Journal of Inflammation Research

Dovepress
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ORIGINAL RESEARCH

A Clinical Predictive Model Based on SOCS3 Promoter Methylation to Predict the Prognosis of Acute-on-Chronic Hepatitis B Liver Failure

Ji-Hui Li[1], Yuna Tang[1], Jing Wang[1], Xue-Fei Wei[1], Na Wang[2], Jing-Wei Wang[2], Hui Lyu[3], Xue-Mei Jiang[4], Hui-Hui Liu[1,5], Kai Wang[1,5,6]

[1]Department of Hepatology, Qilu Hospital of Shandong University, Jinan, Shandong, People's Republic of China; [2]Department of Hepatology, Qilu Hospital of Shandong University (Qingdao), Qingdao, Shandong, People's Republic of China; [3]Department of Severe Liver Disease, Shandong Public Health Clinical Center of Shandong University, Jinan, Shandong, People's Republic of China; [4]Department of Hepatology, Shandong Public Health Clinical Center of Shandong University, Jinan, Shandong, People's Republic of China; [5]Institute of Hepatology, Shandong University, Jinan, Shandong, People's Republic of China; [6]Shenzhen Research Institute, Shandong University, Shenzhen, Guangdong, People's Republic of China

Correspondence: Hui-Hui Liu; Kai Wang, Department of Hepatology, Qilu Hospital of Shandong University and Institute of Hepatology, Shandong University, Jinan, Shandong, People's Republic of China, Email liuhuihui_2017@163.com; wangdoc2010@163.com

Purpose: The study aimed to quantitatively detect the suppressors of cytokine signaling (SOCS) 3 promoter methylation levels, investigate the relationship between SOCS3 methylation and gene expression, and construct a prognosis prediction model combined with clinical indicators for Acute-on-chronic Hepatitis B Liver Failure (ACHBLF).

Methods: A total of 135 ACHBLF patients were enrolled and randomly divided into the training cohort and validation cohort. The SOCS3 mRNA and promoter methylation in peripheral blood mononuclear cells (PBMCs) of ACHBLF patients were quantitative measured. A clinical prediction model was established based on SOCS3 promoter methylation and clinical indicators. The prediction model was evaluated by the area under the receiver operating characteristic curve, the Hosmer-Lemeshow (H-L) goodness-of-fit test, and decision curve analysis.

Results: In this study, compared with ACHBLF survivals, SOCS3 showed lower mRNA levels and higher methylation levels in ACHBLF non-survivals. The SOCS3 methylation rates were negatively correlated with SOCS3 mRNA levels. PT-INR, IL-6, and percentage of the methylation reference (PMR) value (SOCS3) were used to establish a clinical model for predicting ACHBLF patients' prognosis. The results of AUC, the Hosmer-Lemeshow (H-L) goodness-of-fit test and decision curve analysis (DCA) showed that the prediction model had good clinical applicability. The prediction model was visualized.

Conclusion: A prognosis prediction model for ACHBLF was developed based on PMR (SOCS3), PT-INR and IL-6, which may have a good potential clinical application value.

Keywords: acute-on-chronic hepatitis B liver failure, DNA methylation, prediction model, SOCS3

Introduction

Hepatitis B virus (HBV) infections often progress chronically. Some patients with chronic hepatitis B (CHB) are at risk of developing acute-on-chronic liver failure (ACLF), a syndrome characterized by severe systemic inflammation, organ failure, and poor prognosis and that is often closely related to emergencies.[1] The disease develops rapidly and has a high short-term mortality.[2] Although some prognostic prediction scores for ACLF have been developed,[3–5] there are still limitations due to factors such as etiology and clinical characteristics. There is an urgent need for an accurate early prediction model for ACHBLF.

Cytokine storm (CS) is a life-threatening systemic inflammatory syndrome caused by various factors, such as infection, that involve elevated levels of circulating cytokines and immune cell hyperactivation.[6] CS occurrence is prevalent among ACLF patients, as their excessive systemic inflammatory responses may make immunocompromised patients with ACLF susceptible to secondary infections, resulting in higher organ dysfunction and mortality rates.[7] The

Received: 12 November 2024
Accepted: 4 March 2025
Published: 14 March 2025

cytokine expression profiles of patients with ACHBLF are characterized by a diverse array of aberrant interleukin and chemokine signaling pathways.[8,9]

Suppressors of cytokine signaling (SOCS) are a family of proteins—including SOCS1-7 and cytokine-inducible sh2 (CIS) proteins—that negatively regulate cytokine receptor signaling pathways.[10] The most well-known SOCS-regulated cytokine signaling pathway is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction pathway.[11] Several studies have shown that SOCS1 expression is aberrant in ACHBLF patients and is clinically significant.[12,13] SOCS3, which is structurally similar to SOCS1 and a member of the SOCS protein family, regulates the interleukin-6 (IL-6) family.[14–16] Additionally, SOCS3 is involved in regulating related cytokines in inflammatory and infectious diseases.[17,18] Some studies have suggested that SOCS3 plays a crucial role in the pathogenesis and progression of chronic liver diseases, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[19–22] SOCS3 is correlated with the severity of inflammation in patients with ACHBLF and in mouse hepatitis virus strain 3-induced acute liver failure.[23] However, few studies have evaluated the role of SOCS3 in ACHBLF prognosis.

DNA methylation typically occurs within gene CpG-rich promoter regions, often correlating with gene expression levels in diseases.[24] Thus, DNA methylation is frequently utilized as a disease biomarker.[25,26] In patients with ACHBLF, the DNA methylation of GSTP1 and PPAR-γ is changed, which is closely related to the disease's clinical status.[27,28] Aberrant SOCS1 promoter methylation has also been observed in patients with ACHBLF and is associated with clinical features and treatment outcomes.[13,29] Therefore, SOCS3 promoter methylation may serve as a biomarker for predicting ACHBLF patients' prognosis.

In this study, SOCS3 promoter methylation was evaluated in the peripheral blood mononuclear cells (PBMCs) of ACHBLF patients. A predictive model for ACHBLF prognosis was established and validated based on SOCS3 promoter methylation to guide disease treatment.

Methods

Study Population

A total of 135 ACHBLF patients were prospectively screened from December 2017 to October 2023 at the Department of Hepatology, Qilu Hospital of Shandong University, Qilu Hospital of Shandong University (Qingdao), and Shandong Public Health Clinical Center of Shandong University. ACHBLF was diagnosed according to the consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL).[3] The diagnostic criteria for CHB patients were determined according to the 2015 APASL guidelines.[30]

Patients were excluded if they fulfilled one or more of the following criteria: HCC, autoimmune liver diseases, alcoholic hepatitis, hepatitis C virus (HCV), hepatitis E virus (HEV), hepatitis G virus (HGV), known decompensated cirrhosis prior to onset of acute hepatic insult, human immunodeficiency virus (HIV), any other type of immunodeficiency, antioxidant use, interferon therapy, age less than 18 years, and pregnancy. After the patients were enrolled in the study, they were randomly allocated into training and validation cohorts at a ratio of 7:3, and their demographic characteristics and laboratory variables were recorded (Figure 1).

The Medical Ethical Committee of Qilu Hospital of Shandong University approved this study, with the ethical approval number "KYLL-202111-244-2". Informed consent was obtained from all patients. The study was performed in accordance with the 1964 Declaration of Helsinki and later amendments.

Collection and Preparation of Peripheral Blood Mononuclear Cells

Peripheral venous blood (5 mL) was collected from each participant in tubes containing ethylenediaminetetraacetic acid (EDTA). PBMCs were isolated via Ficoll-Paque density gradient centrifugation and used immediately for subsequent experiments or preserved at −80°C until use.

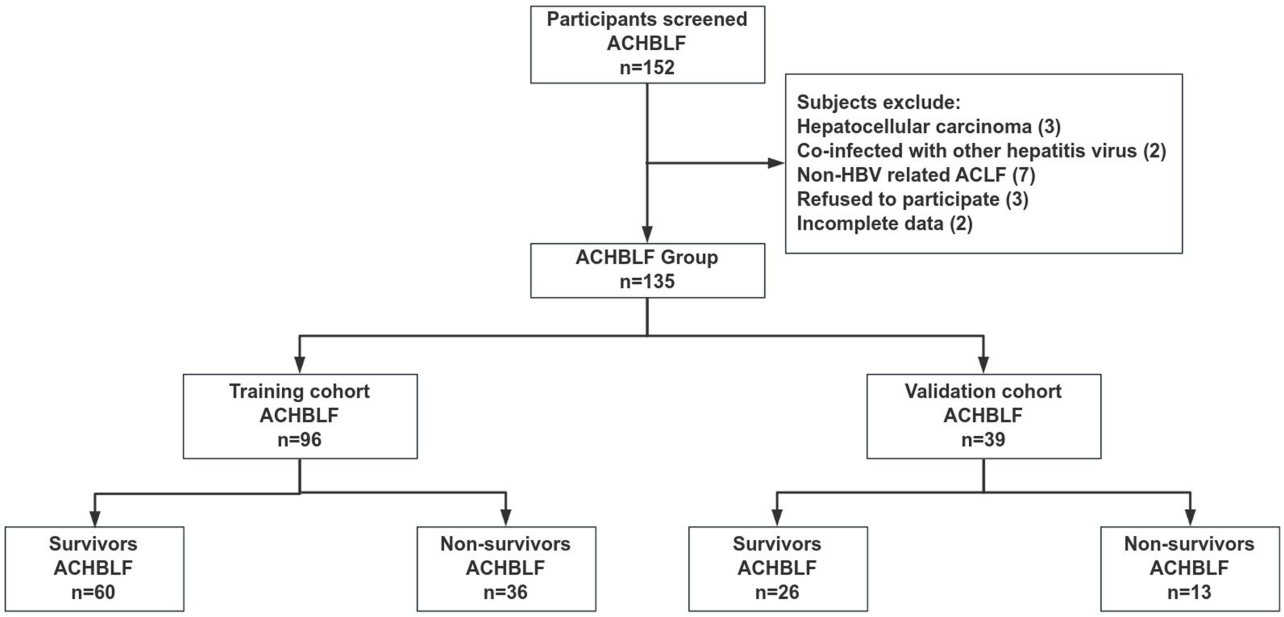

Figure 1 Flow chart of patient enrollment and grouping.

Total DNA Extraction and TaqMan Probe-Based Quantitative Methylation-Specific Polymerase Chain Reaction (MethyLight)

Total DNA was extracted from the PBMCs using TRIzol reagent (Invitrogen). The EZ DNA Methylation-Gold kit (Zymoresearch, Orange, CA) was used for DNA bisulfite modification. MethyLight was performed using the EpiTect MethyLight PCR + ROX Vial Kit (QIAGEN, Hilden, Germany). Two sets of primers and probes for the SOCS3 and GAPDH genes of bisulfite-converted DNA were designed and used. Table 1 lists the SOCS3 and GAPDH gene primers and probe sequences. The manufacturer provided the following standard protocol conditions for the 10 μL reaction system: 95°C for 15 min, followed by 45 cycles of 95°C for 15s and 60°C for 1 min.[31] The MethyLight data were indicated as a percentage of the methylation reference (PMR) value:[27] PMR = 100% × 2 exp. [Sample ΔCt (target gene-control gene) M.SssI-Reference ΔCt (target gene-control gene)].

Measurement of SOCS3 mRNA Expression by Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcription kit was used to reverse transcribed the extracted RNA into complementary DNA (cDNA) for subsequent RT-PCR quantitative detection. The SOCS3 mRNA expression level and ACTB mRNA were then detected by RT-qPCR. The SOCS3 and ACTB mRNA primer sequences are listed in Table 1.

Table 1 Sequences of the Primers and Probes Used

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Probe Oligo Sequence
Methylight			
SOCS3	TTTAGGTTTATAGCGTTTGTATTTC	TCATCTTATCCCCAAATAAACTATATAAT	AATGTTATTGAGGTAGTTTTCGGGTGTTTT
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAAA	ACCACCACCCAACACACAATAACAAACACA
RT-qPCR			
SOCS3	TCTGTCGGAAGACCGTCAAC	CCTTAAAGCGGGGCATCGTA	
ACTB	ATGGGTCAGAAGGATTCCTATGTG	CTTCATGAGGTAGTCAGTCAGGTC	

https://doi.org/10.2147/JIR.S506050

Clinical Parameter Collection

The following potentially predictive indicators were recorded: age, gender, BMI, smoking history, alcohol consumption, diabetes mellitus (DM), hypertension, cirrhosis, ascites, hepatic encephalopathy (HE), bacterial or fungal infections, pneumonia, leukocyte count, neutrophil count, lymphocyte count, platelet count (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin (TBIL), lactate dehydrogenase (LDH), creatinine (Cr), blood urea nitrogen (BUN), sodium, kalium, ammonia, prothrombin time activity (PTA), prothrombin time-international normalized ratio (PT-INR), hepatitis B e antigen (HBeAg), serum HBV-DNA, ferritin, C-reactive protein (CRP), procalcitonin (PCT), IL-6, alpha-fetoprotein (AFP), MELD score, and MELD-Na score. As Table 2 presents, these laboratory parameters were measured using the operating procedures of the Department of Medicine Laboratory, Qilu Hospital, Shandong University.

Table 2 Characteristics of ACHBLF Patients in the Training and Validation Cohorts

Variable	Training Cohort	Validation Cohort	P value
Cases (n)	96	39	
Demographic indicators			
Age (years)	50.52±12.49	48.72±12.12	0.445[a]
Gender (male,%)	69.00 (71.90)	28.00 (71.80)	0.993[b]
BMI (kg/m^2)	22.75 (20.62–25.48)	23.10 (20.55–25.30)	0.730[b]
Smoking history (n,%)	44.00 (45.80)	14.00 (40.00)	0.973[b]
Alcohol consumption (n,%)	53.00 (55.20)	22.00 (56.40)	0.899[b]
Past medical history			
DM (n,%)	30.00 (31.20)	11.00 (28.20)	0.727[b]
Hypertension (n,%)	32.00 (33.30)	12.00 (30.80)	0.773[b]
Cirrhosis (n,%)	70.00 (72.90)	28.00 (71.80)	0.895[b]
Complications			
Ascites (n,%)	58.00 (60.40)	28.00 (71.80)	0.213[b]
HE (n,%)	23.00 (24.00)	13.00 (33.30)	0.264[b]
Bacterial or fungal infections (n,%)	36.00 (37.50)	11.00 (28.20)	0.304[b]
Pneumonia (n,%)	30.00 (31.20)	14.00 (35.90)	0.602[b]
Blood routine indicators			
Leukocyte count (×10^9/L)	6.69 (4.72–9.90)	7.34 (5.66–10.39)	0.282[b]
Neutrophil count (×10^9/L)	4.68 (2.62–7.35)	4.91 (4.04–7.77)	0.282[b]
Lymphocyte count (×10^9/L)	1.26 (0.80–1.64)	1.45 (0.94–1.86)	0.211[b]
PLT (×10^9/L)	96.00 (66.00–136.00)	102.00 (67.50–153.00)	0.600[b]
Liver function indicators			
ALT (U/L)	69.00 (32.50–219.25)	145.00 (43.50–312.50)	0.135[b]
AST (U/L)	87.50 (51.50–179.00)	120.00 (69.00–198.00)	0.214[b]
ALB (g/L)	32.60 (29.23–36.12)	32.60 (31.65–36.10)	0.266[b]
TBiL (μmol/L)	206.75 (145.40–312.05)	254.60 (191.75–362.05)	0.036[b]
LDH (U/L)	237.50 (213.75–275.75)	261.00 (227.00–309.00)	0.071[b]
Renal function indicators			
Cr (μmol/L)	56.00 (46.75–70.00)	56.00 (41.00–65.50)	0.130[b]
BUN (mmol/L)	5.20 (3.54–7.48)	4.70 (3.55–6.95)	0.850[b]

(*Continued*)

Table 2 (Continued).

Variable	Training Cohort	Validation Cohort	P value
Biochemical indicators			
Sodium (mmol/L)	138.00 (134.75–141.00)	138.00 (136.50–140.50)	0.757[b]
Kalium (mmol/L)	3.94±0.64	3.85±0.52	0.432[a]
Ammonia (μmol/L)	57.00 (42.75–71.50)	64.00 (38.50–93.50)	0.468[b]
Coagulation indicators			
PTA (%)	37.00 (30.00–48.25)	45.00 (35.80–53.00)	0.037[b]
PT-INR	1.98 (1.66–2.39)	1.70 (1.52–2.05)	0.031[b]
Hepatitis B indicators			
HBeAg positive (n,%)	61.00 (63.50)	21.00 (53.80)	0.296[b]
HBV DNA (log_{10} IU/mL)	3.73 (2.72–5.42)	4.27 (2.92–6.47)	0.498[b]
Inflammatory indicators			
Ferritin (ng/mL)	611.50 (323.50–994.00)	879.00 (467.00–1343.50)	0.100[b]
CRP (mg/L)	8.61 (5.06–14.81)	10.76 (5.18–21.43)	0.340[b]
PCT (ng/mL)	0.44(0.22–0.80)	0.45 (0.31–0.75)	0.555[b]
IL-6 (pg/mL)	25.78 (10.04–74.13)	31.97 (9.13–56.94)	0.835[b]
Other Indicators			
AFP (ng/mL)	30.52 (5.32–166.17)	16.03 (5.53–136.18)	0.791[b]
PMI (SOCS3,%)	48.94 (28.24–57.95)	41.05 (33.04–54.46)	0.771[b]
Prognostic score			
MELD score	18.62 (14.87–23.58)	18.17 (14.33–21.40)	0.324[b]
MELD-Na score	20.35 (15.42–25.33)	18.67 (15.58–22.66)	0.281[b]
Mortality rate			
28-day mortality (n,%)	32.00 (33.30)	12.00 (30.80)	0.773[b]
90-day mortality (n,%)	36.00 (37.50)	13.00 (33.30)	0.648[b]

Notes: [a]unpaired *t* test. [b]Mann–Whitney *U*-test.

Predictor Selection

To identify the predictors that were used in the prediction model, univariate logistic regression analysis and LASSO regression were performed to select optimal predictors from the clinical parameters in the training cohort using the "glmnet" package.[32] Finally, based on the predictors screened using LASSO regression analysis, multivariate logistic regression analysis was performed to determine the final predictors that were included in the model.

Prediction Model Development and Validation

Based on the multivariate logistic regression analysis, a clinical prediction model was performed to develop a nomogram to discriminate between survivals and non-survivals. To verify and assess the model's clinical accuracy and applicability, the area under the curve (AUC) was calculated by analyzing the receiver operating characteristic (ROC) curve. The Hosmer-Lemeshow (H-L) goodness-of-fit test was used to assess the model's calibration, and decision curve analysis (DCA) was used to assess the model's clinical utility.

Statistical Analysis

Statistical analyses were performed using the following software: SPSS (version 27.0; IBM SPSS Inc., Chicago, IL), GraphPad (GraphPad Prism version 9.5.1), and R (version 4.0.1). Categorical variables were indicated as numbers (proportions). Normally distributed variables were expressed as means ± standard deviations, whereas non-normally distributed variables were expressed as medians with interquartile ranges (P25-P75). Categorical variables were compared using the chi-square test. Between the two groups, normal distribution was compared using the *t*-test, and non-normal distribution was compared using the Mann–Whitney *U*-test. Any p values less than 0.05 were considered statistically significant.

Results

Characteristics of Patients in the Training and Validation Cohorts

In this study, 135 ACHBLF patients were enrolled and allocated at a ratio of 7:3 into either a training (n = 96) or validation (n = 39) cohort. Table 2 shows the patients' demographic characteristics, medical histories, clinical presentations, and laboratory parameters. There were no significant differences between the training and validation cohorts (p > 0.05) in demographic indicators; medical histories; complications; blood routine indicators; liver function indicators, such as ALT, AST, ALB, and LDH; renal function indicators; biochemical indicators; hepatitis B indicators; inflammatory indicators; AFP; PMR (SOCS3); prognostic scores; and 28- and 90-day mortalities. In addition, TBIL and PTA were significantly lower in the training cohort than in the validation cohort (p = 0.036, 0.037), and PT-INR was significantly higher in the training cohort than in the validation cohort (p = 0 0.031).

SOCS3 Promoter Methylation Status in ACHBLF Patients with Different Outcomes

Figure 2A and B presents SOCS3 promoter methylation, expressed as PMR, in ACHBLF patients with different outcomes. For 28-day mortality, SOCS3 methylation was significantly higher in non-survivors with ACHBLF (n = 44,

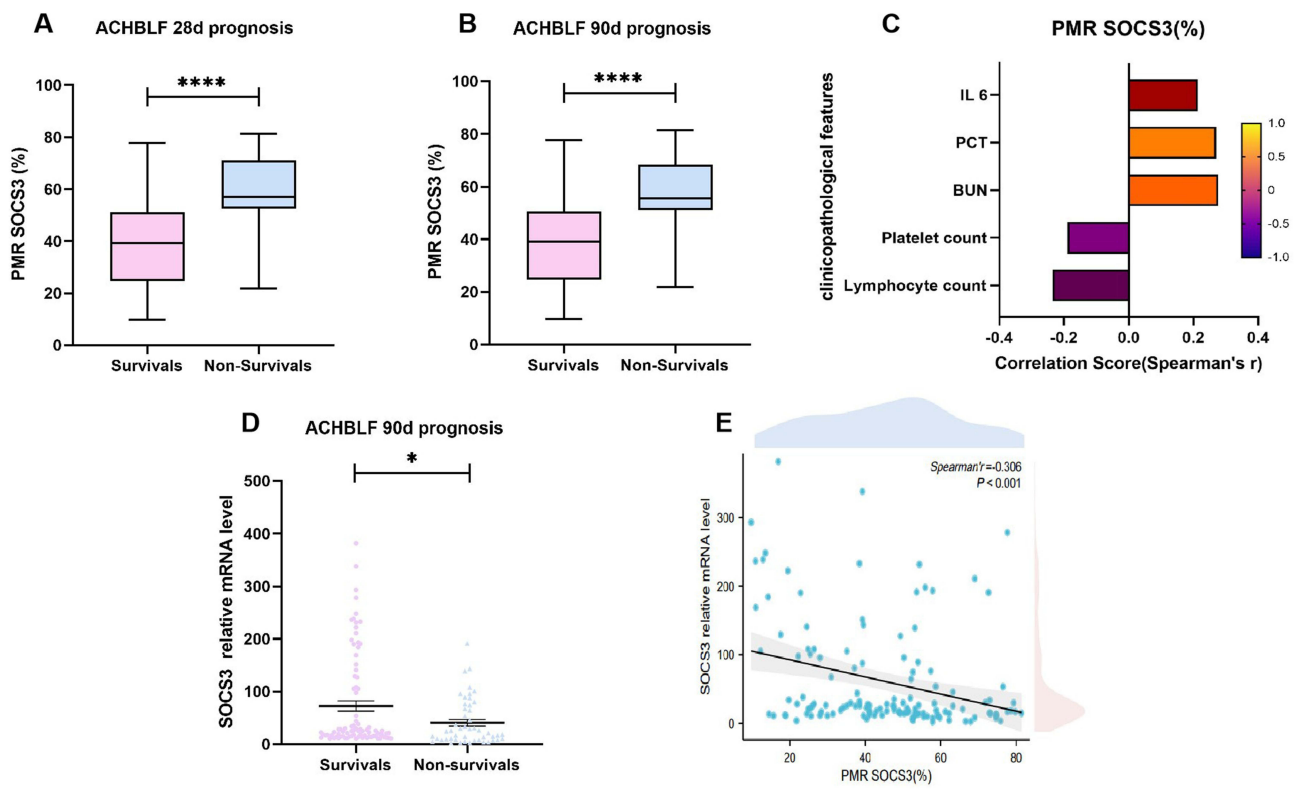

Figure 2 SOCS3 methylation and mRNA level in PBMCs form ACHBLF patients with different prognosis. (**A**) SOCS3 methylation in PBMCs form ACHBLF patients with 28d prognosis. (**B**) SOCS3 methylation in PBMCs form ACHBLF patients with 90d prognosis. (**C**) Correlation between SOCS3 methylation and clinicopathological features in ACHBLF patients. (**D**) SOCS3 mRNA level in PBMCs form ACHBLF patients with 90d prognosis. (**E**) Correlation between SOCS3 methylation and mRNA level in ACHBLF patients. (*p < 0.05, ****p < 0.0001).

58.06 ± 14.45%) than in survivors (n = 91, 39.05 ± 17.07%, p < 0.001) (Figure 2A). For 90-day mortality, SOCS3 methylation was also significantly higher in non-survivors (n = 49, 56.35 ± 15.11%) than in survivors (n = 86, 38.92 ± 17.31%, p < 0 0.001) (Figure 2B).

Associations Between SOCS3 Promoter Methylation Status and Clinicopathological Features in ACHBLF Patients

Figure 2C shows that SOCS3 methylation levels were significantly correlated with Lymphocyte count (Spearman's r = −0.24, p = 0.006), Platelet count (Spearman's r = −0.19, p = 0.03), BUN (Spearman's r = 0.28, p = 0.001), PCT (Spearman's r = 0.27, p = 0.002) and IL-6 (Spearman's r = 0.21, p = 0.01). However, there were no significant correlations between SOCS3 methylation levels and other clinical biochemical indicators (p > 0.05).

SOCS3 Relative mRNA Levels in ACHBLF Patients with 90d Prognosis and Correlations with Promoter Methylation Status

As illustrated in Figure 2D, for 90-day prognosis, SOCS3 relative mRNA levels was significantly higher in survivors (n = 49, 72.61 ± 91.12) than in non-survivors (n = 86, 40.65 ± 44.00%, p = 0 0.026). Additionally, SOCS3 relative mRNA levels were negatively correlated with promoter methylation levels (n=135, Spearman's r = - 0.306, p < 0.001) (Figure 2E).

Baseline Characteristics of Patients with Different Outcomes in the Training and Validation Cohorts

As Table 3 shows, according to the 90-day prognosis, ACHBLF patients in the training (n = 96) and validation (n = 39) cohorts were divided into survivors (n = 60, 26) and non-survivors (n = 36, 13). In the training cohort, the hypertension

Table 3 Baseline Characteristics of ACHBLF Patients with 90-Day Prognosis

Variable	Training Cohort (n=96)			Validation Cohort (n=39)		
	Survivors	Non-survivors	P value	Survivors	Non-survivors	P value
Cases (n)	60	36		26	13	
Age (years)	48.97±12.90	53.11±11.49	0.116[a]	48.88±13.54	48.38±9.12	0.905[a]
Gender (male,%)	25.00 (69.40)	44.00 (73.30)	0.682[b]	18.00 (69.20)	10.00 (76.90)	0.615[b]
BMI (kg/m^2)	22.70 (20.40–26.05)	22.80 (20.78–25.08)	0.803[b]	22.54±3.06	23.88±4.15	0.262[a]
Smoking history (n,%)	17.00 (47.20)	27.00 (45.00)	0.832[b]	13.00 (50.00)	5.00 (38.50)	0.496[b]
Alcohol consumption (n,%)	32.00 (53.30)	21.00 (58.30)	0.633[b]	12.00 (46.20)	10.00 (76.90)	0.068[b]
DM (n,%)	19.00 (31.70)	11.00 (30.60)	0.909[b]	6.00 (23.10)	5.00 (38.50)	0.314[b]
Hypertension (n,%)	15.00 (25.00)	17.00 (47.20)	0.025[b]	8.00 (30.80)	4.00 (30.80)	1.000[b]
Cirrhosis (n,%)	44.00 (73.30)	26.00 (72.20)	0.906[b]	16.00 (61.50)	12.00 (92.30)	0.044[b]
Ascites (n,%)	37.00 (61.70)	21.00 (58.30)	0.746[b]	17.00 (65.40)	11.00 (84.60)	0.208[b]
HE (n,%)	9.00 (15.00)	14.00 (38.90)	0.008[b]	6.00 (23.10)	7.00 (53.80)	0.055[b]
Bacterial or fungal infections (n,%)	19.00 (31.70)	17.00 (47.20)	0.127[b]	7.00 (26.90)	4.00 (30.80)	0.801[b]
Pneumonia (n,%)	15.00 (25.00)	15.00 (41.70)	0.088[b]	9.00 (34.60)	5.00 (38.50)	0.813[b]
Leukocyte count (×10^9/L)	6.25 (4.22–9.82)	7.58 (4.86–11.19)	0.217[b]	7.28 (5.52–10.13)	7.39 (6.07–11.09)	0.882[b]
Neutrophil count (×10^9/L)	3.84 (2.15–6.44)	6.46 (4.02–8.96)	0.016[b]	4.91 (4.00–7.50)	4.91 (4.27–8.89)	1.000[b]
Lymphocyte count (×10^9/L)	1.35 (0.97–1.69)	0.92 (0.62–1.53)	0.012[b]	1.46 (1.13–2.02)	1.36 (0.63–1.64)	0.228[b]
PLT (×10^9/L)	104.00(67.00–145.00)	86.00 (56.00–109.50)	0.054[b]	113.00 (87.75–169.50)	67.00 (43.00–78.00)	0.003[b]
ALT (U/L)	69.00 (25.75–155.25)	72.50 (42.50–233.00)	0.382[b]	139.00 (42.75–365.75)	145.00 (45.00–181.00)	0.789[b]
AST (U/L)	78.50 (47.50–147.50)	102.00 (58.00–240.50)	0.305[b]	131.50 (78.50–228.75)	105.00 (55.00–152.00)	0.257[b]

(*Continued*)

https://doi.org/10.2147/JIR.S506050

Table 3 (Continued).

Variable	Training Cohort (n=96)			Validation Cohort (n=39)		
	Survivors	Non-survivors	P value	Survivors	Non-survivors	P value
ALB (g/L)	32.71±4.71	32.71±5.46	1.000[a]	33.60 (32.23–36.20)	31.80 (30.20–35.10)	0.205[b]
TBiL (μmol/L)	176.60 (133.05–258.75)	259.15 (178.02–332.40)	0.013[b]	260.95±127.49	332.42±121.12	0.102[a]
LDH (U/L)	234.00 (213.50–274.25)	255.00 (214.50–288.00)	0.241[b]	262.00 (228.50–299.50)	261.00 (227.00–314.00)	0.623[b]
Cr (μmol/L)	55.00 (46.75–64.25)	60.00 (46.75–89.25)	0.185[b]	52.96±17.48	56.85±14.52	0.495[a]
BUN (mmol/L)	4.35 (3.27–5.80)	6.40 (4.20–9.00)	0.002[b]	4.35 (3.30–6.70)	5.80 (4.60–7.20)	0.185[b]
Sodium (mmol/L)	138.00 (136.00–141.00)	136.50 (134.00–141.00)	0.415[b]	139.00 (137.25–141.00)	137.00 (132.00–139.00)	0.068[b]
Kalium (mmol/L)	3.95±0.62	3.92±0.68	0.787[a]	3.76±0.51	4.02±0.52	0.150[a]
Ammonia (μmol/L)	56.00 (44.25–69.00)	61.50 (42.50–85.75)	0.493[b]	65.65±31.90	73.69±44.78	0.522[a]
PTA (%)	43.50 (31.75–53.00)	31.00 (27.75–42.00)	0.001[b]	46.00 (36.70–53.00)	45.00 (34.00–52.00)	0.644[b]
PT-INR	1.77 (1.52–2.21)	2.34 (1.80–2.60)	<0.001[b]	1.67 (1.52–1.98)	1.75 (1.57–2.10)	0.239[b]
HBeAg positive (n,%)	40.00 (66.70)	21.00 (58.30)	0.411[b]	14.00 (53.80)	7.00 (53.80)	1.000[b]
HBV DNA (log_{10} IU/mL)	3.98 (2.82–5.76)	3.39 (2.63–5.31)	0.403[b]	3.93 (2.89–5.99)	4.72 (3.61–6.90)	0.512[b]
Ferritin (ng/mL)	563.00 (246.75–801.75)	751.25 (467.10–1474.25)	0.047[b]	821.50 (432.75–1057.25)	1088.00 (500.00–2329.00)	0.356[b]
CRP (mg/L)	7.00 (4.12–11.16)	12.08 (7.99–21.92)	<0.001[b]	6.91 (4.26–10.80)	26.96 (16.49–43.65)	<0.001[b]
PCT (ng/mL)	0.30 (0.14–0.47)	0.82 (0.57–1.09)	<0.001[b]	0.38 (0.26–0.53)	0.75 (0.46–0.89)	0.006[b]
IL-6 (pg/mL)	12.55 (5.56–25.64)	68.98 (43.97–156.04)	<0.001[b]	16.96 (5.95–36.74)	54.59 (32.29–130.50)	0.001[b]
AFP (ng/mL)	30.52 (5.17–100.99)	31.55 (5.32–207.96)	0.700[b]	31.52 (6.61–154.60)	12.62 (4.63–115.28)	0.326[b]
PMI (SOCS3,%)	39.08±17.88	55.62±14.64	<0.001[a]	16.34±5.50	20.32±5.18	0.037[a]
MELD score	16.52 (14.27–20.84)	22.63 (17.91–26.25)	<0.001[b]	17.27±5.18	22.02±6.02	0.015[a]
MELD-Na score	18.73±5.64	23.92±7.99	<0.001[a]	38.55±16.24	58.38±16.78	0.001[a]

Notes: [a]unpaired *t* test. [b]Mann–Whitney *U*-test.

and HE proportions in non-survivors were significantly higher (p = 0.025, 0.008) than in survivors. Neutrophil count, TBIL, BUN, PT-INR, ferritin, CRP, PCT, IL-6, PMR (SOCS3), MELD scores, and MELD-Na scores were significantly higher in non-survivors than in survivors (p < 0.05). In addition, lymphocyte counts and PTA were significantly lower in non-survivors than in survivors (p = 0.012, 0.001). In the validation cohort, the cirrhosis proportion in non-survivors was significantly higher than in survivors (p = 0.044). PLT in non-survivors was significantly lower than in survivors (p = 0.003). Moreover, CRP, PCT, IL-6, PMR (SOCS3), MELD scores, and MELD-Na scores were significantly higher in non-survivors than in survivors (p < 0.05).

Prognostic Predictor Screening

Univariate logistic regression analysis was performed in the training cohort to identify the factors associated with the 90-day prognosis of ACHBLF patients. As Table 4 shows, ten possible factors screened by univariate logistic regression analysis were used as prognostic predictors: hypertension, HE, lymphocyte count, TBiL, BUN, PTA, PT-INR, ferritin,

Table 4 Univariate and Multivariate Logistic Regression Analysis to Screen the Prognostic Factors Based on the Training Cohort

Variables	Univariate Logistic Regression Analysis			Multivariate Logistic Regression Analysis		
	β	OR (95% CI)	P value	β	OR (95% CI)	P value
Age	0.028	1.028(0.993, 1.064)	0.118			
Gender (male)	−0.191	0.826(0.332, 2.056)	0.682			
BMI	−0.026	0.974(0.882, 1.076)	0.607			
Smoking history	0.089	1.094(0.477, 2.505)	0.832			
Alcohol consumption	0.203	1.225(0.532, 2.822)	0.634			
DM	−0.052	0.949(0.388, 2.321)	0.909			
Hypertension	0.987	2.684(1.116, 6.454)	0.027			

(*Continued*)

Table 4 (Continued).

Variables	Univariate Logistic Regression Analysis			Multivariate Logistic Regression Analysis		
	β	OR (95% CI)	P value	β	OR (95% CI)	P value
Cirrhosis	−0.056	0.945(0.374, 2.389)	0.906			
Ascites	−0.139	0.870(0.375, 2.021)	0.747			
HE	1.283	3.606(1.360, 9.563)	0.010			
Bacterial or fungal infections	0.658	1.931(0.824, 4.521)	0.130			
Pneumonia	0.762	2.143(0.886, 5.184)	0.091			
Leukocyte count	0.032	1.032(0.946, 1.126)	0.475			
Neutrophil count	0.086	1.090(0.989, 1.201)	0.081			
Lymphocyte count	−0.820	0.440(0.219, 0.885)	0.021			
PLT	−0.003	0.997(0.990, 1.004)	0.358			
ALT	0.001	1.000(0.999, 1.001)	0.566			
AST	0.001	1.000(0.999, 1.002)	0.639			
ALB	0.001	1.000(0.920, 1.087)	1.000			
TBiL	0.004	1.004(1.001, 1.007)	0.019			
LDH	0.004	1.004(0.999, 1.009)	0.128			
Cr	0.015	1.015(0.999, 1.031)	0.059			
BUN	0.137	1.146(1.028, 1.278)	0.014			
Sodium	−0.029	0.971(0.882, 1.070)	0.554			
Kalium	−0.091	0.913(0.474, 1.757)	0.785			
Ammonia	0.011	1.011(0.997, 1.026)	0.132			
PTA	−0.054	0.948(0.914, 0.982)	0.003			
PT-INR	1.565	4.783(1.910, 11.981)	0.001	1.122	3.072 (1.022, 9.234)	0.046
HBeAg positive	−0.357	0.700(0.298, 1.642)	0.412			
HBV DNA	0.001	1.000(1.000, 1.000)	0.383			
Ferritin	0.001	1.001(1.000, 1.001)	0.044			
CRP	0.022	1.023(0.993, 1.053)	0.141			
PCT	0.524	1.689(0.866, 3.293)	0.124			
IL-6	0.016	1.016(1.007, 1.025)	0.001	0.014	1.014 (1.004, 1.024)	0.005
AFP	0.001	1.001(0.999, 1.003)	0.458			
PMR (SOCS3)	0.059	1.061(1.030, 1.093)	<0.001	0.064	1.066 (1.027, 1.106)	0.001

Abbreviations: β, partial regression coefficient; SE, Standard error; OR, odds ratio; CI, confidence interval.

IL-6, and PMR (SOCS3) ($p < 0.05$). These ten factors were then included in the LASSO regression for 10-fold cross-validation (Figure 3A and B). Three variables were optimal when lambda.1se = 0.146: PT-INR, IL-6, and PMR (SOCS3). Finally, three variables were analyzed using multivariate logistic regression analysis, and all p values were less than 0.05 (Table 4).

Development and Evaluation of ACHBLF Prognosis Prediction Model

Based on the multivariate logistic regression analysis results, PT-INR, IL-6, and PMR (SOCS3) were used to establish the clinical model for predicting ACHBLF patients' 90-day prognosis. The formula is as follows: Logit (P) = −6.716 + 1.122 × PT-INR + 0.014 × IL-6 (pg/mL) + 0.0064 × PMR (SOCS3) (%), where P represents the death risk probability of patients. ROC curves were drawn to evaluate the model's discrimination. In the training cohort, the model's AUC was 0.917 (95% CI: 0.854–0.979) (Figure 4A). The maximum value of the Youden index was 0.767, with a sensitivity of 0.917 and a specificity of 0.850; the corresponding optimal cut-off value was 0.326. In the validation cohort, the AUC was 0.880 (95% CI: 0.761–0.999) (Figure 4B). When the cut-off value was 0.326, the sensitivity was 0.692 and the specificity was 0.846. With the optimal cut-off value was 0.326, the mortality of ACHBLF patients with a model score

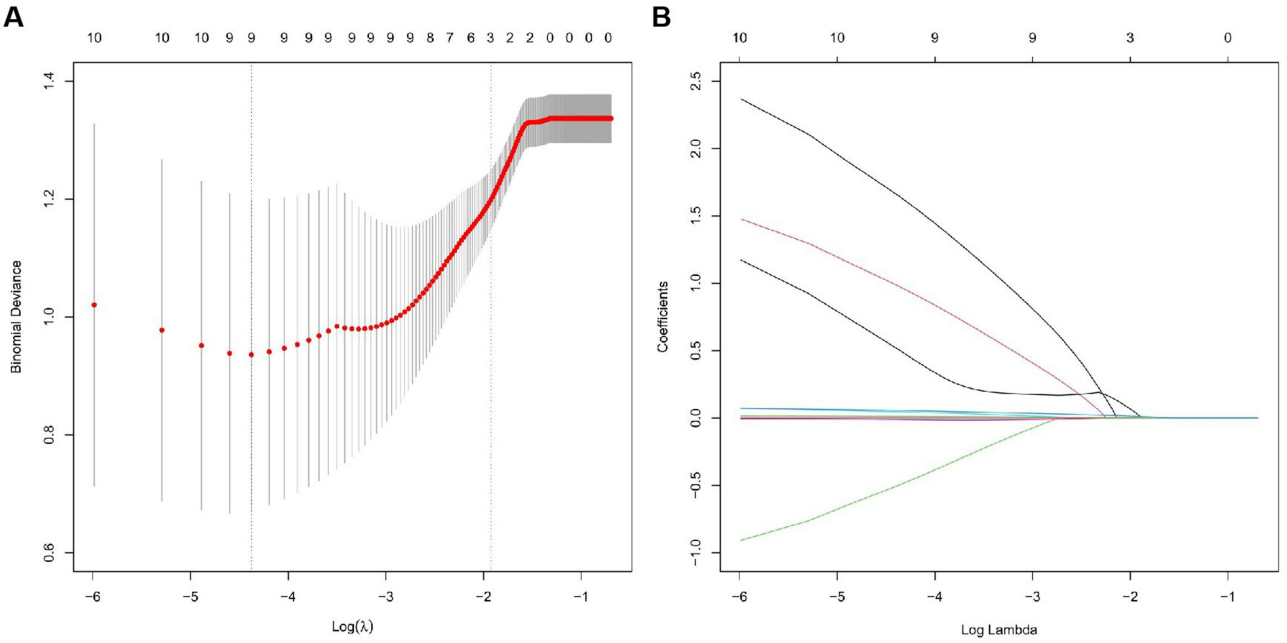

Figure 3 LASSO regression was used for variable selection. (**A**) The coefficient distributions of ten factors were plotted from the log (λ) sequence. The vertical dashed lines are drawn at lambda.min (λ=0.013) and lambda.1se (λ=0.146), respectively. (**B**) The figure shows the relationship between Log lambda and LASSO regression coefficient.

greater than or equal to 0.326 in the training cohort was 78.05%, and the mortality of patients with a model score less than 0.326 was 7.27% (Figure 4C). In the validation cohort, the mortality of ACHBLF patients with a score greater than or equal to 0.326 was 69.23%, and the mortality of patients with a score of less than 0.326 was 15.38% (Figure 4C). In the training cohort, the AUC of the MELD score was 0.733 (95% CI: 0.624–0.842), and the AUC of the MELD-Na score was 0.705 (95% CI: 0.595–0.815). In the validation cohort, the AUC of the MELD score was 0.701 (95% CI: 0.524–0.878), and the AUC of the MELD-Na score was 0.746 (95% CI: 0.559–0.932). The AUC of the model was higher than that of the MELD and MELD-Na scores in the training and validation cohorts.

DCA was used to evaluate the degree of clinical benefit of the prediction model for ACHBLF patients (Figure 5). The results of DCA showed that the threshold probability of ACHBLF patients in the training cohort was in the 0.08–0.99 range, and the net benefit of the model predicting their 90-day mortality risk was high. The threshold probability of the validation cohort was 0.1–0.99.

Finally, the H-L goodness-of-fit test was used to evaluate the consistency between the model's predicted and actual probability of occurrence, and a calibration curve was drawn to visualize the results (Figure 6). In the training cohort, the bias-corrected concordance index (C-index) was 0.918, and the H-L test $\chi 2$ was 7.203 ($p = 0.515$). In the validation cohort, the bias-corrected C-index was 0.879, and the H-L test $\chi 2$ was 3.457 ($p = 0.902$). The results showed that the model's predicted probability was in good agreement with its actual probability of occurrence.

Development of ACHBLF Prognostic Nomogram

As Figure 7 shows, the nomogram was based on the multivariate logistic regression analysis. It integrated the three predictors and used the scale line to express the mutual relationship between the variables in this prediction model. The nomogram could intuitively express the prediction model score and 90-day survival probability.

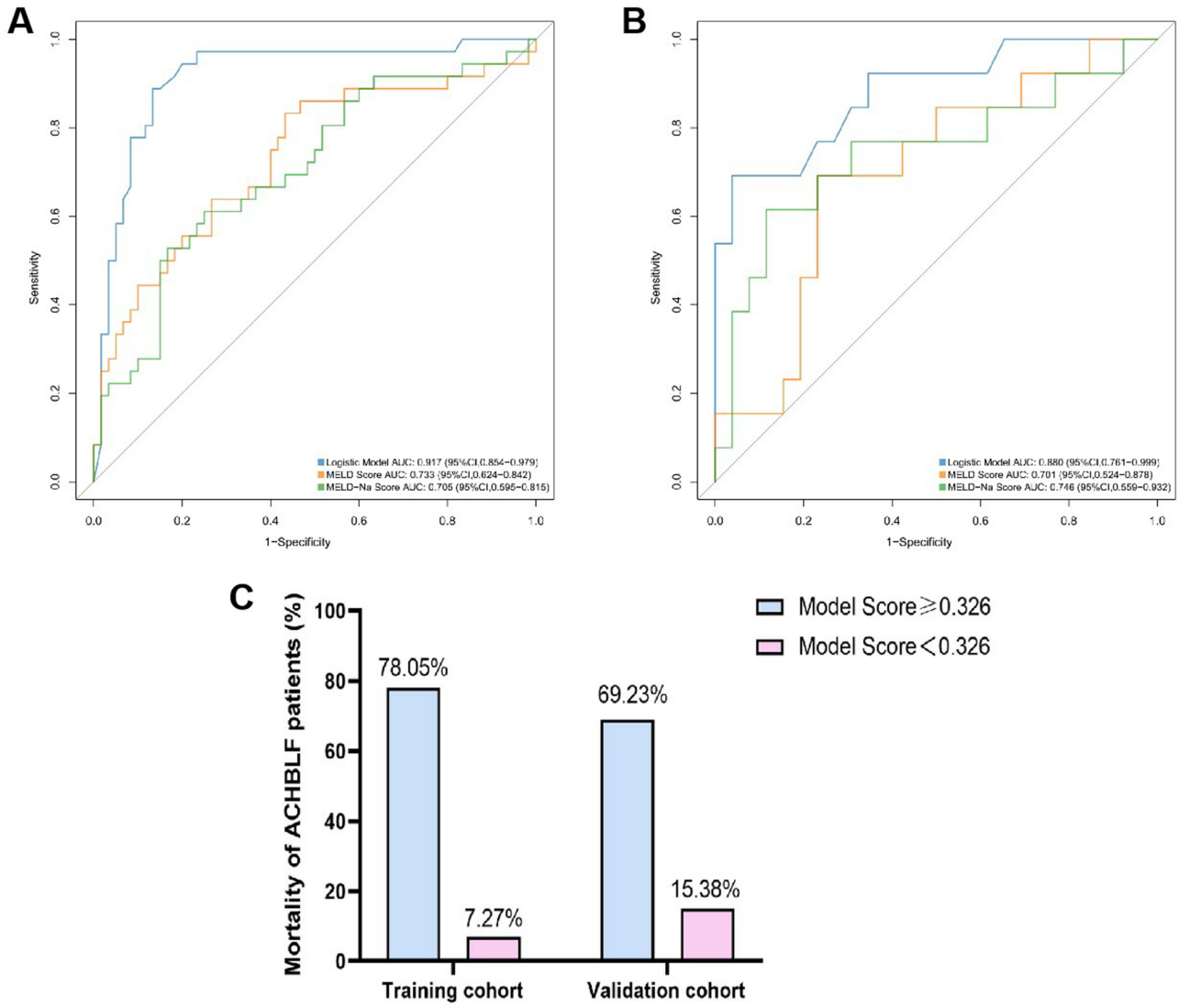

Figure 4 The ROC curve and cutoff value were drawn to evaluate the discrimination of the model. (**A**) ROC curve of the training cohort. (**B**) ROC curve of validation cohort. (**C**) Mortality of ACHBLF patients discriminated by the cutoff value of Model Score in the training and validation cohorts.

Discussion

In this study, we report for the first time that SOCS3 promoter methylation was significantly increased in 28- and 90-day non-survivors with ACHBLF. As a method for detecting gene methylation, MethyLight is highly sensitive, quantitative, fast, and accurate.[33] In addition, CpG island methylation in gene promoter regions results in stable silence gene expression.[34] We hypothesized that SOCS3 promoter methylation affected SOCS3 expression levels, resulting in a reduced anti-inflammatory ability, which affects ACHBLF patients' prognosis. We also observed that the following predictors were associated with prognosis: PT-INR, IL-6, and PMR (SOCS3). In this study, the model's ROC curves were significantly higher than those of the MELD and MELD-Na scores in predicting ACHBLF prognosis. As shown with DCA, the model demonstrated a high degree of predictive performance in both the training and validation cohorts. Finally, a nomogram was drawn to visualize the model.

In ACLF pathogenesis, extensive liver necrosis and severe systemic inflammation cause CS, leading to organ dysfunction and failure; this is a significant cause of the high mortality of patients.[35] Therefore, early prediction is key to reducing CS and improving survival for ACHBLF patients. Severe inflammatory responses in ACHBLF patients are often accompanied by the increased production and release of cytokines, such as IL-1β, IL-6, IL-8, IL-10, and TNF-α.

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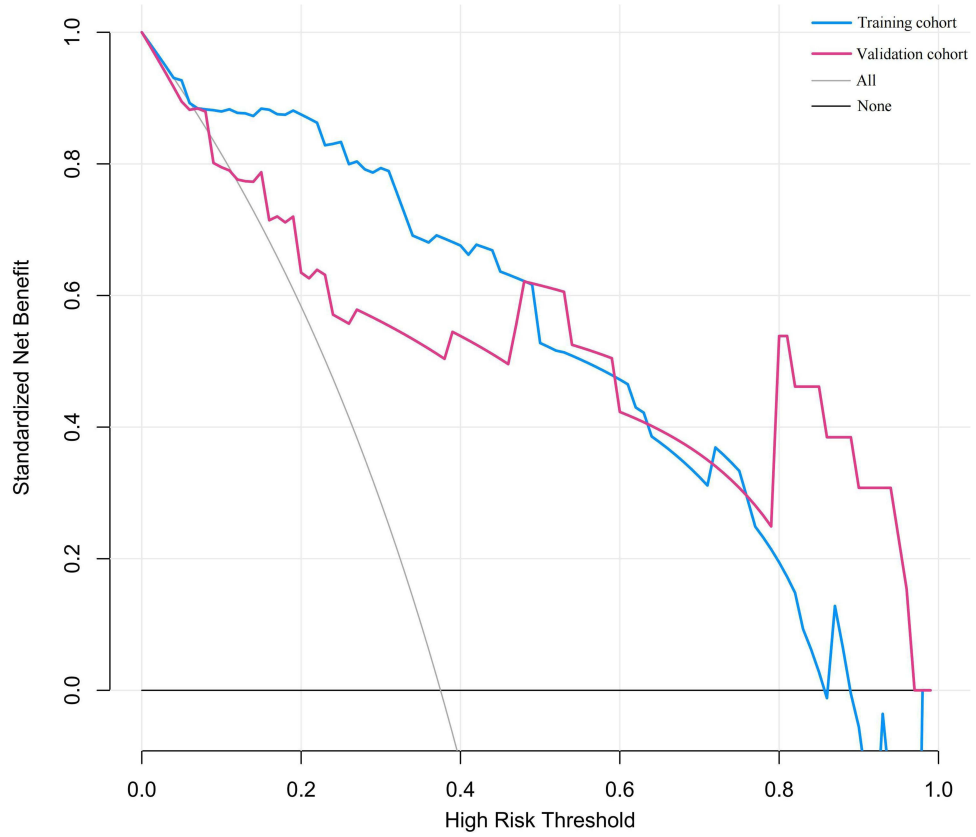

Figure 5 DCA was drawn to evaluate the degree of clinical benefit of the prediction model. The abscissa is the high risk threshold probability and the ordinate is the standardized net benefit.

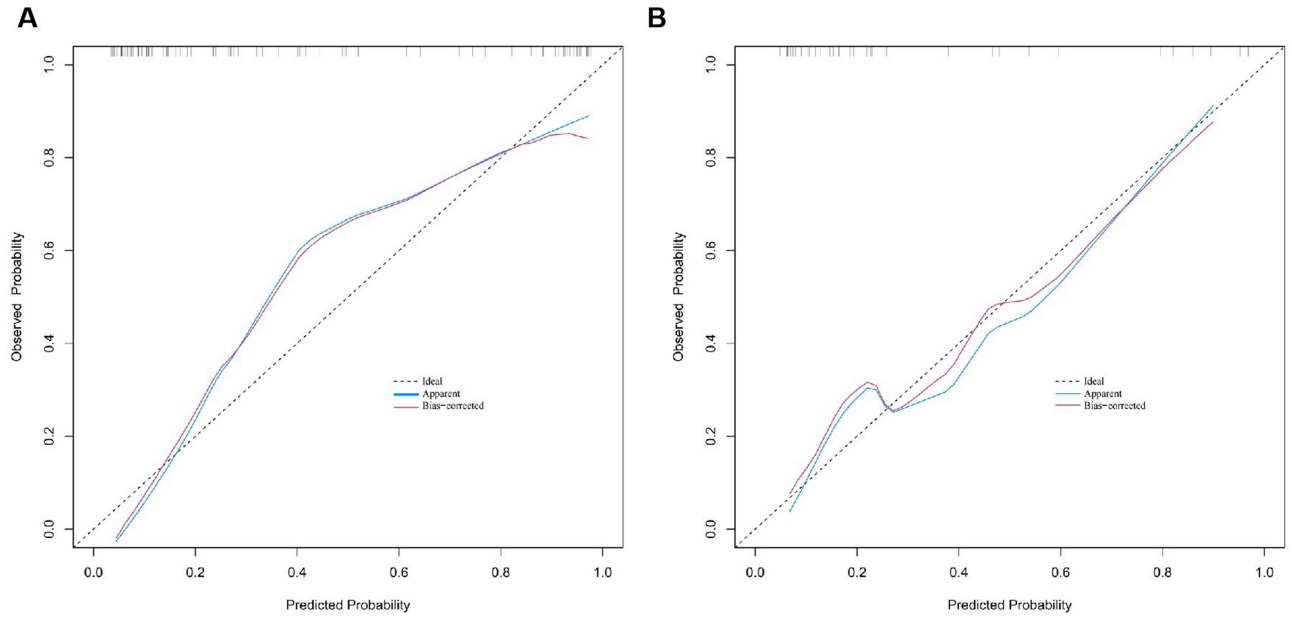

Figure 6 Calibration curves were drawn to assess the calibration of the prediction model. (**A**) Calibration curve of the training cohort. (**B**) Calibration curve of validation cohort.

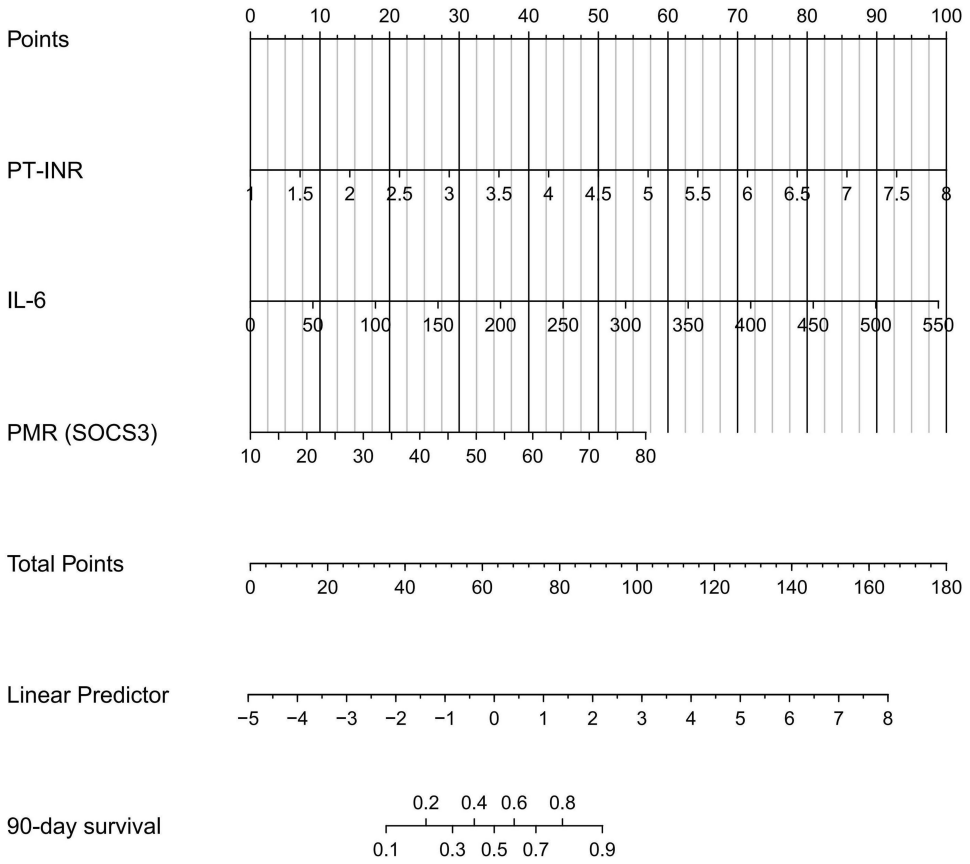

Figure 7 Nomogram for the prediction the 90-day prognosis of ACHBLF patients.

Among these, IL-6 is an important proinflammatory cytokine; inflammation and infection significantly increase its levels, and various studies have shown that a high level of serum IL-6 is an independent risk factor for death in ACHBLF patients.[36,37] In addition, IL-6 is an important inducer of the acute phase response and infection defense in the liver, essential for hepatocyte homeostasis, and a potent hepatocyte mitogen.[38] Therefore, IL-6 plays an important role in the development of inflammation in ACHBLF patients.

The JAK/STAT pathway primarily regulates cytokine signaling, and the SOCS family includes the main signaling molecules that attenuate this pathway.[39] Previous studies have shown that SOCS1 mRNA and SOCS1 methylation are related to ACHBLF patients' prognosis and glucocorticoid treatment.[12,13] Although SOCS1 and SOCS3 belong to the same protein family, they regulate different cytokines. SOCS3 is a major IL-6 signaling inhibitor, as it interacts with gp130, JAK1, JAK2, and TYK2.[14] Yong et al have shown that SOCS3 expression was significantly increased in the liver tissues and PBMCs of ACHBLF patients, and SOCS3, IL-1β, IL-6, and TNF-α were significantly increased in the livers of BALB/cJ mice 72 hours after infection and were closely related to the degree of liver injury.[23] However, the methylation levels of SOCS3 in ACHBLF have not been clearly defined. In this study, we first quantitatively detected SOCS3 promoter methylation in the PBMCs of ACHBLF patients and determined that SOCS3 was hypermethylated in non-survivors. Detecting DNA methylation using PBMCs from blood offers a non-invasive method for early diagnosis, prognosis prediction, dynamic monitoring after treatment, and other clinical research applications for cancer.[40] Therefore, the results of the present study suggest that SOCS3 methylation of PBMCs may be used as a biomarker to evaluate ACHBLF prognosis and to assess the degree of inflammation.

Several studies have reported biomarkers and developed nomograms to predict prognosis in ACHBLF patients. For example, Zhang et al established a nomogram for predicting the 30-day mortality of patients with ACHBLF and bacterial infections.[41] In Bai et al's study, a psoas muscle index-based nomogram was developed to determine cirrhosis risk in non-cirrhotic ACHBLF patients.[42] Yang et al constructed a nomogram for predicting 90-day outcomes in ACHBLF

patients based on five factors: age, TBIL, PTA, lymphocyte (L)%, and monocyte (M)%.[43] These studies involved models constructed to predict ACHBLF prognosis based on selected laboratory indicators and paid little attention to the acute inflammatory response states of patients. Some previous studies have reported that the methylation of specific genes in PBMCs of ACHBLF patients is related to their prognosis and has a certain predictive value,[13,27–29] but no prognosis prediction models that could be applied to clinical practice were constructed. In this study, we focused on SOCS3 promoter methylation in ACHBLF patients. Using quantitative detection and analysis, we constructed a nomogram to predict ACHBLF prognosis based on SOCS3 promoter methylation.

In this study, considering the characteristics of convenience, speed, less trauma, and high feasibility, this prediction model's construction is based on the analysis and screening of ACHBLF patients' clinical characteristics and SOCS3 promoter methylation in their PBMCs. This model is based on three easily accessible clinical variables for the rapid prediction of ACHBLF prognosis. This clinical prediction model can be an important reference for clinical management and treatment, which help inform prognosis prediction.

This study had several limitations. First, the prediction model was conducted with a small sample of multicenter cohorts; thus, it needs to be validated with a larger sample size. Second, because ACLF causes vary by region, such as alcoholic liver disease in Western countries, the prediction model can be further validated in ACLF cohorts with different causes.

In conclusion, SOCS3 promoter methylation was hypermethylated in non-surviving ACHBLF patients. The PMR value of SOCS3 had a high predictive value for ACHBLF prognosis, suggesting that SOCS3 methylation may affect ACHBLF patients' anti-inflammatory abilities. We also constructed a model to predict ACHBLF prognosis based on SOCS3 promoter methylation combined with laboratory indicators and demonstrated its good clinical applicability. This study showed that SOCS3 methylation could be a non-invasive diagnostic biomarker for ACHBLF prognosis. However, the specific role of SOCS3 in the pathogenesis of ACHBLF remains unclear, and its potential as a therapeutic target and prognostic factor requires further investigation.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author on reasonable request.

Ethics Approval

The Medical Ethical Committee of Qilu Hospital of Shandong University approved this study, with the ethical approval number "KYLL-202111-244-2". Informed consent was obtained from all patients. The study was performed in accordance with the 1964 Declaration of Helsinki and later amendments.

Acknowledgment

We thank all patients and their families who participated in the study, and all researchers who participated in the study of liver failure.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National key research and development program of China (2021YFC2301801) and National Natural Science Foundation of China (82272313).

Disclosure

The authors report no conflicts of interest in this work.

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