatory death (DCD) from January 1, 2019, to November 30, 2020, from the department of liver surgery of Renji Hospital affiliated to School of Medicine, Shanghai Jiao Tong University was enrolled for the derivation of the indicator.

The predictive performance of SPP1 was then validated using a prospective cohort made up of 24 Non-ACLF and 43 ACLF patients receiving LT who were recruited at the department of liver surgery of Renji Hospital from January 1, 2021, to May 31, 2022.

Table 1 displayed the clinical features of the derivation cohort and validation cohort. These individuals were monitored for 90 days after LT. During the postoperative phase, samples of liver tissue and peripheral blood were obtained.

This study was approved in advance by the Human Research Committee at Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (approve number: 2018–205), demonstrating that the study conformed to the ethical principles outlined in the 1975 Declaration of Helsinki (6th revision, 2008). All participants or their legal guardians gave their written agreement to participate after receiving all necessary information.

2.2. Definitions

The European Association for the Study of the Liver-Chronic Liver Failure (EASL-CLIF) Consortium criteria have been used to define and grade ACLF patients at the time of admission; these criteria are applicable only to patients with acutely decompensated cirrhosis and organ failures [7]. The perspective defines the function of 6 organ systems, measured by the CLIF Consortium Organ Failure (CLIF-OF) score [2]. There are three tiers of ACLF, each representing a more severe form of the disease: ACLF1, ACLF2, and ACLF3 [16]. AD is characterized by the sudden onset of ascites, hepatic encephalopathy, gastrointestinal bleeding, infection (in individuals with antecedent AD), or any

Table 1

Clinical characteristics of study subjects in the derivation and validation cohort.

Variables	Derivation Cohort (n = 137)		Validation Cohort (n = 67)	
	Non-ACLF (n = 51)	ACLF (n = 86)	Non-ACLF (n = 24)	ACLF (n = 43)
Male (%)	46 (90.2)	76 (88.4)	23 (95.8)	38 (88.4)
Age (yr)	46 (41, 55)	45 (40, 53)	52.5 (47, 63)	47 (39, 55) *
Etiology (%)				
HBV	33 (64.7)	66 (76.7)	19 (79.1)	41 (95.3)
Alcohol	8 (15.7)	10 (11.6)	3 (12.5)	2 (4.7)
Autoimmune hepatitis	5 (9.8)	4 (4.7)	1 (4.2)	–
Other	5 (9.8)	6 (7)	1 (4.2)	–
WBC count ($\times 10^9/L$)	3.5 (2.3, 5.2)	6 (4.5, 8.3) ***	3.8 (2.6, 5.4)	6.8 (4.5, 10.1) ***
Platelet count ($\times 10^9/L$)	54 (45, 89)	57 (34, 90.5)	61 (46, 88.5)	58 (41, 73)
Sodium (mmol/L)	137 (135, 141)	132 (129, 135.5) ***	139 (136, 144)	135 (131, 139) *
ALT (U/L)	98 (85, 108)	472 (419, 518.5) ***	88.5 (78, 109.8)	413 (375, 477) ***
AST (U/L)	88 (79, 97)	365.5 (324.5, 396.3) ***	70.5 (60.5, 86.3)	245 (228, 301) ***
TBil ($\mu\text{mol/L}$)	46 (22.1, 63.5)	566.5 (241.4, 844.3) ***	55.3 (23.4, 79.8)	437.1 (267.5, 525.1) ***
Albumin (g/L)	30.2 (28.2, 33.6)	32.5 (29.8, 35.4) *	34.4 (30.7, 42.2)	34.1 (30.7, 38.1)
Creatinine ($\mu\text{mol/L}$)	60.8 (53, 72.6)	79 (57.7, 117.3) ***	65.5 (59, 82.5)	81 (60.6, 122.1)
International normalized ratio	1.4 (1.2, 1.6)	2.3 (1.9, 3) ***	1.4 (1.2, 1.5)	2.4 (1.8, 2.9) ***
Prothrombin time (s)	17.2 (14.9, 19.8)	24.8 (20.5, 27.7) ***	15.4 (14, 16.8)	26.4 (20.1, 31.1) ***
ACLF grade (%)				
1		55 (64)		21 (48.8)
2		22 (25.6)		14 (32.6)
3		9 (10.4)		8 (18.6)
Organ Failure (%)				
Liver		77 (89.5)		41 (95.3)
Coagulation		36 (41.9)		20 (46.5)
Kidney		7 (8.1)		2 (4.7)
Cerebral		7 (8.1)		8 (18.6)
Respiration		1 (1.2)		2 (4.7)
Circulation		1 (1.2)		3 (7.0)
MELD	10 (7, 13)	28.5 (21, 34) ***	12.5 (8, 14)	24 (21, 28) ***
CLIF-C OFs	6 (6, 7)	9 (9, 10) ***	6 (6, 6)	10 (9, 11) ***
CLIF-C ACLFs	27.9 (23.5, 32.3)	40.4 (36.8, 45.8) ***	29.5 (25.4, 34.6)	43.9 (39.2, 49.6) ***

Data are expressed as the median (25% percentile, 75% percentile) and n (%).

*P Value (<0.05) ** P Value (<0.01) ***P Value (<0.001).

combination of these disorders [7,17]. Patients with AD but no ACLF (Non-ACLF) comprise 3 types: (a) those with AD but no organ failure or dysfunction; (b) organ failure sufferers, whether they have one or several and (c) individuals suffering single non-kidney organ failure but no evidence of kidney or brain malfunction [18,19]. MELD, CLIF-OF, CLIF-C AD, and CLIF-C ACLF scores have been used to assess the degree of liver disease [4].

2.3. Isolation of PBMCs

Peripheral blood obtained prior to LT was centrifuged (500g, 5 min) to remove plasma, then diluted with equal volumes of phosphate-buffered saline (PBS) and layered on top of Ficoll-Isolate in a 15 ml tube (Ficoll: Blood 1:1). After horizontal centrifugation (800g, 20 min), PBMCs were concentrated in the second layer from the top to the bottom, collected in another 15 ml tube, and centrifuged (800g, 5 min) to remove the supernatant after being mixed with 10 ml Dulbecco's phosphate buffer (DPBS). After being lysed with 1x Red Blood Cell Lysis Buffer and centrifuged (500g, 10 min), the cell pellet was resuspended in culture media.

2.4. Isolation of hepatic CD45⁺ T cells

According to the manufacturer's directions, hepatic CD45⁺ cells were extracted from liver slices taken from the surgery using MACS MicroBeads (Miltenyi Biotec). Briefly, liver sections were dissected, and then enzymatically digested using the Multi Tissue Dissociation Kit 1 (Miltenyi Biotec). In order to separate CD45⁺ T cells, we utilized CD45 MicroBeads to label them magnetically before loading them onto a MACS Column (Miltenyi Biotec) for magnetic separation. Liquid nitrogen was used to promptly freeze the isolated CD45⁺ T cells, and they have been kept frozen at -80 °C until transcriptomics analysis could be conducted.

2.5. Transcriptomics analysis

Using the RNAmicro kit (Qiagen, Germany), total RNA was extracted from PBMCs and hepatic CD45⁺ T cells. The integrity of the RNA was tested by gel electrophoresis and a Qubit (Thermo, Waltham, MA, USA). Shanghai Neo-Biotechnology Co., Ltd. analyzed the RNA-bulk sequencing data. The threshold of DEGs was established as $P < 0.01$ and absolute fold change ≥ 2 . The Gene Ontology (GO) database was then used to select DEGs for investigation of their enrichment in functions and signaling pathways. Pathways were considered substantially enriched if they contained at least two related genes and had a P value < 0.05 .

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA isolated from PBMCs and CD45⁺ T cells was isolated with Trizol reagent, reverse transcribed into cDNA, and put to use for SYBR Green qRT-PCR in accordance with the manufacturer's procedures. The Ct value of GAPDH was used as a standard to normalize the relative expression of SPP1 mRNA in each sample. The qRT-PCR analysis primers were as follows: SPP1-F: CTCCATTGACTCGAAGCAGCTC; SPP1-R: CAGGTCTGCGAACTTCTTAGAT; GAPDH-F: GGAGCGA-GATCCCTCCAAAT; GAPDH-R: GGCTGTTGTCATACTTCTCATGG.

2.7. Immunohistochemical (IHC) staining

Liver specimens were taken from patients undergoing LT for either ACLF or Non-ACLF due to HBV, alcohol, and autoimmune hepatitis. The anti-SPP1 antibody (R&D Systems, Minneapolis, MN) was used for IHC staining, and the proportion of SPP1 positive cells was counted using Image J software.

2.8. Detection of serum SPP1

The serum SPP1 concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's recommended procedure.

2.9. Statistical analysis

Statistical analysis was conducted using SPSS v20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). Kolmogorov-Smirnov analysis was applied to check for the normality distribution. Non-normally distributed variables were expressed as median (25% percentile, 75% percentile), and categorical values were reported as frequencies. For continuous variables, comparisons were done using Student's t -test, one-way analysis of variance, Mann-Whitney U test, and Kruskal-Wallis H test. Chi-square and Fisher's exact tests were used to compare frequency distributions. The rank correlation was analyzed using the Spearman technique. Kaplan-Meier curves were used to analyze the cumulative 90-day survival of ACLF patients after LT. Univariate and multivariate Cox regression was used to

identify independent risk factors for 90-day mortality of ACLF patients. Z-test with DeLong's test allowed us to compare the receiver operating characteristic (ROC) curves of different scores. Statistical significance was defined as a two-tailed $P < 0.05$ for all analyses.

3. Results

3.1. Analysis for transcriptomics data

The complete research design is depicted in Fig. 1A, which was created with the help of web-based applications (<https://biorender.com/>). mRNA sequencing profiling of PBMCs and hepatic CD45⁺ T cells from enrolled ACLF patients receiving LT and NC was used to acquire DEGs. Volcano plots comparing patients with ACLF and NC revealed a total of 868 DEGs in PBMCs and 371 DEGs in hepatic CD45⁺ T cells when the significance level was set to FDR filter ($P < 0.01$ with a difference of 2-fold or more), as shown in Fig. 1B, 1C. 10 downregulated DEGs and 3 upregulated DEGs were detected in PBMCs and hepatic CD45⁺ T cells, respectively, as seen in the overlapping circles in the Venn diagrams (Fig. 1D, 1E). Furthermore, based on the overall expression patterns of DEGs of PBMCs and hepatic CD45⁺ T cells, these individuals could be identified in an unsupervised clustering analysis, with the top 50 DEGs being shown in Fig. 1F and 1G. Fig. 1B, 1C, 1F, and 1G illustrated that among these DEGs, SPP1 was massively increased in both PBMCs and hepatic CD45⁺ T cells from ACLF patients, compared to NC. These findings suggested that SPP1 was a potential indicator for ACLF when transcriptomics analysis was employed to screen the immune response of the ACLF process.

3.2. Identification of the significance of SPP1 in patients with ACLF

Through pathway enrichment analysis, we were able to deduce that SPP1 was engaged in the cell cycle, cell proliferation, and cellular response to the lipid in systematic inflammation of ACLF, providing new insight into the bioprocess of PBMCs involved in SPP1 (Fig. 2A). However, SPP1-related pathway enrichment analysis was overrepresented in the inflammatory response, response to stimulus, and response to lipid (Fig. 2B). SPP1-related pathways were enriched in the inflammatory response, response to stimulus, and lipid response, prominently included in hepatic immune reaction (Fig. 2B). These PBMCs and hepatic CD45⁺ T cells were also tested by qRT-PCR for variations in SPP1 expression, and the results proved that SPP1 was activated in ACLF patients (Fig. 2C, 2D). SPP1 IHC labeling revealed dramatically enhanced expression in liver sections of ACLF patients in contrast to Non-ACLF patients with various etiology of disease, including HBV, alcohol, and autoimmune hepatitis (Fig. 2E). These findings supported the notion that SPP1 expression was elevated in ACLF, which was attributed to cellular responses of lipid, crucial for immune system reactions in PBMCs and hepatic CD45⁺ T cells.

3.3. Alterations of SPP1 levels according to the condition of ACLF in the derivation cohort

Markedly increased levels of SPP1 were discovered in ACLF patients in comparison to those without ACLF, and these levels kept rising with deteriorating ACLF severity (Fig. 3A, 3B). Table 1 displayed that among the first retrospective cohort ($n = 137$), liver failure was the most frequent organ failure (89.5%), followed by coagulation (41.9%), kidney (8.1%), and cerebral failure (8.1%). Patients with ACLF waiting for LT seldom had circulatory or respiratory failure (1.2% and 1.2%, respectively). Regarding ACLF patients, the more the number of failing organs, the higher the pre-LT SPP1 levels in circulation (Fig. 3C). Furthermore, SPP1 levels were also considerably greater in ACLF patients with liver failure prior to LT compared to them without liver failure (Fig. 3D). Pre-LT SPP1 levels were found higher in ACLF patients with coagulation failure (Fig. 3E), kidney failure (Fig. 3F), and cerebral

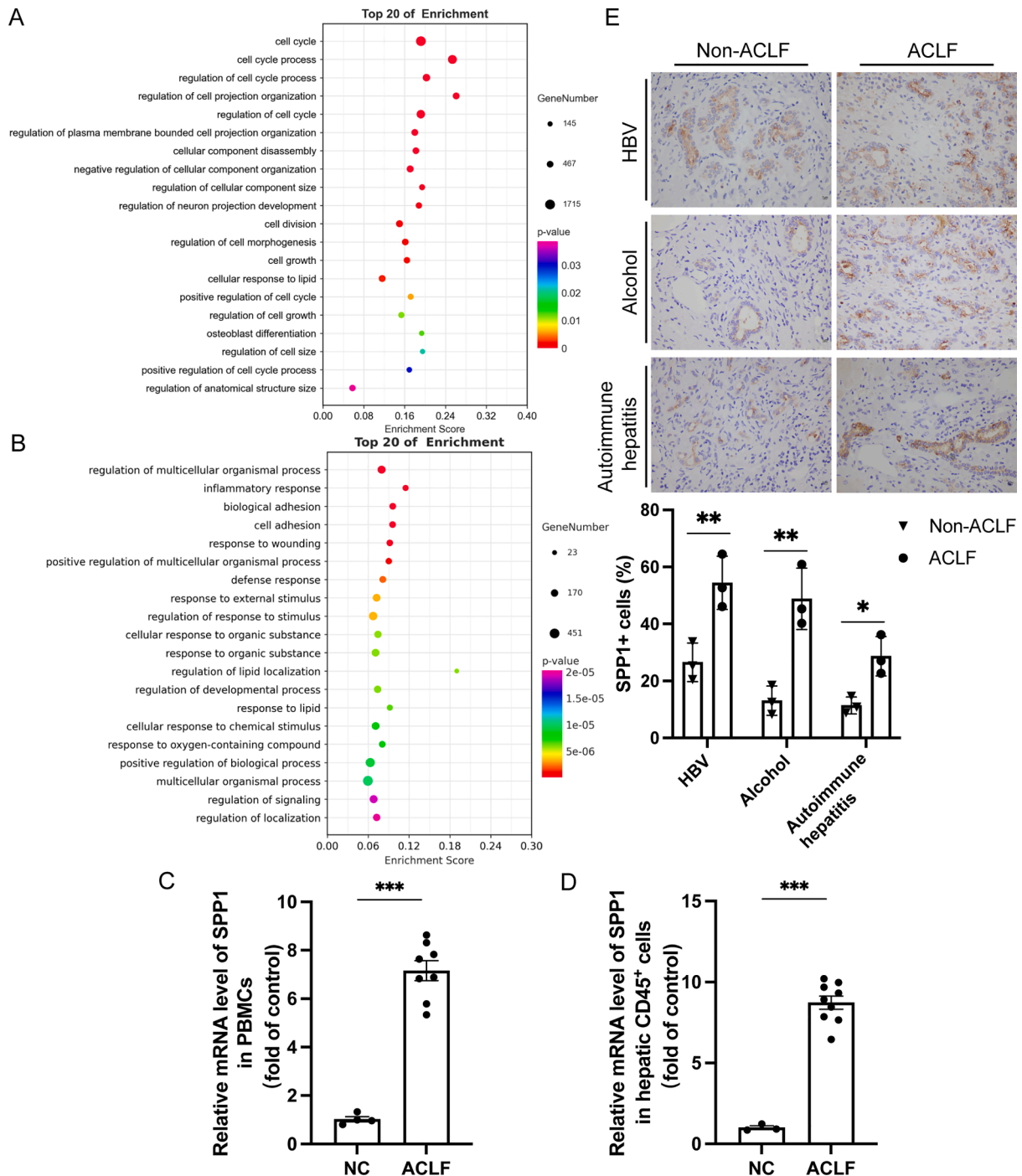


Fig. 2. Identification of the significance of SPP1 in patients with ACLF. (A) Top 20 enriched GO pathways in PBMCs between NC and ACLF patients. (B) Top 20 enriched GO pathways in hepatic CD45⁺ T cells between NC and ACLF patients. (C) qRT-PCR analysis of SPP1 in PBMCs between NC and ACLF patients. (D) RT-PCR analysis of SPP1 in hepatic CD45⁺ T cells between NC and ACLF patients. (E) IHC staining of SPP1 in liver tissues of ACLF patients compared with Non-ACLF patients regardless of the etiology. The proportion of SPP1-positive cells was quantified. *P < 0.05. **P < 0.01. ***P < 0.001.

failure (Fig. 3G) than in those without. Collectively, serum SPP1 concentrations were associated with the severity of ACLF, especially the existence of organ failure.

3.4. Alterations of SPP1 levels according to the condition of ACLF in the validation cohort

When comparing ACLF and Non-ACLF patients in the validation

cohort, it is worth noting that SPP1 concentrations were considerably higher in ACLF patients, with the rise being maximal in ACLF2 and ACLF3 patients compared to ACLF1 patients (Fig. 4A, 4B). SPP1 levels consistently rose when patients with ACLF encountered a growing number of malfunctioning organs (Fig. 4C). SPP1 consistently rose as the number of ACLF patients' organs climbed (Fig. 4C). SPP1 expression was correlated with organ failure, increasing dramatically in ACLF patients encountering liver failure, coagulation failure, kidney failure, or cerebral failure

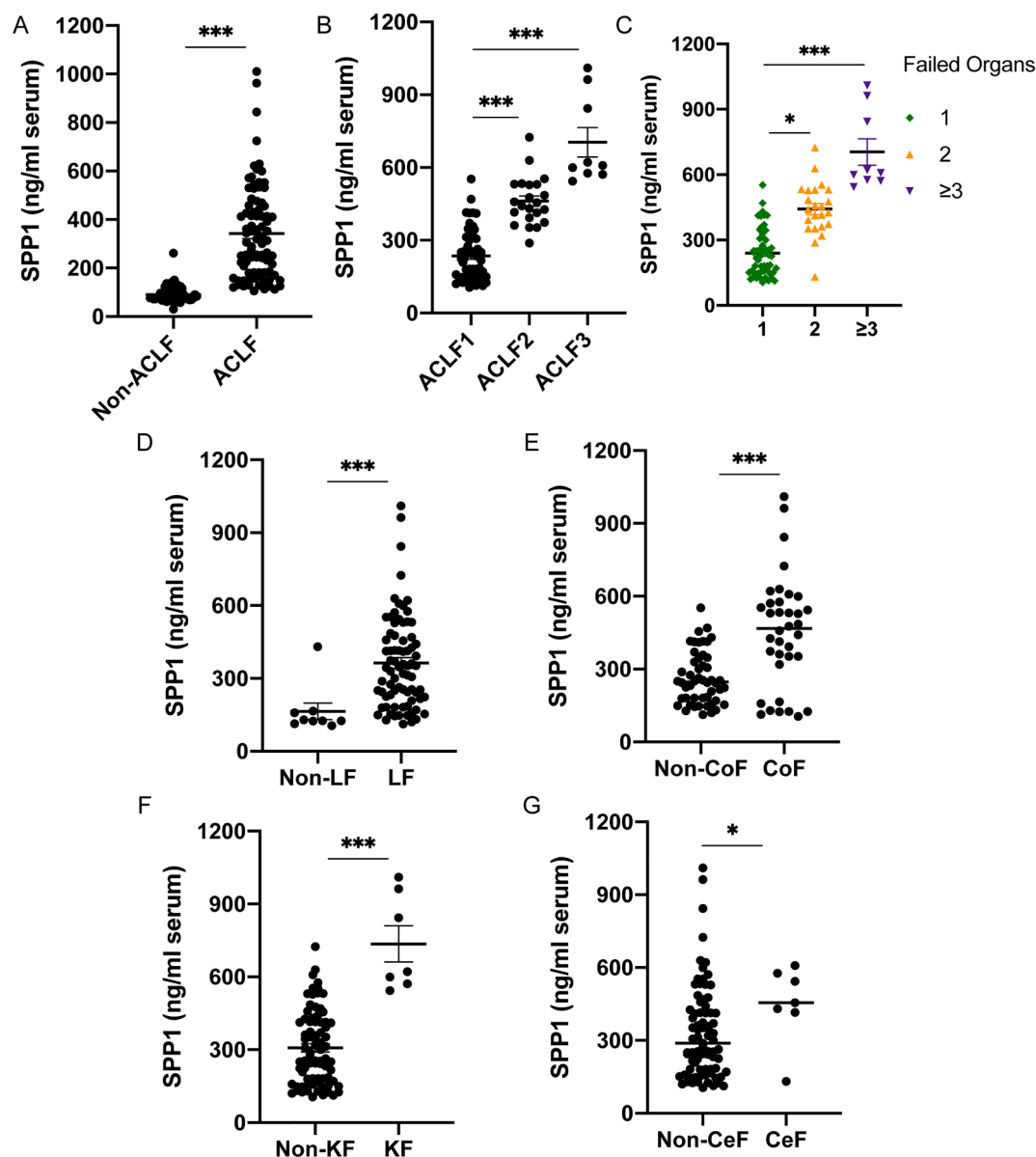


Fig. 3. Alterations of SPP1 levels according to the condition of ACLF in the derivation cohort. (A) SPP1 distribution in patients with or without ACLF. (B) Serum SPP1 levels in ACLF1, ACLF2, and ACLF3 patients. (C) SPP1 distribution along with increased numbers of organ failure in ACLF. SPP1 distribution in ACLF patients with and without (D) liver failure, (E) coagulation failure, (F) kidney failure, and (G) cerebral failure. LF, liver failure; CoF, coagulation failure; KF, kidney failure; CeF, cerebral failure; * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

compared to those who did not have any of these complications (Fig. 4D–4G). Consistent with the derivation cohort, these validation cohort results confirmed a linkage between SPP1 levels and the extent of ACLF, including organ failure.

3.5. Association of SPP1 levels and 90-day post-LT survival of ACLF patients

To further examine the predictive relevance of SPP1 levels in LT therapy of ACLF, we investigated the relationship between SPP1 levels and 90-day survival of ACLF patients after LT. Compared to the surviving group, the non-survivors had considerably greater pre-LT SPP1 levels (Fig. 5A). When patients with ACLF were classified by survival and non-survival outcomes of 90-day post-LT survival in the derivation cohort and validation cohort, the SPP1 levels prior to LT progressively climbed with the growing number of failed organs (Fig. 5B, 5C). Additionally, patients in the survival group who had only one failing organ

had lower pre-LT SPP1 levels compared to those with two or more failed organs. SPP1 levels for indicating 90-day post-LT mortality had an area under the ROC curve (AUC) of 0.743, sensitivity of 0.667, and specificity of 0.676 at an appropriate cut-off value of 345.89 ng/mL in the derivation cohort (Fig. 5D). In the validation cohort, the AUC of SPP1 levels for 90-day mortality was 0.806, with a sensitivity of 0.833 and a specificity of 0.784 at an optimal cut-off value of 455.72 ng/mL (Fig. 5E). Using the appropriate cut-off value, we subdivided ACLF patients in the derivation cohort into high-SPP1 and low-SPP1 sub-groups and found that 4 patients (8.5%) with low-SPP1 expired within 90 days, whereas 11 patients (28.2%) with high-SPP1 died within the same time frame. The low-SPP1 group had a generally higher cumulative survival rate than those in the high-SPP1 group ($P = 0.02$; Fig. 5F). For the validation cohort, the appropriate cut-off value was used to categorize 43 ACLF patients into high-SPP1 and low-SPP1 sub-groups. 1 patient (3.2%) with low SPP1 and 5 patients (41.7%) with high SPP1 were observed to decrease within 90 days. The cumulative survival rate of the low-SPP1

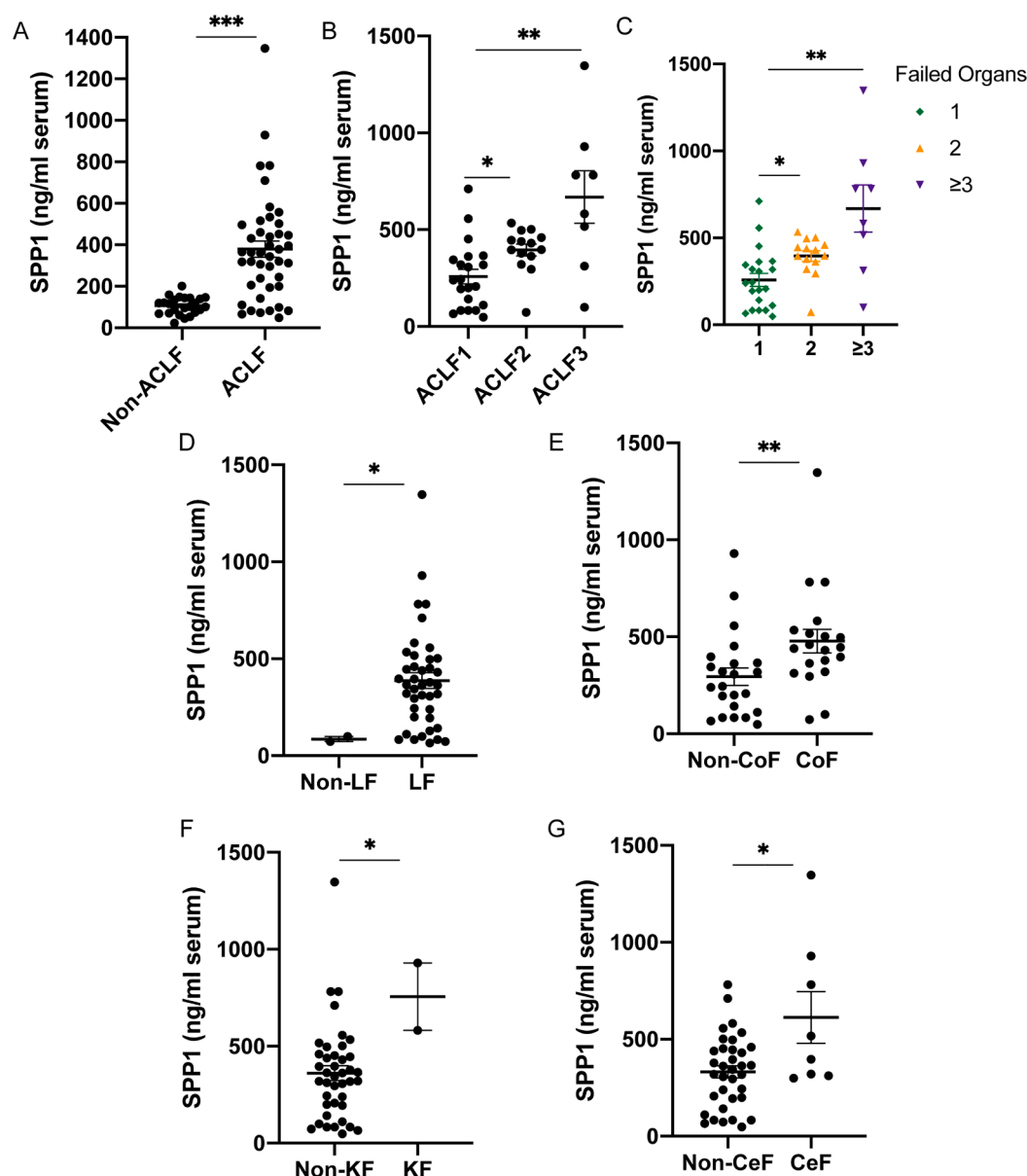


Fig. 4. Alterations of SPP1 levels according to the condition of ACLF in the validation cohort. (A) Circulating SPP1 distribution in patients with ACLF and Non-ACLF. (B) Serum SPP1 distribution in ACLF1, ACLF2, and ACLF3 patients. (C) SPP1 distribution along with growing numbers of organ failure in ACLF. SPP1 distribution in ACLF patients with and without (D) liver failure, (E) coagulation failure, (F) kidney failure, and (G) cerebral failure. LF, liver failure; CoF, coagulation failure; KF, kidney failure; CeF, cerebral failure; * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

group was significantly higher than that of the high-SPP1 group ($P < 0.001$; Fig. 5G). In particular, we compared the SPP1 mRNA in PBMCs from 16 HBV-ACLF patients (8 survivors and 8 controls) in an external GEO database (GSE168048) using Agilent SurePrint G3 Human Gene Expression ver. 3.8x60K Microarrays to evaluate the significance of SPP1 levels for 28-day prognosis. SPP1 mRNA was found to be considerably higher in the non-survival group compared to the survival group, agreeing with our previous data (Fig. 5H). These data indicated the association between high SPP1 levels prior to LT and a poor 90-day post-LT prognosis of ACLF patients.

3.6. The prognostic performance of SPP1-based models in predicting 90-day post-LT mortality of ACLF patients

To further investigate the application of SPP1 in the clinic, we

employed SPP1 levels in collaboration with three commonly accepted clinical models of ACLF under the guidance of the multivariate Cox regression analysis to evaluate their capacity to predict 90-day post-LT mortality of ACLF patients, further exploring the clinical application of SPP1. As said in Fig. 6A-C, for ACLF patients in the derivation cohort, the AUC of SPP1 + MELD was dramatically higher than that of MELD (0.81 [95% CI, 0.690–0.912] vs. 0.73 [95% CI, 0.585–0.868], $P = 0.04$). Another statistical significance of AUC was generated between SPP1 + CLIF-C ACLF and CLIF-C ACLF (0.83 [95% CI, 0.731–0.926] vs. 0.75 [95% CI, 0.620–0.888], $P = 0.045$). SPP1 + CLIF-C OF had a somewhat better AUC than CLIF-C OF score, but there was no statistical discrepancy (0.78 [95% CI, 0.655–0.906] vs. 0.754 [95% CI, 0.62–0.888], $P = 0.10$). To further verify the prognostic significance of SPP1, the ACU analysis was also conducted in the validation cohort in Fig. 6D-E. These results indicated that SPP1 was an essential prognostic factor for 90-day

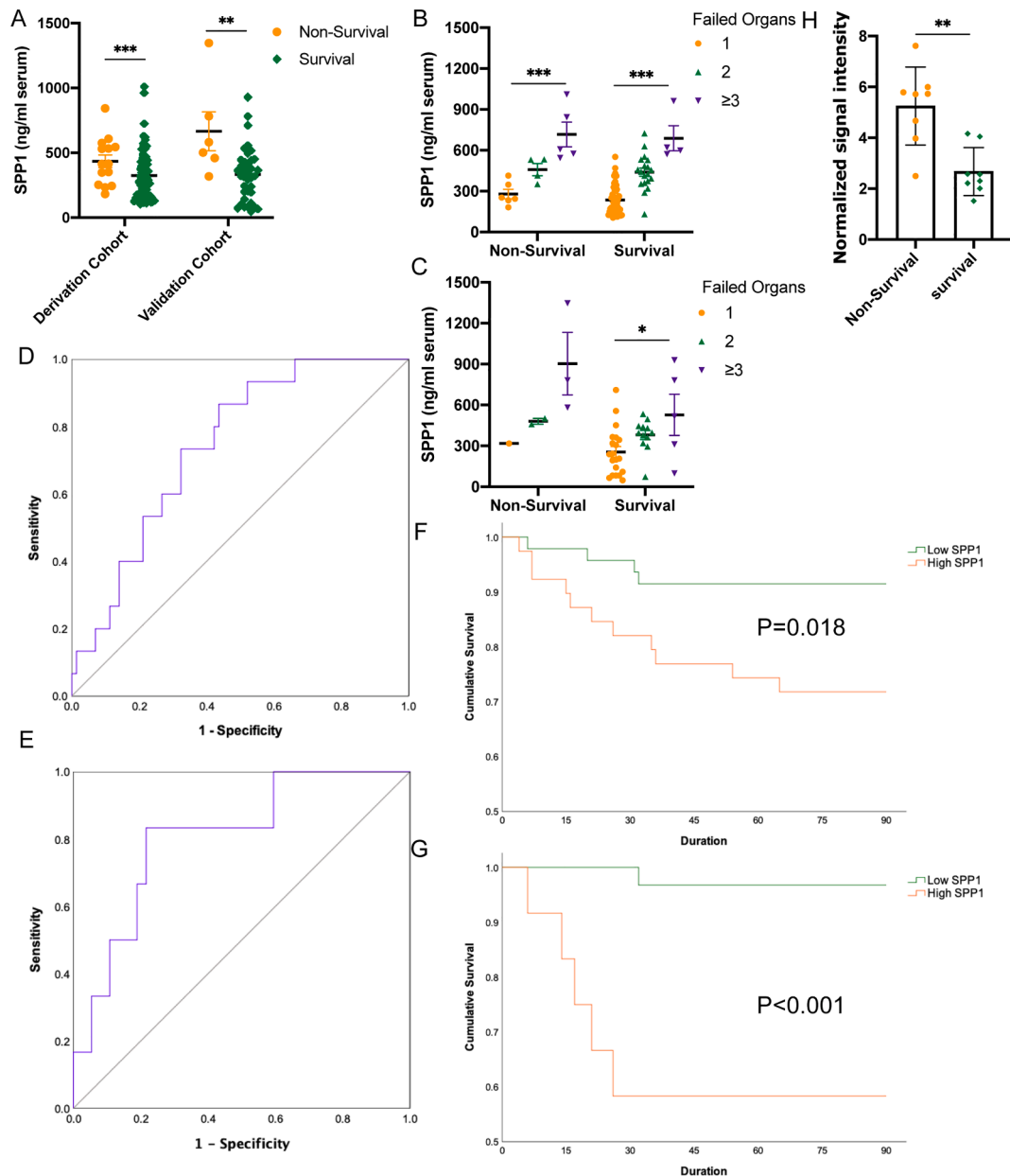


Fig. 5. Association of SPP1 levels and 90-day post-LT survival of ACLF patients. (A) SPP1 distribution in ACLF patients in both the derivation and validation cohorts. SPP1 distribution along with increased numbers of organ failure in ACLF survivors and non-survivors in the derivation cohort (B) and the validation cohort (C). Predictive performance of serum SPP1 for 90-day post-LT mortality in ACLF patients in the derivation cohort (D) and the validation cohort (E). Cumulative 90-day post-LT survival rates of ACLF patients in the high- and low-SPP1 groups in the derivation cohort (F) and the validation cohort (G). (H) External validation of GEO database (GSE168048) on the prognostic value of SPP1 for ACLF patients.

post-LT mortality in ACLF patients, complementing the MELD and CLIF-C ACLF scores.

4. Discussion

A few studies have utilized proteomic, or transcriptome approaches to identify the molecular basis for ACLF [15,20]. This research, however, relied on plasma or PBMCs, and, consequently, their findings related to blood or systemic inflammation. Additionally, these investigations did not include the hepatic microenvironment, the trigger of ACLF, failed to attempt to distinguish between ACLF survivors and non-survivors of LT, and also did not evaluate the prognostic value of any candidate indicator for LT in a reasonable manner. In summary, further work was needed to fully characterize the molecular signature of ACLF. Therefore, we performed this study with the aim of identifying

effective indicators for the prognosis of ACLF patients undergoing LT.

Clustering of genes data revealed that 868 PBMCs DEGs and 371 hepatic CD45⁺ T cells DEGs could reliably discriminate ACLF from NC, suggesting that these genes might predict the incidence of ACLF. Further, DEGs overlapping suggested that SPP1 was presumably the primary regulator for PBMCs and hepatic CD45⁺ T cells, leading to substantial alterations in DEGs during ACLF, as validated by qRT-PCR and IHC staining. Liver pathological processes such as inflammation, immunology, fibrosis, and cancer are linked to either the secretory or the intracellular forms of SPP1 [21]. Proven evidence has indicated that acetaminophen (APAP)-induced hepatic SPP1 production was predominantly localized to the necrotic region, and was created by dying or dead hepatocytes, making it an essential DAMP [22].

The accumulation of data suggested that ACLF was facilitated by an excessive systemic inflammatory response, leading to organ dysfunction

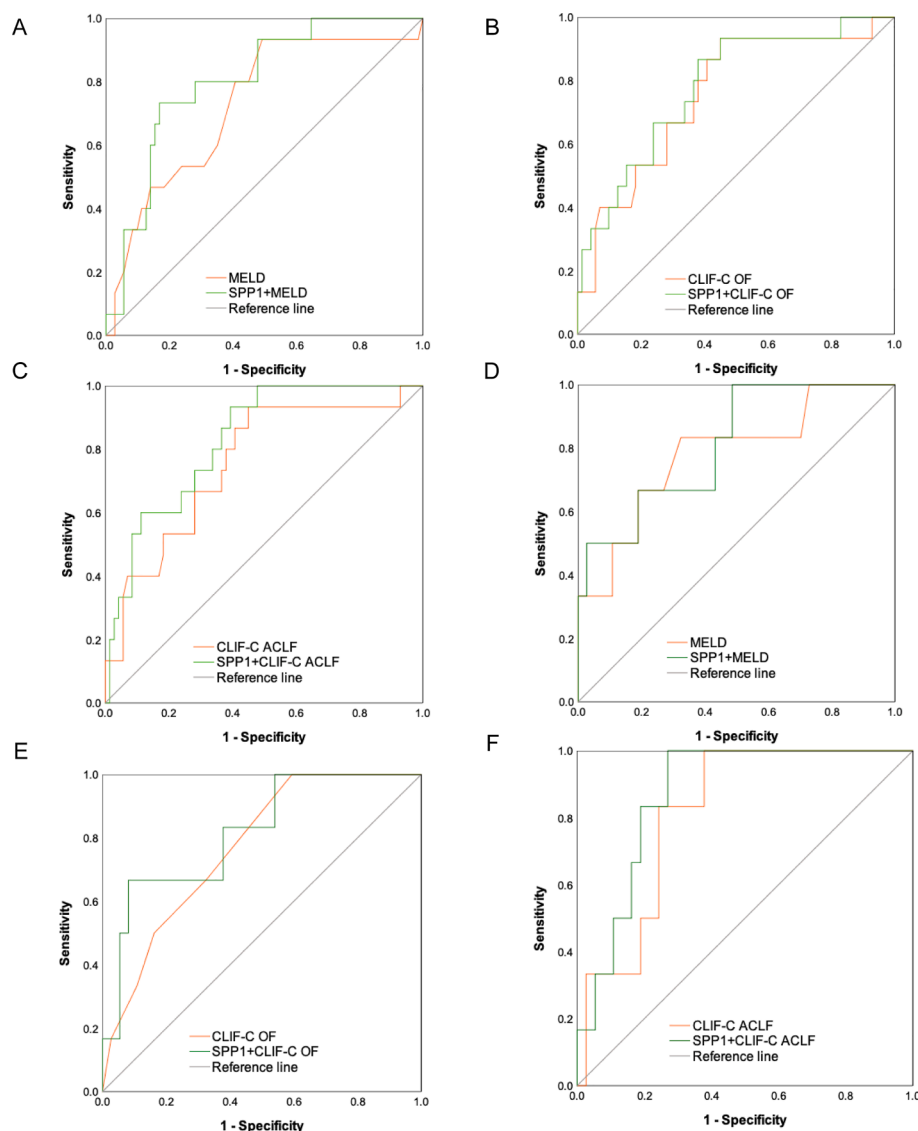


Fig. 6. The prognostic performance of SPP1-based models in predicting 90-day post-LT mortality of ACLF patients. The prognostic performance of SPP1 in the combination of MELD (A), CLIF-C OF (B), and CLIF-C ACLF (C) scores for 90-day post-LT survival of ACLF patients in the derivation cohort, respectively. The prognostic performance of SPP1 in the combination of MELD (D), CLIF-C OF (E), and CLIF-C ACLF (F) scores for 90-day post-LT survival of ACLF patients in the validation cohort, respectively. MELD, model for end-stage liver disease; CLIF-C OF, Chronic Liver Failure Consortium Organ Failure; CLIF-C ACLF, sChronic Liver Failure Consortium ACLF.

or failure if it was out of control [19,23,24]. Under uncontrolled immune modulation, the prolonged inflammatory response can exacerbate tissue damage and induce decompensation [25]. SPP1 was found to be involved in numerous PBMCs transcriptome activities in the current investigation, including cell cycle, cell proliferation, and cellular component organization, as revealed by GO pathway enrichment. It was demonstrated that recombinant SPP1 stimulated cardiac cell growth, activated cardiomyocyte (CM) cell-cycle re-entry, induced nuclear translocation of the cytoplasmic yes-associated protein 1 (YAP1), and increased transcription of cell cycle genes and transcription factors, all of which were essential for infarct healing [26]. Applying to GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis, Huo et al. have identified that SPP1, predicting overall survival of hepatocellular cancer, was substantially connected with DNA damage repair molecular subtype, revealing that cell cycle-related pathways were elevated [27]. Here, we hypothesized that SPP1 was involved in PBMCs restoration as ACLF progressed; this hypothesis might be further verified *in vitro* and *in vivo*.

Moreover, SPP1 may take part in the inflammatory response and reaction to lipids in hepatic CD45⁺ T cells, both of which were attached to immunological disturbances and metabolic disorders, suggested by the current Go functional enrichment analysis. Th1 and Th17 cell immunity, which are derived from CD4⁺ T cells, have been reported to be

related to ACLF [28,29]. The conclusion that the innate immune system was aroused and the adaptive immune system was exhausted in the development of HBV-ACLF has been supported by the evidence that the frequency of Tfh cells decreased markedly in ameliorated ACLF patients, but not in non-ameliorated patients [30]. Alterations in lipid metabolism and oxygen balance in liver tissues were also referred to SPP1 during ACLF advancement; these changes were linked to the development of extrahepatic organ failure, an increased risk of succumbing, and much more severe disease [31]. However, further research is needed to determine the underlying mechanism by which SPP1 modulates the lipid metabolism of immune cells.

The incidence of alcoholic cirrhosis and Non-alcoholic steatohepatitis (NASH)-induced cirrhosis may be mirrored in plasma SPP1, based on recent research [32–34]. Preliminary clinical studies discovered a positive correlation between SPP1 levels and liver necrosis grade in cases of acute liver failure (ALF) [35]. This paper examined Chinese cohorts of patients with ACLF undertaking LT to display that circulating SPP1 levels might operate as an index; their levels may be considered alongside the MELD score and the CLIF-C ACLF score in determining whether or not LT should be attempted. In both the derivation and validation cohorts, we discovered a statistically significant difference in serum SPP1 between Non-ACLF and ACLF patients, leading us to hypothesize that a higher level of serum SPP1 is connected with illness

state, as marked as ACLF grade and organ failure in ACLF patients. The results of the ROC research suggest that serum SPP1 was a reliable signal for determining which ACLF patients were unlikely to survive the LT. More intensive care was required for individuals whose serum SPP1 levels rose to 345.89 ng/ml or more. ACLF patients with high SPP1 levels may not be recommended to undergo LT due to the high 90-day post-LT mortality rate. Likewise, SPP1 + MELD and SPP1 + CLIF-C ACLF scores were proposed, with considerably better prognostic capabilities than MELD and CLIF-C ACLF scores separately. Collectively, our study not only described the inflammatory response during ACLF from the transcriptome analysis but also identified SPP1 as a potential indicator for ACLF patients undergoing LT, demonstrating substantial implications in facilitating the clinical practice of ACLF and organ allocation for LT.

Nonetheless, there are several restrictions imposed by such a study. Our study began by aiming at the potential role of SPP1 in the prognosis of ACLF patients. Therefore, deeper studies are required to clarify the underlying pathogenic mechanism of SPP1 on immunocyte metabolism. There are a considerable number of patients with ACLF awaiting LT at the department of liver surgery in Renji Hospital in China, although the number of patients and samples was restricted given the difficulties of sample collection. Given the magnitude of such a LT center, the sample size we used here is comparably meaningful. This study was conducted place at a single center, hence its findings required to be validated in multicenter investigations.

In conclusion, our results demonstrated that SPP1 expression was crucial in ACLF patients, with possible explanations including its roles in shaping damage repair on PBMCs and in the modulating inflammatory response and lipid metabolism on hepatic T cells. The incorporation of SPP1 into existing ACLF prognostic models boosted their prediction power, and ACLF patients with high circulating SPP1 levels who underwent LT fared worse than those without. Taken together, our analysis revealed SPP1 served as a pivotal possible signal for patients with ACLF.

Ethical approval

Institutional Review Board and Human Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China was obtained. (Approval number: 2018–205).

Data availability statement

The data supporting this study's findings are available on request from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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