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ORIGINAL RESEARCH

Clinical Model Based on DR3 Promoter Methylation for Predicting Short-Term Mortality in Patients with Acute-on-Chronic Hepatitis B Liver Failure

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Purpose: Death receptor 3(DR3) is a key factor in the regulation of immune response and inflammatory diseases. The study aimed to quantitatively assess the levels of DR3 promoter methylation, investigate the correlation between DR3 promoter methylation and its expression, and develop a prognosis prediction model incorporating clinical indicators for acute-on-chronic hepatitis B liver failure (ACHBLF).

Methods: DR3 expression in peripheral blood mononuclear cells (PBMCs), as well as methylation levels, were detected using Methylight and quantitative polymerase chain reaction(qPCR) in a total of 362 patients and volunteers. Univariate, LASSO regression, and multifactorial analyses were performed to identify factors associated with 90-day outcomes in patients with ACHBLF. A clinical prediction model was constructed using DR3 promoter methylation levels and clinical parameters. Receiver Operating Characteristic Curve (ROC) was used to evaluate the model's discriminative ability. The Hosmer-Lemeshow (H-L) goodness-of-fit test and Decision Curve Analysis (DCA) were employed to assess the model's calibration and clinical practicability. The SHapley Additive explanations (SHAP) method was employed to interpret the top-performing model.

Results: The results showed that DR3 methylation levels were significantly lower in ACHBLF patients. Furthermore, non-survivors exhibited lower DR3 methylation levels than survivors and higher mRNA levels than survivors. A clinical model incorporating prothrombin activity (PTA), procalcitonin (PCT), and the percentage of methylation reference (PMR) value of DR3 was developed to predict ACHBLF prognosis. The model demonstrated good performance in predicting 3-month mortality. The goodness-of-fit test and DCA confirmed the model's robust calibration and clinical applicability.

Conclusion: Abnormal DR3 promoter methylation exists in patients with ACHBLF. The integration of PMR DR3, PTA, and PCT into a short-term prognostic model holds significant promise for clinical application in predicting ACHBLF outcomes.

Keywords: DR3, ACHBLF, methylation, prognosis, noninvasive model

Introduction

Acute-on-chronic liver failure (ACLF) is an acute decompensation of chronic liver disease accompanied by a complex syndrome characterized by intense systemic inflammation associated with proinflammatory induction and end-stage organ failure.[1,2] In Asian countries, the main cause of ACLF remains hepatitis B virus (HBV) infection.[3] The excessive immune response induced by HBV exacerbation is the main factor that drives the progression of chronic hepatitis B (CHB) and/or (liver cirrhosis) LC to ACLF and exacerbates the short- and medium-term mortality of patients.[4] In the past, the MELD score was widely used to assess the severity and predict mortality in ACHBLF. However, in recent years,

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an increasing number of studies have revealed significant limitations in the MELD score for predicting the prognosis of ACHBLF.[5,6] Currently, the SOFA score is commonly used to guide the prognosis of ACHBLF,[7] but this scoring system are less sensitive in assessing infection and inflammatory responses and may not accurately identify patients with early sepsis, which increases the difficulty of clinical application.[8] Therefore, it is very important to find accurate and sensitive non-invasive indicators, establish an effective clinical evaluation model to guide clinical management and reduce the high short-term mortality of ACHBLF patients.

Systemic inflammatory responses play a key role in the pathogenesis of ACLF, with the main pathophysiological processes resulting are changes in tissue homeostasis through inflammatory responses to pathogen or damage-related molecular patterns and systemic oxidative stress responses, and/or inflammation caused by the pathogen itself through tissue tolerance dysfunction Cytokines regulate the immune response and inflammation through a variety of signaling pathways.[9] DR3, also known as the death receptor 3 promoter, is an important member of the TNFR protein superfamily and is primarily expressed in lymphocytes.[10,11] DR3 can synergistically promote T cell activation by binding to its relevant ligands, activate NF-κB, and induce the production of apoptosis suppressor proteins (eg, c-IAP), thereby promoting the transcription and release of inflammatory factors and participating in the regulation of immune responses and the development of inflammatory diseases.[12,13] Studies have shown that the DR3 gene is associated with various diseases, such as liver cirrhosis, rheumatoid arthritis, colon cancer, and asthma.[14–17] Additionally, DR3 signaling promotes the secretion of GM-CSF, increases the infiltration of myeloid cells (eosinophils, macrophages, and neutrophils), and contributes to tissue inflammatory damage.[18] Therefore, the expression pattern and prognostic potential of DR3 in ACHBLF need to be further elucidated.

DNA methylation is one of the most important epigenetic regulatory mechanisms and is often associated with the long-term stabilization of transcriptional silencing and loss of gene function, which can regulate gene expression without altering the DNA sequence, thus significantly affecting the course of disease.[19,20] Therefore, it is considered an ideal biomarker for disease detection and prognostic prediction. Recent studies have revealed that DNA methylation has the potential to serve as a complementary biomarker in peripheral blood mononuclear cells (PBMCs) for cancer, and specific alterations in DNA methylation have been identified in peripheral immune cells from several diseases. In addition, we also found significant differences in DNA methylation profiles of PBMCs from patients with chronic hepatitis and HCC.[21,22]

In this study, we examined methylation levels of DR3 promoters in peripheral blood mononuclear cells (PBMC) of ACHBLF patients, CHB and HCs, and assessed differences in methylation expression at 1, 2, and 3 months. At the same time, we also detected the mRNA expression level of DR3 in PBMC. Finally, with 90 days as the end point, a prognostic model of ACHBLF based on DR3 promoter methylation was screened, constructed and validated, aiming to provide guidance for clinical treatment.

Materials and Methods

Study Population

We collected 362 study samples from four hospitals, including the Department of Hepatology, Qilu Hospital of Shandong University, between September 2019 and December 2023. These samples comprised 172 patients with ACHBLF, 94 patients with CHB, and 40 healthy volunteers. As shown in Figure 1, ACHBLF patients were randomly divided into derivative and validation groups on a 7:3 basis, with exclusion criteria including refusal to participate in the study, pregnant women and children, or other causes of chronic/acute liver disease. Based on the criteria proposed by the Asia Pacific Association for the Study of the Liver (APASL) Guidelines (updated in 2019), patients with ACHBLF were enrolled: ACLF is an acute hepatic insult manifesting as jaundice (serum bilirubin≥5 mg/dL (85 μ/L) and coagulopathy (INR≥1.5 or prothrombin activity < 40%) complicated within 4 weeks by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis, and is associated with a high 28-day mortality.[1]

All patients with ACHBLF were admitted for standard care and none of them received a liver transplantation in this study. All ACHBLF patients were followed up 1, 2, and 3 months after study initiation. Patients with CHB were defined as chronic necrosis of the liver caused by persistent hepatitis B virus (HBV) infection - inflammatory disease. This disease often leads to varying degrees of liver inflammation and necrosis, HBsAg positivity for at least 6 months. Healthy

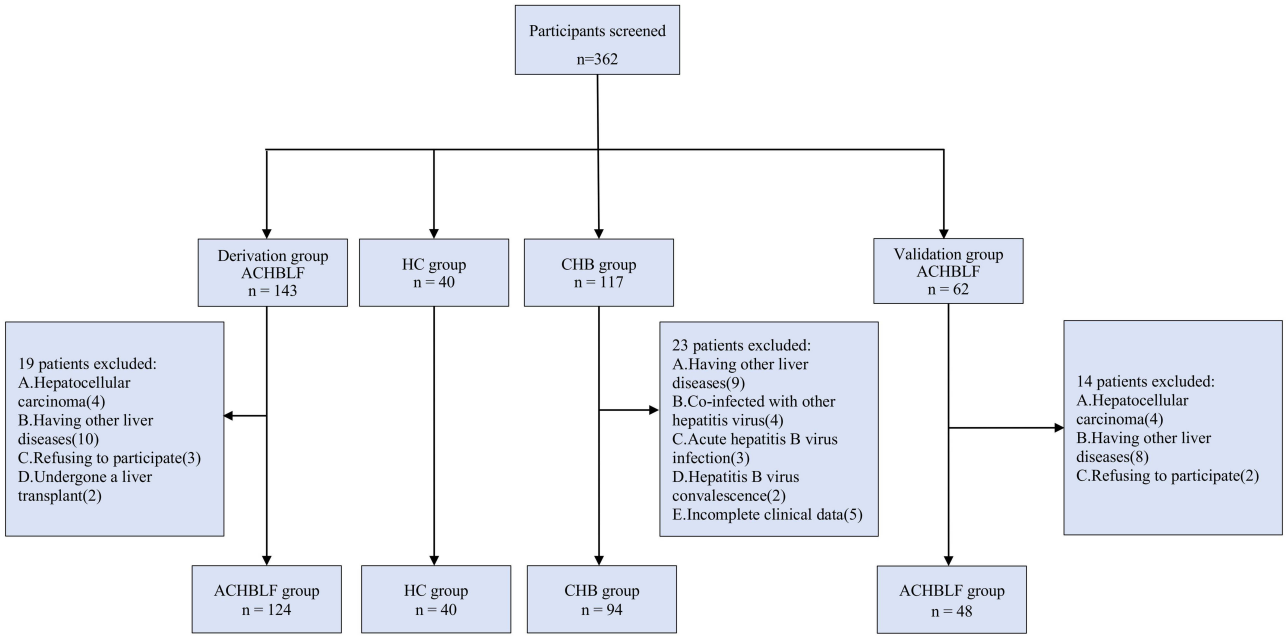

Figure 1 Flow diagram describing the participant selection process.

volunteers who had negative viral hepatitis tests, and no evidence of other liver or malignant disease served as normal controls. The observational end point was 90 days. Demographic, clinical, and laboratory data were recorded after enrollment. After admission, the patient's symptoms and signs are closely monitored, and regular examinations and return visits are made according to the patient's individual clinical condition. The experiment obtained the prior informed consent of all patients and was reviewed and approved by the Medical Ethics Committee of Shandong University Qilu Hospital (Ethics review number: 2021-S923).

Extraction of PBMCs and DNA Extraction

On the first day after diagnosis, 5mL of peripheral blood and plasma were collected from all subjects, Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifuge, and monocytes at the intermediate interface were collected and washed twice with PBS. DNA was extracted from PBMCs using TRIzol Reagent (Invitrogen). The extracted DNA was eluted in 50–100 μL of sodium hydroxide and the concentration was detected.

Methylation Fluorescence Detection Technology (MethyLight)

DNA bisulfate modification was performed using EZ DNA methylation gold kit (Zymo research, Orange, CA, USA). MethyLight was quantitatively detected using the EpiTect MethyLight PCR + ROX vial kit (included positive and negative controls) and two sets of primers and probes specifically designed for bisulfite converted DNA. The β-actin gene was used as a reference set for normalization. The sequences of specific primers and probes for DR3 and β-actin gene are listed in Table 1. Then the system was prepared and methylation-specific PCR was performed.[23] In addition, in order to ensure the repeatability of the experiment, we set up 3 biological replicates, and each sample was repeated for 3 times. The MethyLight results were calculated according to the following formula. The percentage methylation reference value (PMR) is MethyLight data.

PMR = 100% × 2 exp. [Delta Ct (target gene-control gene) Sample- Delta Ct (target gene-control gene) M.SssI-Reference][23]

Real-Time PCR

Total RNA was extracted by adding 1mL TRIzol to PBMCs, and the concentration was determined by PrimerScript™RT reagent Kit (Perfect Real Time; Beans, Japanese). The kit uses instructions to reverse transcribe RNA into cDNA. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was then performed. The primer pairs β-actin

Table 1 The Sequence of Prime and Probes

Gene	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
DR3 β-actin	TATTTTGTGTTTTTGGTCGTAGTAGGTA TGGTGATGGAGGAGGTTTAGTAAGT	CCCTCTACTCGACCTAAACCTAA AACCAATAAAACCTACTCCTCCCTTAAA
	Probe oligo sequence	
DR3 β-actin	ACGACAACGAACAAACCAAATAAAACACGCGAA ACCACCACCCAACACACAATAACAAACACA	

and DR3 were synthesized by Shanghai Sangon Biotech and the sequences are listed in Table 1. These results were determined using the comparative (2-ΔCt, ΔCt = Ct(target)-Ct(β-actin)) method. Plasma cytokine levels were determined using ELISA (enzyme-linked immunosorbent assay), a competitive method for measuring sample content. Measure absorbance at 450nm and calculate according to the standard curve of the manual.

Predictor Selection and Prediction Model Development and Validation

To determine the predictors used in the predictive model, we first screened the optimal predictors from the clinical parameters of the training cohort through single-factor logistic regression analysis and LASSO regression analysis, using the "glmnet" software package.[24] Then, based on the predictors selected by LASSO regression analysis, multivariate logistic regression analysis was performed to determine the final predictors included in the model. Based on multivariate logistic regression analysis, the clinical prediction model was established. To verify and evaluate the clinical accuracy and applicability of the model, we calculated the area under the curve (AUC) by analyzing the receiver operating characteristic curve (ROC curve). In addition, Hosmer-Lemeshow (H-L) goodness of fit test was used to evaluate the calibration of the model, and decision curve analysis (DCA) was used to evaluate the clinical utility of the model. Finally, the Shapley Additive explanations (SHAP) algorithm provides consistent and locally accurate values for each variable in the best-performing predictive model, further deepening our understanding of model performance.

Statistical Analysis

SPSS 29.0 and R (version 4.1) software were used for statistical analysis of the data, and GraphPad Prism 10 was used to date visualization. $P<0.05$ (two-sided) was considered to indicate statistical significance. Quantitative variables are reported as median, and categorical variables are reported in numbers (proportions). The comparison of categorical variables was conducted using Student's *t*-test, Mann–Whitney *U*-test and Kruskal–Wallis *H*-test.

Result

Clinical Baseline Characteristics Of patients with ACHBLF

In our study, in all 172 ACHBLF patients were enrolled and we used randomization as a simple and straightforward internal cross-validation method, assigned to the training (n=124) or validation (n=48) cohort in a ratio of 7:3. The flowchart for inclusion is shown in Figure 1. Comparison of clinical data between the training cohort and the validation cohort for gender, age, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), prothrombin time (PT), platelet (PLT), international normalized ratio (INR), prothrombin time activity (PTA), creatinine (Cr), alpha-fetoprotein (AFP), log10[HBV-DNA], ammonia, hepatic encephalopathy (HE), white blood cell (WBC) count, procalcitonin (PCT), ascites, bacterial infections, smoking history, drinking history, MELDs, PMR and 3-month mortality. As shown in Table 2, there is no significant difference. (All $P>0.05$).

The Expression Level of DR3 Methylation in All Groups and Follow-up Periods Was Different and Correlated with Clinicopathological Features

Methylation of the PMR values of DR3 promoter in all groups and three follow-up periods of participants is presented in Figure 2. DR3 methylation levels were significantly lower in patients with ACHBLF (median 56.13%, interquartile spacing

Table 2 The Clinical Characteristics of ACHBLF Patients in the Derivation Cohort and Validation Cohort

Variables	Derivation Cohort	Validation Cohort	P value
N	124	48	
Male (%)	84 (67.7)	37 (77.1)	0.229
Age (years)	50.00 (40.00–59.25)	49.00 (43.0–56.00)	0.713
ALT (U/L)	91.50 (36.75–234.50)	95.00 (43.50–299.25)	0.534
AST (U/L)	96.00 (60.50–169.50)	110.00 (56.50–240.50)	0.539
TBIL (μM)	235.55 (159.05–316.85)	238.55 (147.15–397.20)	0.488
ALB (g/L)	32.70 (29.95–35.62)	31.95 (29.73–35.32)	0.558
PLT (10^9/L)	101.50 (63.00–139.00)	81.00 (52.00–148.25)	0.255
PT (s)	20.55 (18.60–26.88)	24.10 (19.67–28.42)	0.066
INR	1.79 (1.60–2.31)	2.04 (1.71–2.41)	0.097
PTA (%)	41.00 (31.00–48.00)	36.50 (28.75–45.00)	0.056
Cr (μM)	55.00 (42.50–65.00)	60.00 (47.00–69.25)	0.212
AFP	28.60 (6.64–135.85)	14.03 (4.70–59.63)	0.079
Log10[HBV-DNA]	4.18 (3.24–5.31)	3.77 (3.33–5.82)	0.834
Ammonia (μM)	56.00 (46.00–79.00)	59.50 (48.00–92.00)	0.326
WBC (10^9/L)	7.62 (5.31–11.01)	7.46 (5.34–9.98)	0.803
PCT (pg/mL)	0.50 (0.31–0.96)	0.47 (0.29–0.98)	0.887
Ascites (%)	77 (62.1)	33 (68.8)	0.415
HE (%)	26 (21.0)	12 (25.0)	0.567
Bacterial Infections (%)	70 (56.5)	27 (56.2)	0.981
Smoking history (%)	42 (33.9)	23 (47.9)	0.088
Drinking history (%)	53 (42.7)	28 (58.3)	0.066
MELDs	18.09 (15.59–22.85)	20.42 (16.43–23.90)	0.094
PMR DR3	56.87 (48.42–63.62)	55.24 (49.59–60.24)	0.31
Mortality, n (%)	54 (43.5)	27 (56.2)	0.134

Notes: Quantitative variables were expressed as medians (25th, 75th percentage). Categorical variables were expressed as number (%).

48.39–63.27%) than in CHB (median 60.61%, interquartile spacing 56.34–68.05%; P<0.0001) and the DR3 methylation level in CHB patients was significantly lower than that in HCs (median 74.44%, interquartile spacing 65.93–82.27%; P<0.0001). (Figure 2A). After 3-month of follow-up, 81 of 172 patients with ACHBLF had died. At the 28-day, 2-month, and 3-month follow-ups, the 1-month mortality rate of ACHBLF was 33.72% (58/172). Survivors (median 60.22%, interquartile range 55.32–66.12%) had significantly higher DR3 methylation levels than non-survivors (median 46.21%, interquartile range 39.20–51.83; P<0.0001) (Figure 2B). The 60-day mortality rate of ACHBLF was 43.60% (75/172). Non-survivors (median 48.22%, interquartile range 41.32–54.86%) had significantly lower DR3 methylation levels than survivors (median 60.76%, interquartile range 55.59–66.79%; P<0.0001) (Figure 2C). Finally, the 90-day mortality of ACHBLF was 47.09% (81/172). Non-survivors (median 48.78%, interquartile range 41.46–56.04%) had significantly lower DR3 methylation levels than survivors (median 61.32%, interquartile range 55.68–67.08%; P<0.0001) (Figure 2D). Relationship between DR3 promoter methylation status and clinicopathological features in ACHBLF patients.

Figure 2E shows that DR3 methylation level and international INR (Spearman's r=−0.35, P<0.001), PTA (Spearman's r =0.28, P=0.002), TBIL (Spearman's r=−0.48, P<0.001), Cr (Spearman's r=−0.19, P=0.01) and PCT (Spearman's r=−0.61, P<0.001). There was no significant correlation between DR3 methylation level and other clinical biochemical indexes (P<0.05).

The Expression of DR3 mRNA in PBMCs of ACHBLF Patients and Correlation with Promoter Methylation Status

DR3 mRNA levels were compared between 90-day survivors (n=91) and non-survivors (n=81) of ACHBLF patients. As shown in Figure 3A, the results showed that DR3 mRNA levels were significantly higher in non-survivors than in survivors

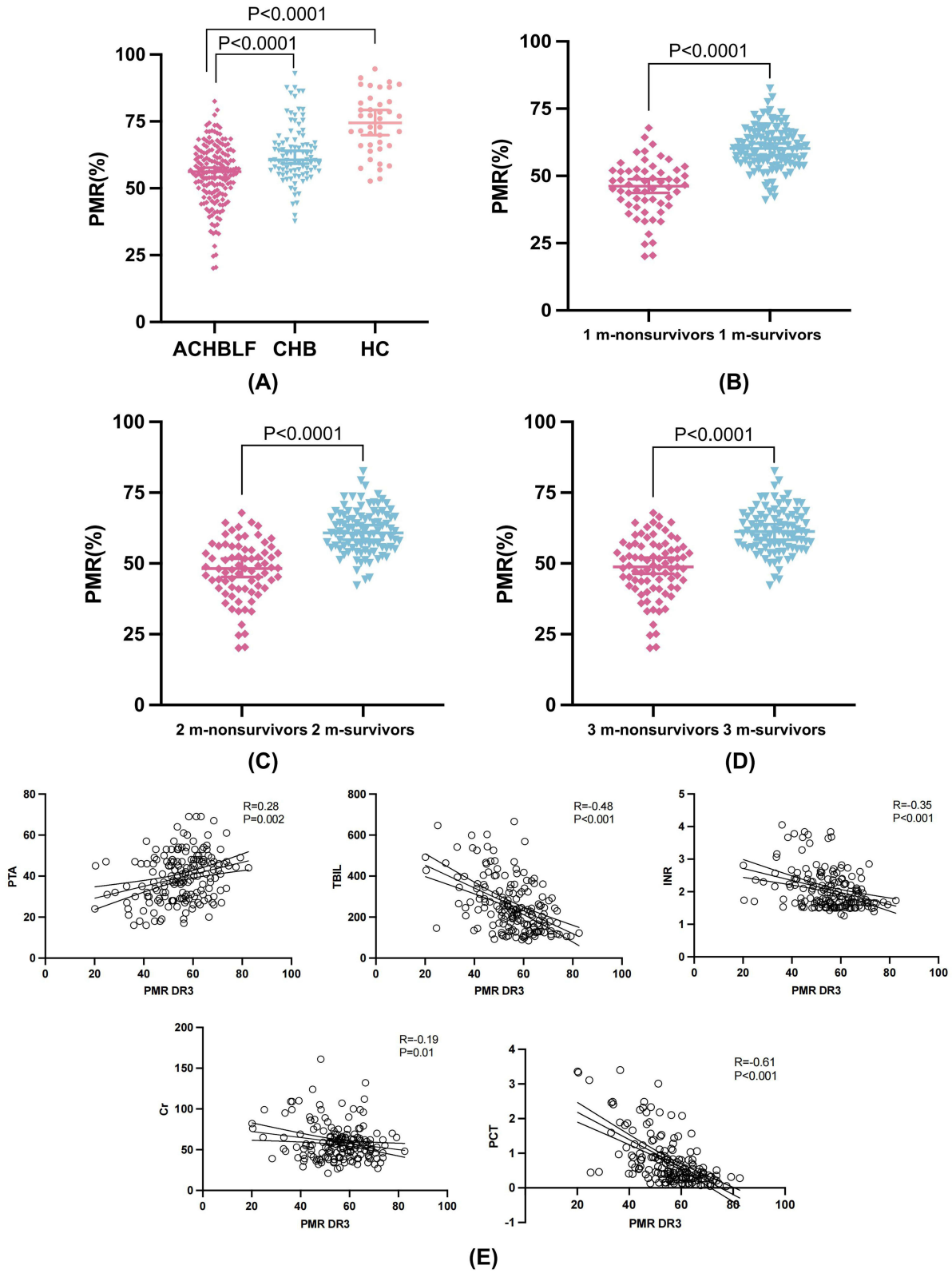

Figure 2 The DR3 methylation levels in all groups and different follow-up periods. **(A)**PMR values of DR3 promoter in PBMC of ACHBLF, CHB and HC group. (P<0.0001). **(B)**PMR values of DR3 promoter were significantly higher in survivors than non-survivors at the end of 1-month follow-up. (P<0.0001). **(C)**PMR values of DR3 promoter were significantly higher in survivors than non-survivors at the end of 2-month follow-up. (P<0.0001). **(D)**PMR values of DR3 promoter were significantly higher in survivors than non-survivors at the end of 3-month follow-up. (P<0.0001). **(E)**DR3 methylation is associated with clinicopathological features.

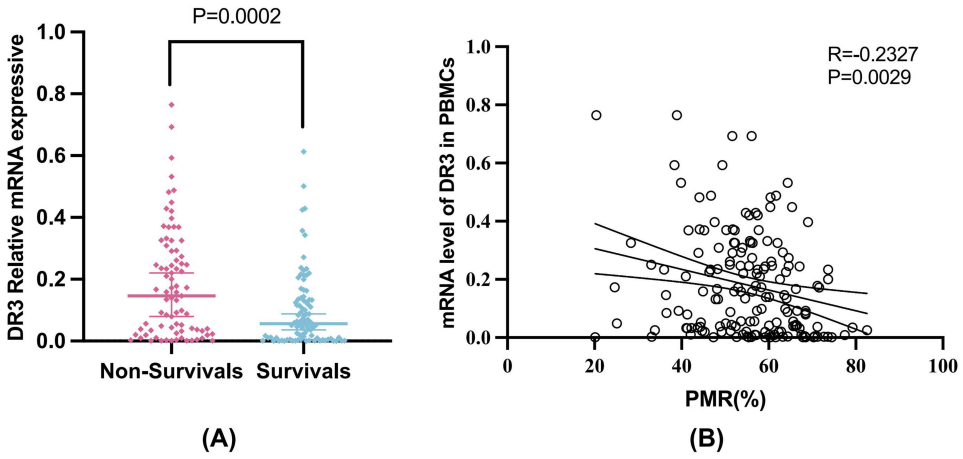

Figure 3 The expression level of DR3 methylation in all groups and follow-up periods was different and correlated with clinicopathological features. **(A)**The expression level of DR3 was detected by RT-PCR. (P=0.0002). **(B)**The methylation level of TL1A promoter of PBMCs in ACHBLF patients was significantly negatively correlated with mRNA expression levels. (Spearman's r=−0.2327, P=0.0029).

(P=0.0002). In addition, correlation analysis showed that DR3 mRNA expression in PBMCs of ACHBLF patients was significantly negatively correlated with its promoter methylation level (Spearman's r=−0.2327, P=0.0029) (Figure 3B).

Baseline Characteristics of Patients with Different Outcomes Were Trained and Validated in the Cohort

According to the 90-day prognosis, ACHBLF patients in the training group (n=124) and validation group (n=48) were divided into survival group (n=70,21) and non-survival group (54,27), as shown in Table 3. In the training cohort, the scores of TBIL, PTA, INR, WBC, PCT, bacterial infection, HE and MELDs in the non-survival group were significantly higher those in the survivors than in survivors (P<0.05). In addition, PLT, PMR (DR3) and PTA of non-survivors were significantly lower than those in survivors (P=0.009, P<0.001, 0.001). In the validation cohort, the performance of TBIL, blood ammonia, Cr, PCT, PMR (DR3), and MELDs in non-survivors was consistent with that in the training cohort. (P<0.05).

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

Variables	Derivation Cohort			Validation Cohort		
	Survival	Non-Survival	*P*	Survival	Non-Survival	*P*
N	70	54		21	27	
Male (%)	46 (65.7)	38 (70.4)	0.582	16 (76.2)	21 (77.8)	0.897
Age (years)	49.50 (40.00–55.75)	51.00 (44.00–60.00)	0.287	48.00 (36.00–56.00)	50.00 (48.00–56.00)	0.142
ALT (U/L)	93.00 (32.25–216.75)	86.00 (38.00–241.50)	0.689	95.00 (36.00–363.00)	136.00 (47.50–253.50)	0.486
AST (U/L)	96.00 (55.75–179.75)	92.50 (64.00–163.00)	0.63	89.00 (61.00–169.00)	141.00 (49.50–263.00)	0.603
TBIL (μM)	204.25 (130.20–261.88)	264.10 (205.28–361.97)	0.001	214.10 (137.40–245.40)	322.90 (168.75–473.45)	0.033
ALB (g/L)	33.20 (29.85–35.77)	32.10 (30.20–35.30)	0.681	33.70 (29.80–39.00)	31.50 (29.75–33.20)	0.117
PLT (10^9/L)	108.00 (67.75–161.75)	78.50 (50.00–118.00)	0.009	81.00 (67.00–174.00)	78.00 (48.50–103.00)	0.197
PT (s)	19.15 (17.42–21.03)	26.35 (21.90–31.28)	<0.001	22.50 (19.60–25.50)	26.90 (19.90–30.65)	0.067
INR	1.66 (1.51–1.86)	2.29 (1.78–2.73)	<0.001	1.97 (1.69–2.20)	2.21 (1.74–2.46)	0.149
PTA (%)	47.00 (41.00–53.00)	31.50 (26.00–37.75)	<0.001	38.00 (31.00–46.00)	31.00 (28.00–44.20)	0.061
Cr (μM)	53.00 (43.25–62.00)	56.00 (41.50–76.75)	0.203	54.00 (41.00–63.00)	65.00 (48.50–87.50)	0.047
AFP	31.52 (6.12–124.82)	26.35 (7.23–136.64)	0.811	14.13 (4.90–188.20)	9.25 (3.68–37.08)	0.216
Log10[HBV-DNA]	3.93 (3.21–4.84)	4.37 (3.26–5.38)	0.195	3.58 (3.34–5.82)	4.01 (3.37–5.82)	0.685
Ammonia (μM)	53.50 (45.00–73.00)	61.00 (49.50–84.75)	0.132	55.00 (47.00–69.00)	77.00 (50.00–97.00)	0.027
WBC (10^9/L)	6.47 (4.58–10.12)	8.76 (6.48–11.530	0.014	6.72 (5.30–9.54)	7.79 (5.72–11.98)	0.377

(*Continued*)

Table 3 (Continued).

Variables	Derivation Cohort			Validation Cohort		
	Survival	Non-Survival	*P*	Survival	Non-Survival	*P*
PCT (pg/mL)	0.36 (0.24–0.55)	0.90 (0.55–1.64)	<0.001	0.30 (0.25–0.49)	0.81 (0.45–1.73)	0.001
Ascites (%)	42 (60.0)	35 (64.8)	0.584	14 (66.7)	19 (70.4)	0.784
HE (%)	7 (10.0)	19 (35.2)	0.001	3 (14.3)	9 (33.3)	0.131
Bacterial Infections (%)	34 (48.6)	36 (66.7)	0.044	12 (57.1)	15 (55.6)	0.912
Smoking history (%)	28 (35.7)	17 (31.5)	0.621	10 (47.9)	13 (48.1)	0.971
Drinking history (%)	30 (42.9)	23 (42.6)	0.976	12 (57.1)	16 (59.3)	0.883
MELDs	16.52 (14.49–18.23)	22.38 (18.25–25.06)	<0.001	17.32 (14.80–20.44)	22.33 (19.88–26.8)	0.006
PMR DR3	61.88 (56.44–67.41)	48.30 (41.39–55.62)	<0.001	58.29 (55.47–66.27)	51.10 (45.25–56.54)	<0.001

Notes: Quantitative variables were expressed as medians (25th, 75th percentage). Categorical variables were expressed as number (%).

Screening of Prognostic Risk Factors in Patients with ACHBLF

Univariate logistic regression analysis was performed for clinical characteristic variables included in the training cohort to identify factors associated with 90-day outcomes in ACHBLF patients. As shown in Table 4, 11 possible factors were selected as prognostic predictors through preliminary analysis: PLT, HE, TBIL, Cr, PTA, PT-INR, PCT, MELDs, bacterial Infection, and PMR (DR3) (P<0.05). These 11 factors were then incorporated into the LASSO regression for 10-fold cross-validation (Figure 4A and B). The three variables of PTA, PCT and PMR (DR3) are optimal. 1se = 0.065. Multivariate logistic regression analysis was performed on these three variables, and the P-values were all less than 0.05 (Table 4).

Table 4 Based on Univariate and Multivariate Logistic Regression Analysis of Potential Predictors of Prognosis in ACHBLF Patients

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*p*	OR (95% CI)	*p*
Male	1.239(0.577,2.662)	0.583		
Age	1.020(0.990,1.050)	0.193		
ALT	1.000(1.000,1.001)	0.467		
AST	1.000(0.999,1.002)	0.631		
TBIL	1.005(1.002,1.009)	0.002		
ALB	1.001(0.927,1.082)	0.975		
PLT	0.994(0.988,1.000)	0.039		
PT	1.296(1.174,1.432)	0.000		
INR	15.081(5.133,44.303)	0.000		
PTA	0.846(0.800,0.896)	0.000	0.843(0.782,0.908)	0.000
Cr	1.019(1.000,1.037)	0.045		
AFP	1.000(1.000,1.000)	0.512		
Log10[HBV-DNA]	1.223(0.957,1.561)	0.107		
Ammonia	1.009(0.998,1.020)	0.123		
WBC	1.058(0.984,1.137)	0.130		
PCT	10.133(3.730,27.528)	0.000	4.350(1.212,15.616)	0.024
Ascites	1.228(0.589,2.562)	0.584		
HE	4.886(1.871,12.761)	0.001		
Bacterial Infections	2.118(1.016,4.415)	0.045		
Smoking history	0.827(0.389,1.758)	0.622		
Drinking history	0.989(0.483,2.028)	0.976		
MELDs	1.333(1.193,1.489)	0.000		
PMR DR3	0.838(0.788,0.893)	0.000	0.864(0.792,0.943)	0.001

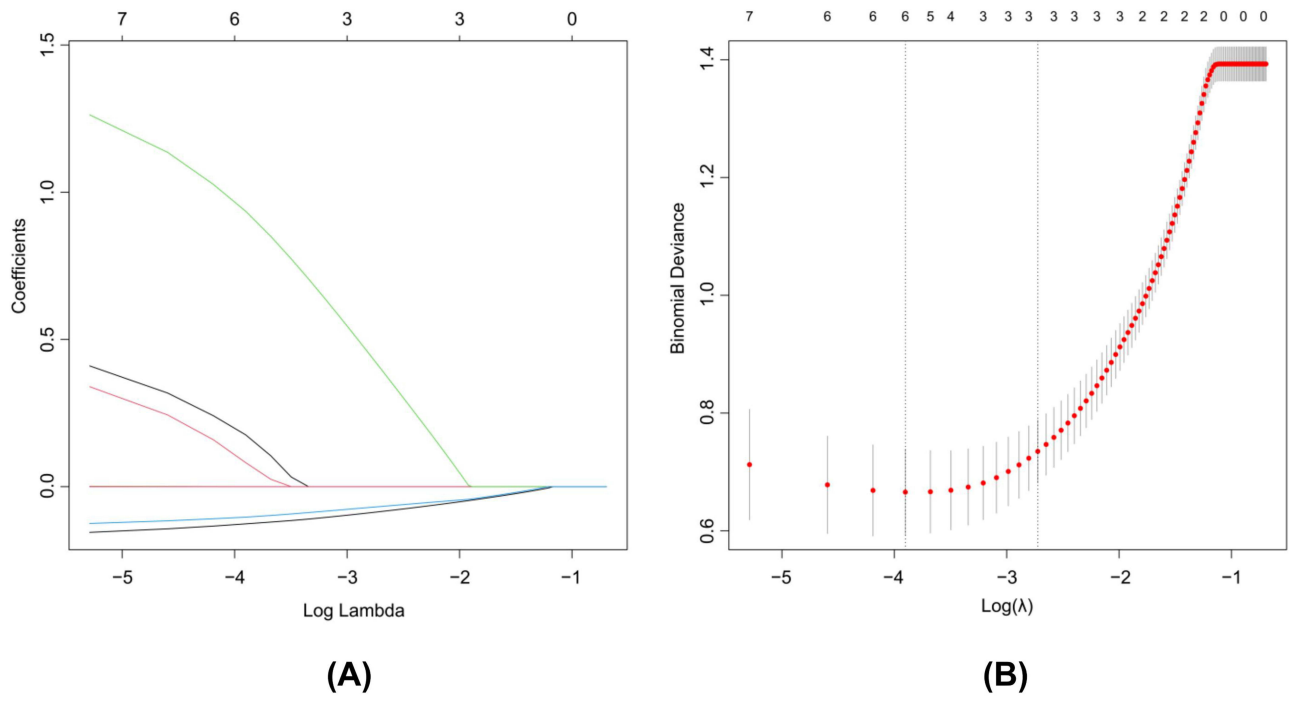

Figure 4 Screening of variables based on Lasso regression. **(A)**The variation characteristics of the coefficient of variables. **(B)**The selection process of the optimum value of the parameter λ in the Lasso regression model by cross-validation method.

Establishment and Evaluation of Short-Term Prediction Model for ACHBLF Patients

Finally, based on the results of multivariate logistic regression analysis, PTA, PCT and PMR (DR3) were used to establish a clinical model to predict the 90-day prognosis of patients with ACHBLF. The formula is as follows:

$$Logit(P)=8.672–0.17\times PTA\ (\%)\ +1.470\times PCT\ (pg/mL)-0.146\times PMR\ (\%),$$

where P is the patient's death risk probability. As shown in Figure 5, the ROC curve was drawn to evaluate the discriminability of the model. In the training cohort, the model had an AUC of 0.859 (95% CI: 0.7955–0.923), and the validation group model had an AUC of 0.832 (95% CI: 0.721–0.944) (Figure 5A and B). The model is stable at around 0.83. When the AUC index or ratio of validation set and test set performance is greater than 0.8, and the difference is less than 0.1, it indicates that the overall effect in the model is good, reaching the practical level, and the model fitting can be considered successful. The maximum value of Jordan index was 0.669, the sensitivity was 0.79, and the specificity was 0.879. The corresponding optimal critical value is 0.5386. When the optimal cut-off value was 0.5386, the mortality rate of ACHBLF patients with model score greater than or equal to 0.5386 in the training cohort was 83.3%, and the mortality rate of ACHBLF patients with model score less than 0.5386 was 6.04% (Figure 5C). In the validation cohort, the mortality rate of ACHBLF patients with a score greater than or equal to 0.5386 was 70.37%, and that of ACHBLF patients with a score less than 0.326 was 16% (Figure 5C).The LR models performed better than the SVM (AUC: 0.822; 95% CI: 0.746–0.923) and the MELDs (AUC: 0.756; 95% CI: 0.666–0.847) in the training set, In the validation set, LR model (AUC: 0.832; 95% CI: 0.721–0.944) was better than support vector machine (AUC: 0.820; 95% CI: 0.704–0.936) and MELDs (AUC: 0.735; 95% CI: 0.586–0.885), consistent with the performance of the training group. These results show that logistic regression models can be used for classification modeling of data sets.

The AUC indicator focuses on evaluating the model's differentiation, ie prediction accuracy and does not indicate whether the model is clinically usable.[3] Therefore, we wanted to further evaluate the clinical significance of the model for predicting adverse events, and analyzed the calibration curves through a decision curve analysis (DCA). The calibration chart verifies that there is a good prediction accuracy between the actual probability and the predicted probability in the

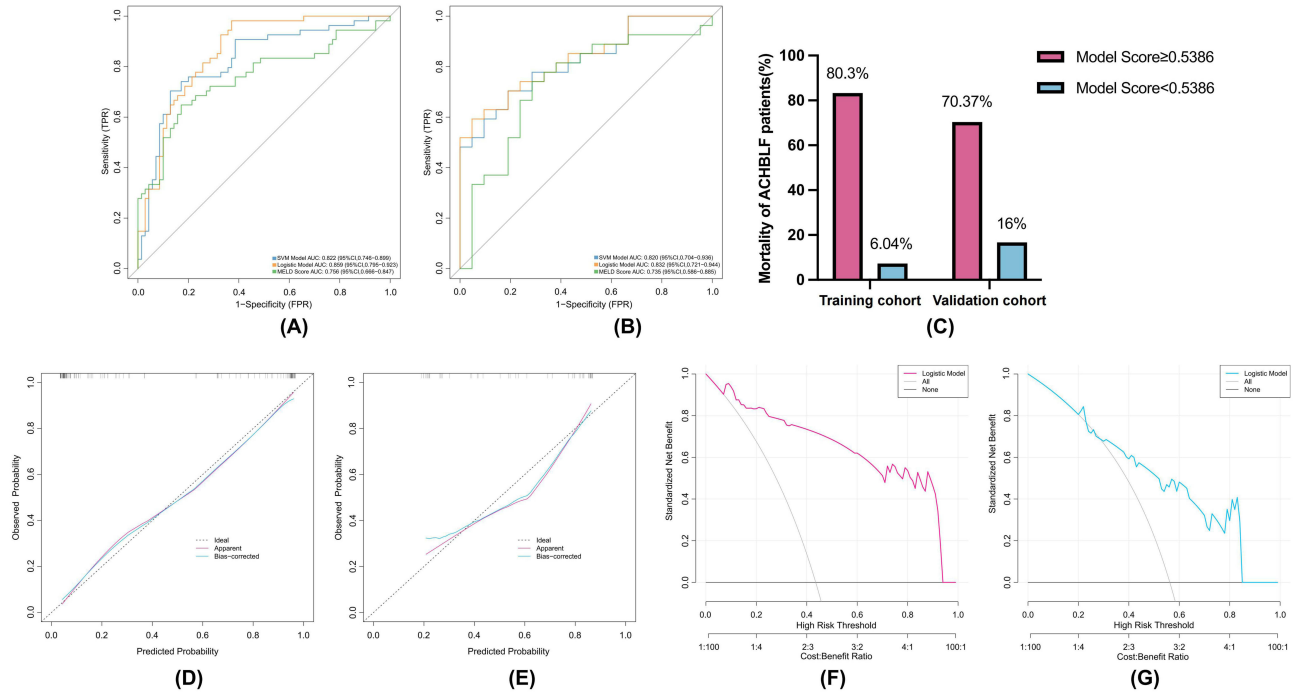

Figure 5 Training, validation and testing of ML models. (**A** and **B**)Trained and validated subject working characteristic curves of ACHBLF patient prediction models in the cohort. **(C)**Mortality of ACHBLF patients in the training and validation groups distinguished by model score cutoff values. (**D** and **E**)Calibration curves for training and validating predictors in the cohort. The X-axis represents the probability of prediction and the Y-axis represents the probability of observation. The dashed line represents the perfect prediction of the ideal model, and the solid line represents the calibration diagram of the predicted model. (**F** and **G**)DCA of predictive models in training and validation queues. The horizontal coordinate of DCA decision curve analysis is the threshold value and the vertical coordinate is the net benefit. The red and blue lines represent the net benefit to patients when the model is used in a clinical intervention.

derivation and validation cohorts (Figure 5D–G). The results showed that the thresholds values in the training cohort were within 0.08–0.99, and the investigatory values in the validation cohort were within 0.21–0.99. All patients could obtain clinical benefits from predicting the occurrence of adverse events and receiving clinical intervention. In both the training and validation cohort, LR showed a higher net benefit. H-L goodness of fit test was used to evaluate the calibration degree of the model, that is, the consistency between the model prediction and the actual probability of occurrence, and a correction curve was drawn to visualize the results. The results show that the predicted probability of the model is in good agreement with the actual probability.

Visualization of Prognostic Factors Associated with ACHBLF

The SHAP summary plot (Figure 6A and B) and dependency plot (Figure 6C–H) depict the contributions of the six predictors in the LR model after Lasso screening. A SHAP value above zero indicates an increased risk of death within 90 days, while a value below zero indicates a reduced risk. Among them, PCT, PMR DR3 and PTA became the three most influential variables in predicting ability. Specifically, higher PCT score, lower PMR DR3 score, and an elevated PTA resulted in a greater likelihood of death.

Discussion

This study is the first to demonstrate that the methylation levels of DR3 in PBMCs of ACHBLF patients are significantly lower than those in CHB patients and HCs. Additionally, within the ACHBLF group, the DR3 methylation levels were notably reduced in non-survivors at 1, 2, and 3 months, accompanied by increased DR3 mRNA expression levels, which showed a significant correlation. This may be attributed to the fact that DNA methylation can regulate mRNA expression levels by influencing gene transcriptional activity. These findings suggest that DR3 methylation levels may be a potential prognostic marker for ACHBLF. Furthermore, we observed that the following predictors were associated with prognosis: PCT, PTA, and PMR DR3. In this study, ROC curve analysis was used to evaluate the model's predictive performance for

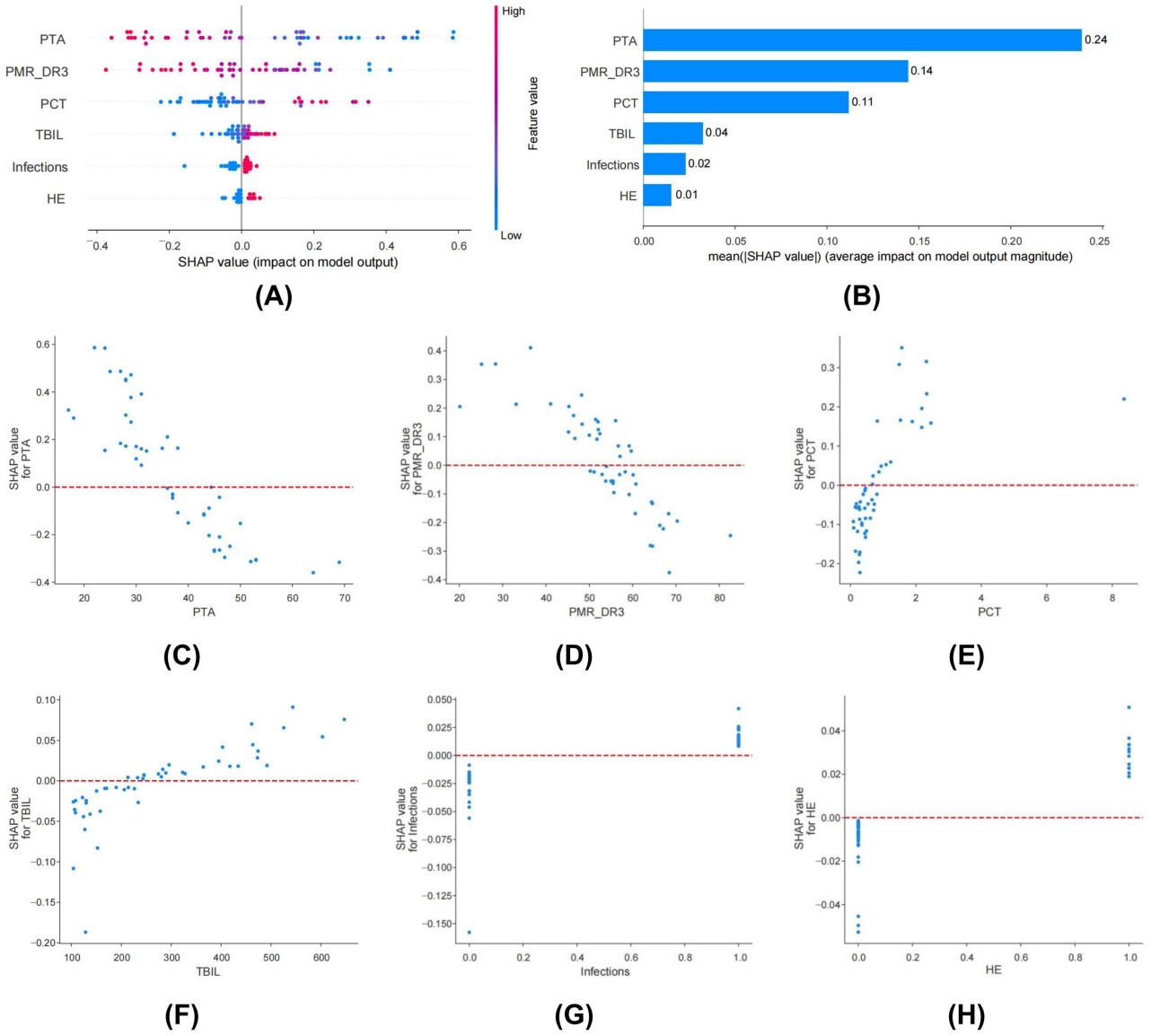

Figure 6 SHAP-based interpretation for the LR model. **(A)**The Bees-warm diagram describes the effects of six features on all model samples. **(B)**The features are sorted according to the absolute average of the Shapley values. **(C-H)**SHAP correlates show the relationship between predicted risks and characteristic values PCT, PMR DR3, PTA, TBIL, Infections, and PTA.

ACHBLF prognosis, which significantly outperformed the MELD score. As demonstrated by DCA, the model exhibited high predictive performance in both the training and validation cohorts. Finally, the SHAP summary plot revealed that higher PCT score, lower PMR DR3 score, and higher PTA were associated with a greater likelihood of death.

The proinflammatory state of local liver inflammation and systemic inflammatory response syndrome (SIRS) are important causes of end-stage death in patients with ACHBLF.[25] PCT is key to early recognition of severe infections, sepsis and multiple organ failure, reflecting the severity of systemic inflammatory responses.[26] Multiple studies have shown that high serum PCT levels are an independent risk factor for death in patients with ACHBLF, which is consistent with our findings.[27] Therefore, PCT plays an important role in the development of inflammation in patients with ACHBLF.

Previous studies have found that DR3 is involved in recruiting caspase-8 to induce cell apoptosis, which exacerbates tissue damage and inflammation.[28] It also binds with related ligands to activate JAK-STAT and other inflammatory signaling pathways to co-regulate inflammatory response.[18] And in diseases such as inflammatory bowel disease, DR3 has been shown to be highly expressed on non-immune cells (IECs and fibroblasts), promoting endothelial cell adhesion and exacerbating

inflammatory responses.[29] DNA methylation, a type of epigenetic modification, has been widely used as a biomarker for diagnosis and prognosis of disease.[30,31] DR3 is involved in the occurrence and development of RA, PBC and other inflammatory diseases, and its expression is significantly increased in liver cancer cells.[32] Therefore, in order to explore its expression in ACHBLF patients and determine its prognostic value in ACHBLF patients by detecting methylation levels. We examined the expression levels of DR3 mRNA and methylation in ACHBLF patients, and found that it showed high expression and low methylation in the non-survival group. In addition, its evaluation efficiency is better than that of MELD score. Compared with MELD score, the change of MELD score is more significant in the case of severe liver damage, and DNA methylation can often be detected at an early stage.[33] These results suggest that DR3 may be involved in the occurrence of ACHBLF, the pathophysiological mechanism related to prognosis and the degree of inflammation. Besides suggest that DR3 methylation of PBMCs can be used as a biomarker to evaluate the prognosis of ACHBLF, which provides a new non-invasive detection method for predicting the prognosis of liver failure.

At present, biomarkers and models for predicting the prognosis of patients with ACHBLF have been reported. For example, Wang et al established predictive outcomes for liver transplantation in patients with ACHBLF.[34] Yang et al constructed a 3-month prognosis prediction for ACHBLF patients based on five factors: age, TBIL, PTA, lymphocyte% and monocyte%.[35] These studies built models to predict the prognosis of ACHBLF based on laboratory indicators and paid little attention to the acute inflammatory response status of patients. Previous studies have reported that peripheral blood specific gene methylation in patients with ACHBLF is related to prognosis, which has certain predictive value,[27,36] but no prognosis prediction model has been established that can be applied in clinic.

In this study, we examined DR3 promoter methylation in ACHBLF patients for the first time. Through quantitative detection and analysis, based on DR3 promoter methylation, we constructed a model to predict the prognosis of ACHBLF. We are considered the characteristics of convenience, low trauma and high feasibility, the prediction model was constructed based on the analysis and screening of clinical characteristics of ACHBLF patients and DR3 promoter methylation in PBMCs. Three easily accessible clinical variables were used to rapidly predict ACHBLF prognosis. In addition, it showed superior clinical utility and showed a high net gain in decision curve analysis, evidence that underscores the potential of the LR model for use in clinical care to aid decision making and allocate resources more efficiently.

Notably, this study has several limitations and inadequacies and is subject to further improvement. First of all, the sample size of this study still needs to be further expanded, the lack of clinical data may slightly affect the results of the analysis, and the small number of patients may slightly affect the performance of machine learning techniques. Therefore, the predictive power of the constructed model DR3 promoter methylation levels should be tested in a larger context. In addition, the possibility of false-positive clinicopathological features should not be ignored. Finally, further analysis of the molecular mechanism of DR3 in ACHBLF is necessary.

In summary, we found that DR3 promoter methylation levels are hypomethylated in non-surviving ACHBLF patients, and its methylation level may be a potential prognostic marker for ACHBLF. We also found that both DR3 mRNA expression levels were elevated in patients with ACHBLF, which may influence the prognosis of patients with ACHBLF through pro-inflammatory responses. In addition, LR models based on DR3 methylation levels were developed and validated to predict 3-month risk of death in ACHBLF patients. The identified predictors, PCT, PTA, and PMR DR3, can guide clinical decision making and resource allocation for ACHBLF patient management. The formula of the model is

Logit (P) = 8.672–0.17 x PTA (%) + 1.470 x to 0.146 x PMR PCT (pg/mL) (%).

The study further highlights that DR3 methylation level may be an important diagnostic biomarker indicating the prognosis of ACHBLF, however, the pathogenesis of DR3 promoter methylation in ACHBLF and its use as a clinical therapeutic target need to be further studied.

Data Sharing Statement

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

Ethics Approval

The experiment obtained the prior informed consent of all patients and was reviewed and approved by the Medical Ethics Committee of Shandong University Qilu Hospital (Ethics review number: 2021-S923). The study was performed in accordance with the 1964 Declaration of Helsinki and later amendments.

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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.

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Disclosure

The authors declare no conflicts of interest in this work.

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