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ORIGINAL ARTICLE

Hypomethylation of thymosin $\beta 4$ promoter is associated with glucocorticoid therapy in patients with acute-on-chronic hepatitis B-induced liver failure

He Wang[a,b], Yu Qian[a], Jing-Wen Wang[a], Yu Fang[a], Yu-Chen Fan[a,c], Hui-Hui Liu[a] and Kai Wang[a,c,*]

[a]*Department of Hepatology, Qilu Hospital of Shandong University, Jinan 250012, China;* [b]*Department of Hepatology, Qingdao Sixth People's Hospital, Qingdao 266000, China;* [c]*Institute of Hepatology, Shandong University, Jinan 250012, China*

*Corresponding author: Tel: +86-531-82169593; Fax: +86-531-86927544; E-mail: wangdoc876@126.com; wangdoc2010@163.com

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Background: We aimed to determine whether the methylation status of thymosin $\beta 4$ (T$\beta 4$) promoter reflects the severity of acute-on-chronic hepatitis B liver failure (ACHBLF) and whether glucocorticoids affect this status.

Methods: Fifty-six patients with ACHBLF, 45 with chronic hepatitis B (CHB) and 32 healthy controls (HCs), were retrospectively enrolled. Methylation-specific PCR and real-time PCR were used to detect T$\beta 4$ methylation frequency and mRNA level. The expression of T$\beta 4$ was measured before and after glucocorticoid treatment in patients with ACHBLF. Clinical and laboratory parameters were obtained.

Results: T$\beta 4$ mRNA expression of patients with ACHBLF was lower than in patients with CHB or HCs, but the methylation frequency was higher. T$\beta 4$ promoter methylation frequency was correlated with serum total bilirubin, prothrombin activity and model for end-stage liver disease score. Moreover, T$\beta 4$ promoter methylation frequency decreased and demethylation occurred during glucocorticoid therapy. After glucocorticoid therapy, T$\beta 4$ mRNA expression and liver function were better in patients with low levels of methylation than in those with higher levels. After 90 d, the survival of patients with low levels of methylation was significantly higher than those with high levels.

Conclusions: Patients with ACHBLF who have low levels of T$\beta 4$ methylation may show a more favorable response to glucocorticoid treatment.

Keywords: acute-on-chronic hepatitis B liver failure, glucocorticoid therapy, methylation, thymosin $\beta 4$ promoter.

Introduction

Acute-on-chronic hepatitis B liver failure (ACHBLF) is a clinical syndrome that is characterized by acute, rapid deterioration of liver function in patients with chronic liver disease associated with hepatitis B virus (HBV) infection. HBV-induced liver failure is an immune-mediated process that is caused by a variety of cells and inflammatory molecules.[1] ACHBLF progresses rapidly and has a high mortality rate of 50 to 90%.[2–4] It has been reported that the prognosis of ACHBLF is poor, and its 3-mo mortality rate is >50% if liver transplantation (LT) is not performed.[5] Therefore, it is essential to develop more effective therapies for liver failure. LT is recognized as the primary rescue therapy, but organ shortages limit its applicability.[6] At present, many fundamental and clinical studies are exploring the feasibility of using glucocorticoids for the therapy of liver failure.[7] Glucocorticoid therapy is recommended as a first-line treatment strategy for severe alcoholic hepatitis and hepatic encephalopathy,[8] and it is attracting attention as a possible therapy for ACHBLF because of its rapid beneficial effect.[9,10]

Thymosin $\beta 4$ (T$\beta 4$) is an important G-actin sequestering protein that contains 43 amino acids and was first isolated from bovine thymus.[11,12] Recently, a variety of biological functions of T$\beta 4$ have been reported, such as the promotion of wound healing, inflammation, tumor metastasis, angiogenesis and tissue regeneration.[13–17] In addition, studies have shown that T$\beta 4$ protects against acute CCl4-induced liver injury in mice.[18] Some clinical studies have also shown that in patients with liver disease, the serum T$\beta 4$ level positively correlates with liver function[19,20] and negatively correlates with the oxidation status of the liver.[21] Han et al.[22] found that reductions in serum T$\beta 4$ are related to the

severity of liver failure, but up to now there has been little quantitative assessment of the relationship between the expression of Tβ4 and the severity of ACHBLF in patients. Furthermore, the changes in Tβ4 expression that accompany glucocorticoid therapy have not been characterized.

DNA methylation plays a significant role in genome regulation,[23] occurring predominantly at CpG islands, and it is the most studied human epigenetic modification. Emerging evidence suggests that methylation plays a vital role in immune regulation. Our previous studies have shown that ACHBLF is associated with aberrant methylation of several genes.[24–27] Aberrant methylation of Tβ4 has been found to be frequent in patients with chondrosarcoma or hepatocellular carcinoma (HCC).[28,29] In addition, previous studies have shown that changes to the DNA, including demethylation, occur following glucocorticoid treatment.[30–32] Therefore, we hypothesized that glucocorticoid therapy might affect Tβ4 promoter methylation, and therefore the assessment of methylation may be useful for screening the efficacy for those patients with ACHBLF who undergo glucocorticoid therapy.

To test this hypothesis, we assessed the Tβ4 promoter methylation status and the expression of Tβ4 mRNA in peripheral blood mononuclear cells (PBMCs) from patients with ACHBLF or chronic hepatitis B (CHB) and healthy controls (HCs), and then measured the change in Tβ4 methylation and Tβ4 mRNA expression before and after glucocorticoid therapy in patients with ACHBLF. In this way, we assessed the relationship between Tβ4 promoter methylation status and the efficacy of glucocorticoid treatment.

Materials and Methods

Participants

Fifty-six patients with ACHBLF were recruited at the Department of Hepatology, Qilu Hospital of Shandong University from March 2017 to May 2020, as were 45 patients with CHB and 32 HCs. Patients with ACHBLF were selected using the following criteria, which were based on those of the Asia-Pacific Association for the Study of the Liver[33]: (1) a history of chronic hepatitis, with the presence of serum hepatitis B surface antigen (HBsAg) for >6 mo; (2) progressive jaundice (serum total bilirubin [TBIL]≥ 170 μmol/L); (3) an international normalized ratio (INR)≥1.5; and (4) a prothrombin activity (PTA)<40%. The exclusion criteria were as follows: (1) coinfection with hepatitis A, C, D or E virus; (2) pregnancy; (3) another liver disease, such as alcoholic liver disease, drug-induced hepatitis, Wilson disease or autoimmune hepatitis; (4) infection with cytomegalovirus, HIV or Epstein-Barr virus; (5) liver tumor; or (6) a history of diabetes, cardiac disease or nephrosis.

None of the participants had used glucocorticoids for at least 6 mo prior to the study, but had undergone conservative treatment, such as with other hepatoprotective medication, nutritional support and other non-specific therapy. Nucleoside analogs were administered to inhibit viral replication in participants when their serum HBV-DNA levels were high. These were entecavir (29 participants; 0.5 mg daily) and tenofovir (27 participants; 300 mg daily).

Once a diagnosis of ACHBLF had been confirmed, the participants began administering glucocorticoids (methylprednisolone or prednisolone). To ensure that the effects would be comparable, the dose of methylprednisolone (MSPL) was converted to the equivalent dose of prednisone (PSL) on the basis of its anti-inflammatory potency. A dose of 1 mg/kg/d MSPL (average: 80 mg/d) or 0.75 mg/kg/d PSL (average: 60 mg/d) was administered for the first 3 d. Then the dose was gradually tapered off by 0.25 mg/kg/d during the remaining three 3-d cycles. MSPL 0.75 mg/kg/d (average: 60 mg/d) or PSL 0.5 mg/kg/d (average: 40 mg/d) was administered for the second 3 d. MSPL 0.5 mg/kg/d (average: 40 mg/d) or PSL 0.25 mg/kg/d (average: 20 mg/d) was given for the third 3 d. According to the improvement of liver function, the total dose of MSPL or PSL was gradually reduced by 5 or 10 mg at least every 4 d, and then was completely withdrawn until the end of the 28th day. The patients with ACHBLF were followed for at least 90 d and the primary outcome was defined as survival at the end of this period.

DNA extraction from PBMCs and sodium bisulfite modification

Whole blood (5 ml) was obtained from each participant and anticoagulated using EDTA. Gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden) was used to isolate PBMCs. A QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was then used to extract genomic DNA from the PBMCs. Twenty microliters of genomic DNA per participant were stored at −20°C for later use.

The amount of hydrogen sulfite required to modify the DNA was calculated on the basis of the concentration of each genomic DNA sample. Approximately 20 μl of the extracted DNA was modified using sodium bisulfite and an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Then 20 μl of modified DNA was immediately used as a template for methylation-specific PCR (MSP) or stored at −20°C for further study. Methylated cytosines are unaffected by bisulfite, whereas unmethylated cytosines are converted to uracil.

MSP

MSP primers for Tβ4 were obtained from the previous study.[28] The primer sequences were as follows: MF: 5′-GTT TTC GGA TGT CGT TTC GAG AC-3′ and MR: 5′-ACG ACG AAC GCA ACT TTA TAA ACG-3′; UF: 5′-TAG GTT TTT GGA TGT TUTT TTT GAG AT-3′ and UR: 5′-AAA TAC AAC AAA CAC AAC TTT ATA AAC A-3′. The reaction mixture comprised 1 μl of modified DNA, 0.5 μl of each primer (10 μM), 12.5 μl of Premix Taq (Zymo Research) and 10.5 μl of nuclease-free water (total 25 μl).

The PCR protocol comprised an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 60 s, then a final extension at 72°C for 7 min. The Tβ4 amplicons were electrophoresed on a 2% agarose gel, stained with Gel Red (Biotium, CA, USA) and visualized under UV illumination. Water was used as a negative control and each reaction was repeated three times.

RNA isolation from PBMCs and RT-qPCR

RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

The concentrations of the RNA samples were measured using an Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY, USA), 1 μg of RNA was reverse transcribed to cDNA using a First-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) and 20 μl aliquots of cDNA were stored at −20°C. The mRNA expression of Tβ4 was measured using RT-qPCR. PCR was performed on a Light Cycler (Roche Diagnostics, Mannheim, Germany) using SYBR Green PCR mix (Takara, Japan). The primer sequences were as follows: Tβ4 F: 5-AAA CCC GAT ATG GCT GAG AT-3′, R: 5′-TGC TTC TCC TGT TCA ATC GT-3′; β-actin F: 5′-ATG GGT CAG AAG GAT TCC TAT GTG T-3′, R: 5′-CTT CAT GAG GTA GTC AGT CAG GTC-3′. The PCR reaction conditions were as follows: initial denaturation at 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s, then a final extension at 72°C for 30 s. The reaction mixture contained 1 μl of cDNA, 0.4 μmol of each specific primer, 10 μl of SYBR Green premix and 8.2 μl of ddH_2O (20 μl total). Each PCR reaction was repeated three times and the expression of Tβ4 was determined using the $2^{-\Delta\Delta Ct}$ method.

Measurement of laboratory parameters

Serum biochemical markers of liver damage and function were measured using an automatic biochemical analyzer (Cobas c311, Roche Diagnostics Ltd) and comprised alanine aminotransferase (ALT), TBIL, albumin (ALB), cholinesterase (CHE), pre-albumin (pre-ALB) and creatinine (Cr). PTA was measured using an ACL TOP 700 (Instrumentation Laboratory, Bedford, MA, USA). Hepatitis B s Antigen (HBsAg) and hepatitis B e Antigen (HBeAg) were measured using an electrochemiluminescence assay (Roche Diagnostics Ltd). HBV-DNA load was measured using ABI 7300 PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. In addition, the model for end-stage liver disease (MELD) score was calculated to estimate the severity of ACHBLF as follows: MELD score=3.78×ln (bilirubin [mg/dl])+11.2×ln (INR)+9.57×ln (Cr [mg/dl])+6.43 (etiology:0 if cholestatic or alcoholic, 1 otherwise).

ELISA

The plasma level of Tβ4 was measured using an ELISA kit (Lengton Bioscience Co. Ltd, Shanghai, China), according to the manufacturer's protocol. The concentration of Tβ4 in each sample was calculated using the standard curve and ELISA Calc (R&D San Diego, CA, USA).

Statistical analysis

PASS software 15.0 (NCSS statistical software, Kaysville, UT, USA) was used to estimate the group size. A sample size of 89 achieves 90% power to detect an effect size (W) of 0.3787 using α-2 degrees, of freedom χ^2 test with a significance level (alpha) of 0.05. Considering 20% of the cases of loss of follow-up and rejection, a total of at least 112 subjects was needed. Therefore, we enrolled 133 patients (32 HCs, 45 CHB, 56 ACHBLF) to participate in this study.

The results were analyzed using SPSS 22.0 (IBM, Inc., Armonk, NY, USA). Continuous variables are presented as median (centile 25; centile 75) and categorical variables are expressed as number (%). Differences between groups were analyzed using the Mann–Whitney U-test, Kruskal–Wallis test or χ^2 test, as appropriate. The relationships between variables were evaluated using Pearson's or Spearman's correlation. All the statistical analyses were two-sided and $p<0.05$ was considered to indicate statistical significance.

Results

General characteristics of the participants

A total of 168 participants were collected for the study, but 20 patients with ACHBLF and 15 patients with CHB were excluded because they had hepatocellular carcinoma, pregnancy or another liver diseases. Finally, 56 patients with ACHBLF, 45 patients with CHB and 32 HCs were studied. The selection process of all participants is shown in Figure 1. The clinical characteristics of all the participants are shown in Table 1. There were no differences between the participants with ACHBLF or CHB and HCs with regard to gender distribution ($p=0.55$) or age ($p=0.607$). However, significant differences existed with regard to TBIL, ALT, ALB, PTA, CHE, pre-ALB, Cr, blood urea nitrogen and β2M (all $p<0.01$). There were no significant relationships of ACHBLF and CHB with HBeAg ($p=0.80$; χ^2 test) or HBV-DNA load ($p=0.30$; χ^2 test). The enrolled patients with CHB were divided into four groups according to their ALT levels, and their detailed clinical characteristics are shown in Table S1.

PBMC expression and the plasma concentration of Tβ4 in participants with ACHBLF or CHB and HCs

The frequency of methylation of the Tβ4 promoter was measured in the PBMCs of participants with ACHBLF or CHB and HCs before glucocorticoid treatment. As shown in Figure 2A, the methylation frequency in participants with ACHBLF (36/56; 64%) was significantly higher than that in participants with CHB (14/45; 31%; $\chi^2=10.99$; $p=0.001$) and HCs (0/31). Furthermore, the frequency of methylation was higher in participants with CHB than in HCs ($\chi^2=11.82$, $p<0.001$). Representative results of agarose gel electrophoresis are shown in Figure 2D.

To measure the Tβ4 mRNA expression in the PBMCs of the participants, RT-qPCR was performed using *ACTB* as the reference gene (Figure 2B). We found that the mean relative Tβ4 mRNA expression was much lower in participants with ACHBLF than in those with CHB ($Z=-4.79$, $p<0.001$) or in HCs ($Z=-5.99$, $p<0.001$). In addition, the Tβ4 mRNA expression in the PBMCs of participants with CHB was also lower than that in HCs ($Z=-3.15$, $p<0.001$).

The plasma levels of Tβ4 were measured by ELISA. In the plasma of participants with ACHBLF or CHB and HCs, the concentrations of Tβ4 were 7.14 (5.50–9.37), 14.00 (11.39–15.30) and 26.33 (22.14–29.95) μg/ml, respectively. As shown in Figure 2C, these concentrations significantly differed (ACHBLF vs CHB, $Z=-7.124$, $p<0.001$; CHB vs HCs, $Z=-6.956$, $p<0.001$; and ACHBLF vs HCs, $Z=-7.763$, $p<0.001$).

Relationships of Tβ4 promoter methylation and mRNA expression with the clinicopathological characteristics of participants with ACHBLF

The relationships between Tβ4 promoter methylation and the clinical characteristics of participants with ACHBLF were assessed using the Mann–Whitney U and χ^2 tests. As shown in Table 2, the

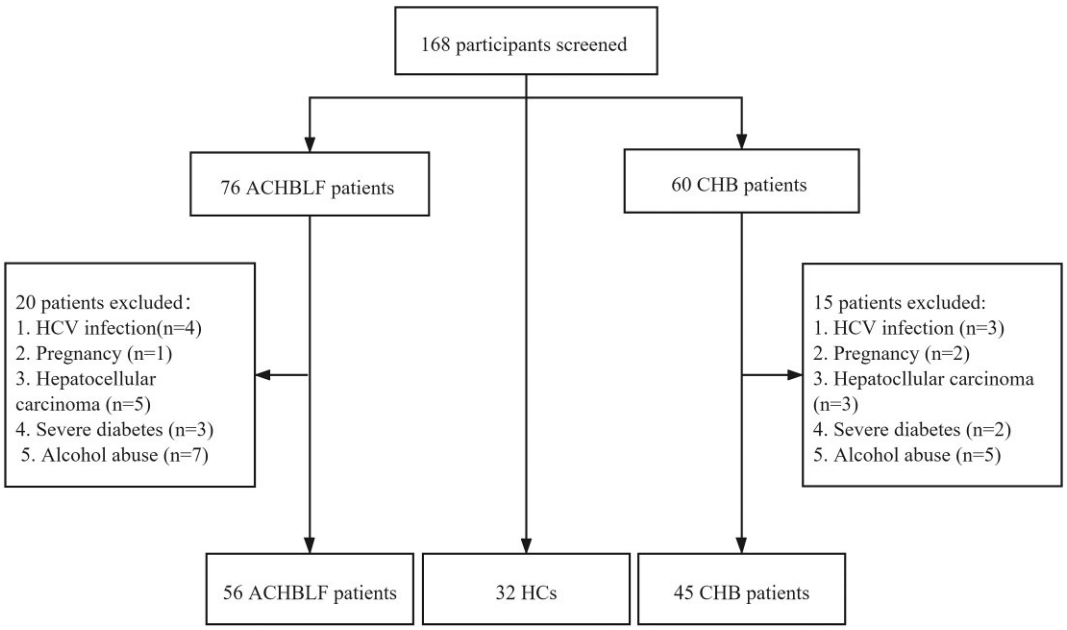

Figure 1. Flowchart depicting the selection process of the participants.

frequency of methylation of the Tβ4 promoter was significantly correlated with TBIL (Z=−2.907, p=0.004), PTA (Z=−4.933, p<0.001) and the MELD score (Z=−2.454, p=0.014). However, there were no correlations between Tβ4 promoter methylation and age, gender, ALT, ALB, Cr, CHE, pre-ALB, HBeAg or HBV-DNA (p>0.05). Moreover, Tβ4 promoter methylation had no significant correlation with encephalopathy, ascites, hepatorenal syndrome (HRS) and spontaneous bacterial peritonitis (SBP) (p>0.05). Table 3 shows the results of multivariate logistic regression analysis, which shows that TBIL (r=1.009, p=0.048) and PTA (r=0.671, p=0.024) were associated with higher Tβ4 promoter methylation, but that HBeAg, DNA, ALB, MELD, encephalopathy, ascites, HRS and SBP were not.

In addition, there was an inverse relationship between the methylation of Tβ4 and its mRNA expression (r=−0.649, p<0.001). To identify possible contributors to the severity of ACHBLF, we analyzed the relationships between Tβ4 mRNA expression and the clinical characteristics of participants with ACHBLF. The results showed that Tβ4 mRNA expression was significantly negatively correlated with TBIL (r=−0.341, p=0.010) and the MELD score (r=−0.271, p=0.043). In addition, there was a positive correlation between Tβ4 mRNA expression and PTA (r=0.416, p=0.001). However, there were no significant correlations between Tβ4 mRNA expression and gender, age, ALT, ALB, CHE, pre-ALB, Cr, HBeAg, HBV-DNA, encephalopathy, ascites, HRS and SBP in participants with ACHBLF (p>0.05).

Effects of glucocorticoid treatment in participants with ACHBLF

On the basis of the above findings, TBIL, PTA and MELD score were used to evaluate the effects of glucocorticoid treatment in participants with ACHBLF. We compared the data for each of these parameters at various time points during the treatment. Figure 3A–C show that there were no differences in TBIL, PTA or MELD score from day 0 to day 7 of the treatment. However, on day 28 of the treatment, TBIL and MELD score were lower than on day 7, whereas PTA was higher (Z=14.02, p<0.001; Z=82.95, p<0.001; and Z=19.07, p<0.001; respectively).

There was a gradual increase of the Tβ4 mRNA expression during treatment (0.76±0.46, 0.95±0.57 and 1.73±1.20 on day 0, 7 and 28, respectively), and it was significantly higher on day 28 than on day 7 (Z=17.53, p<0.001) (Figure 3D). However, the overall methylation frequency of the Tβ4 promoter showed an opposing trend (64.3, 57.13 and 37.5%, respectively; χ^2=8.650, p=0.013) (Figure 3E). After day 7 of treatment, Tβ4 promoter demethylation was detected in four of the participants with ACHBLF. On day 28, when the glucocorticoid therapy ended, there were 11 further participants with ACHBLF who showed demethylation. As shown in Figure 3E, the methylation frequency of the Tβ4 promoter on day 28 (37.5%) was lower than that on day 7.

The plasma concentration of Tβ4 was significantly higher on day 28 than on day 7 of the treatment (Z=−5.549, p<0.001), but there was no difference between baseline and day 7 (Z=−1.874, p=0.061) (Figure 3F).

Effect of Tβ4 promoter methylation status on the prognosis of participants with ACHBLF

The participants with ACHBLF were allocated to two groups on the basis of their DNA methylation status at baseline. The clinicopathological features of the two groups are compared in Table 2. The methylation status of Tβ4 was significantly correlated with TBIL, PTA and MELD score. The MELD score is widely used to assess the prognosis of patients with ACHBLF. Ninety days after the start of glucocorticoid treatment, the survival ratio for

Table 1. Clinical characteristics of the enrolled participants

Parameter	ACHBLF (n=56)	CHB (n=45)	HCs (n=32)	p-value
Gender (male/female)	29/27	24/21	18/14	0.92‡
Age (y)	50.5 (39.3–58.5)	47.0 (49.0–55.5)	45.5 (39.0–56.0)	0.607†
TBIL (μmol/L)	352.0 2(284.42–454.91)	30.11 (26.12–42.43)	16.52 (12.81–18.35)	<0.001†
ALT (U/L)	680.71 (530.82–886.34)	176.6 3(106.90–233.23)	18.92 (14.13–26.43)	<0.001†
ALB (g/L)	25.71 (22.43–28.72)	36.52(33.72–39.51)	40.32 (38.62–43.60)	<0.001†
pre-ALB (mg/L)	110.52 (90.21–131.72)	240.33 (210.51–298.22)	294.64 (268.80–320.20)	<0.001†
PTA (%)	30.02 (28.42–34.24)	75.63 (69.93–79.22)	98.45 (91.41–112.52)	<0.001†
Cr (μmol/L)	59.23(67.01–70.61)	59.31 (56.83–62.92)	53.45 (50.14–56.20)	<0.001†
BUN(mmol/L)	7.35 (6.23–9.04)	7.04 (6.15–8.06)	5.65 (4.52–6.56)	<0.001†
β2M (mg/L)	2.71 (2.37–3.17)	2.32 (1.93–2.61)	2.29 (1.94–2.53)	<0.001†
CHE (U/L)	1998 (1304–2540)	4898 (4027–5265)	6216 (5622–6762)	<0.001†
HBeAg (+/−)	35/21	27/18	NA	0.80†
HBV-DNA (+/−)	38/18	26/19	NA	0.30†
Encephalopathy	19	NA	NA	—
Ascites	40	NA	NA	—
HRS	13	NA	NA	—
SBP	32	NA	NA	—
MELD score	20.2 (19.0–21.9)	NA	NA	—

Abbreviations: ACHBLF, acute-on-chronic hepatitis B liver failure; ALB, albumin; ALT, alanine aminotransferase; β2M, β2 microglobulin; BUN, blood urea nitrogen; CHB, chronic hepatitis B; CHE, cholinesterase; Cr, creatinine; HBeAg, hepatitis B e antigen; HC, healthy control; HRS, hepatorenal syndrome; MELD, model for end-stage liver disease; NA, not available; pre-ALB, pre-albumin; PTA, prothrombin activity; SBP, spontaneous bacterial peritonitis; TBIL, total bilirubin. Data are expressed as median (IQR) or N. †, Kruskal–Wallis H test; ‡, χ^2 test.

participants with low methylation was 16/20 (80%), which was significantly higher than that for participants with higher levels of methylation (18/36, 50%, p=0.026). The mean survival time (calculated from hospital admission) of methylated patients was 60.43 d (SE 5.547; 95% CI 49.56 to 71.33) and for unmethylated patients it was 79.10 d (SE 5.403; 95% CI 68.51 to 89.69, p=0.026) (Figure 4).

ROC curves were used to measure the predictive values of Tβ4 promoter methylation and MELD score for the 3-mo mortality of patients with ACHBLF. At the end of the 90-d follow-up period, the AUCs of Tβ4 methylation were significant higher than those of MELD score (AUC 0.857, SE 0.0586, CI 0.738 to 0.936 vs AUC 0.648, SE 0.0801, CI 0.509 to 0.771, p<0.05) (Figure 5).

Discussion

In the present study, we have demonstrated that the methylation of the Tβ4 promoter closely correlates with the severity of ACHBLF and that glucocorticoids affect the Tβ4 promoter methylation status in patients with ACHBLF. To evaluate the utility of Tβ4 as a biomarker, we first performed RT-qPCR and MSP to measure the mRNA expression of Tβ4 and the frequency of methylation of its promoter in PBMCs from patients with ACHBLF or CHB and HCs. Next, we measured Tβ4 expression before and after glucocorticoid treatment in patients with ACHBLF, and assessed the change in Tβ4 promoter methylation status and demethylation during glucocorticoid treatment. The results show that the frequency of methylation of the Tβ4 promoter positively correlates with the severity of ACHBLF. In addition, the changes in Tβ4 methylation status during the glucocorticoid treatment of patients with ACHBLF imply that glucocorticoids may affect this parameter. Therefore, Tβ4 promoter methylation can act as a biomarker for predicting disease severity and provide a better option for glucocorticoids therapy in ACHBLF patients.

ACHBLF is characterized by complex pathophysiology, but it is caused by HBV infection and systemic inflammation is an important driver.[34] Acute hepatitis caused by HBV infection directly or indirectly activates Kupffer cells, which results in greater release of pro-inflammatory mediators, regulatory cytokines and eicosanoids, as well as lysosomal and proteolytic enzymes. This altered milieu causes immune cells, endotoxins and Lipopolysaccharide (LPS) to accumulate in the liver. Furthermore, the release of nitric oxide, reactive oxygen species (ROS) and inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin (IL)-6, IL-8, IL-10, IL-12 and interferon-γ) leads to inflammation and hepatocyte death. Subsequently, persistent hepatocyte injury and sepsis leads to multiple organ dysfunction syndrome and organ failure.[3,35–37]

Tβ4 is a thymic hormone that controls cell morphogenesis and motility by regulating the dynamics of the actin cytoskeleton. It also plays an anti-inflammatory role by downregulating the expression of nuclear factor-kappa B and IL-8, which is induced by TNF-α.[38] Shah et al.[39] demonstrated that Tβ4 has an anti-inflammatory effect by inhibiting ethanol- and LPS-induced

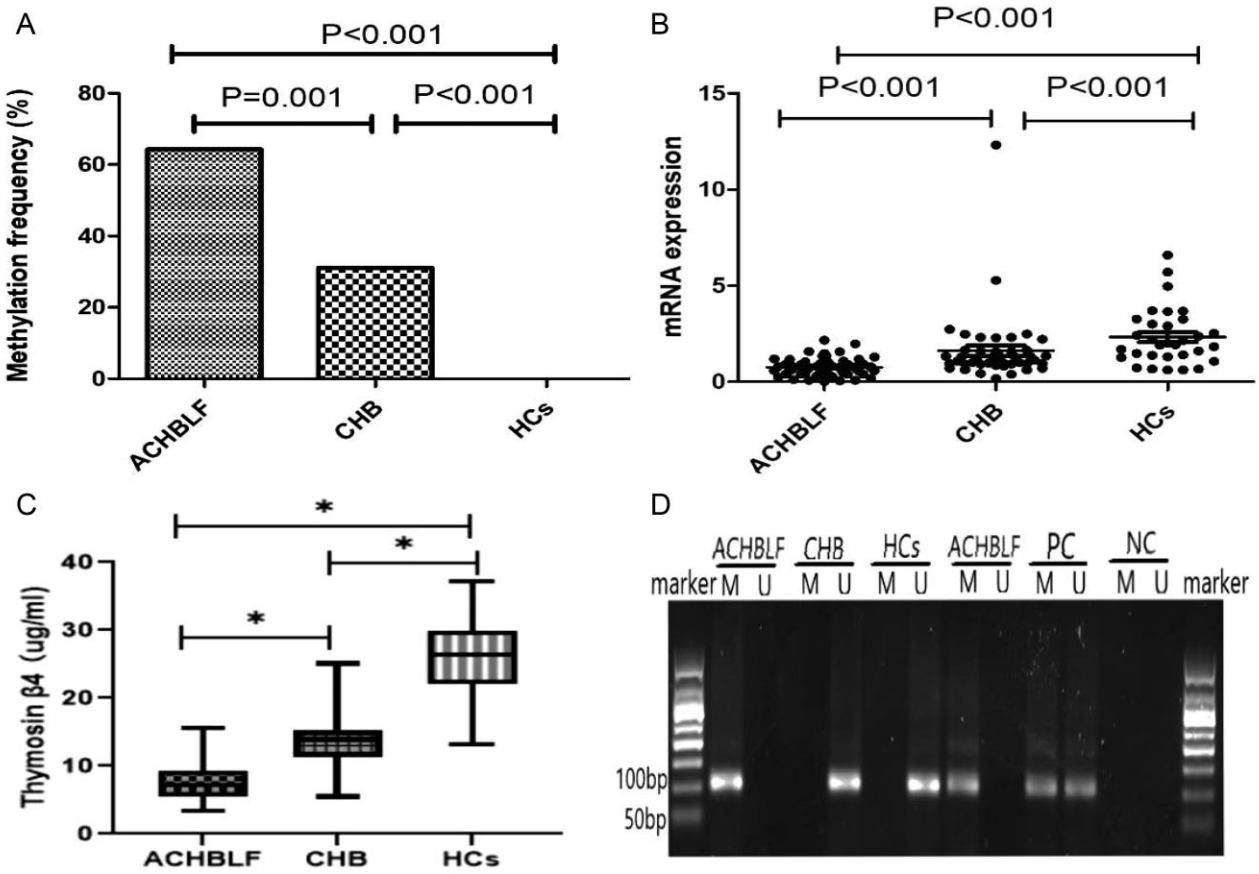

Figure 2. PBMC expression and the plasma concentration of thymosin $\beta 4$ (T$\beta 4$) in participants with ACHBLF or CHB and HCs. (A) The frequency of methylation of the T$\beta 4$ promoter in peripheral blood mononuclear cells (PBMCs) in participants with acute-on-chronic hepatitis B liver failure (ACHBLF) or chronic hepatitis B (CHB) and healthy controls (HCs). (B) T$\beta 4$ mRNA expression in the PBMCs of each group of participants. (C) Plasma T$\beta 4$ concentration in participants in each group. (D) Representative T$\beta 4$ methylation obtained using methylation-specific PCR. A 50 bp DNA ladder marker is shown. M, methylated sequence; NC, negative control; PC, positive control; U, unmethylated sequence.

pro-inflammatory cytokine release. Furthermore, T$\beta 4$ treatment has been shown to reduce the release of ROS and inflammation.[40] In addition, growing evidence shows that T$\beta 4$ is involved in antioxidant, anti-inflammatory and antifibrotic responses to liver injury.[18,39,41,42] It has also been reported that the serum T$\beta 4$ concentration is closely related to the severity of hepatic injury.[22,43] To the best of our knowledge, the serum T$\beta 4$ concentration alone has been measured in patients with ACHBLF. However, the relationship between the methylation status of the T$\beta 4$ promoter and the severity of ACHBLF has not been studied.

Using a relatively quantitative means of analysis, we have shown that T$\beta 4$ mRNA expression is higher in patients with ACHBLF than in those with CHB or in HCs, whereas the opposite trend was identified with respect to methylation frequency. Spearman correlation showed that T$\beta 4$ promoter methylation negatively correlates with mRNA expression ($r=-0.649$, $p<0.001$). DNA methylation, which is one of the most important epigenetic modifications in eukaryotes, is an area of substantial research interest.[44] Methylation can silence gene expression and may therefore play a role in aberrant gene expression in disease.[45] In the present study, we have shown that hypermethylation of the T$\beta 4$ gene promoter is linked to low expression of the gene in PBMCs in patients with ACHBLF, and this is consistent with aberrant methylation of the promoter leading to gene silencing, which might promote disease progression. These findings are consistent with those reported by Ohata et al.,[28] who demonstrated that alterations in DNA methylation induce aberrant expression of T$\beta 4$. More importantly, we observed that the frequency of T$\beta 4$ promoter methylation significantly correlates with TBIL, PTA and the MELD score, which reflect liver function.[46] Therefore, T$\beta 4$ methylation status may reflect the severity of ACHBLF.

Glucocorticoids have been used for the treatment of liver failure for many years.[7,47] Glucocorticoids are immunomodulators that can rapidly suppress excessive inflammatory reactions and immune responses, and thereby prevent or delay primary liver injury.[48] Previous studies have shown that glucocorticoids reduce inflammation in the liver and prevent liver cell necrosis, especially in the early stages of severe hepatitis.[10,49] During mammalian development, changes in DNA methylation patterns are frequent, and involve both de novo methylation and

Table 2. Baseline characteristics of participants with ACHBLF who had high and low levels of Tβ4 promoter methylation

Parameter	Tβ4 methylation status		Statistic	p-value
	Methylated (n=36)	Unmethylated (n=20)		
Age (y)	49.5 (37.3–55.0)	53.0 (42.5–63.5)	Z=−1.412	0.158†
Gender (male/female)	17/19	12/8	χ^2=0.841	0.359‡
TBIL (μmol/L)	398.41 (322.92–486.75)	288.14 (205.82–371.66)	Z=−2.091	0.004†
ALT (U/L)	701.02 (568.4–887.4)	599.20 (434.03–866.72)	Z=−1.026	0.305 †
ALB (g/L)	25.32 (21.46–28.51)	27.31 (25.46–29.13)	Z=−1.411	0.158†
PTA (%)	28.94 (23.02–30.35)	35.46 (32.43–37.25)	Z=−4.933	0.000†
Cr (μmol/L)	64.45 (58.87–70.32)	68.55 (60.81–71.12)	Z=−1.248	0.212†
pre-ALB (mg/L)	105.12 (90.64–123.72)	121.21 (89.23–135.91)	Z=−0.761	0.447†
CHE (U/L)	2047 (1307–2550)	1989 (1293–2568)	Z=−0.205	0.837†
HBeAg (+/−)	23/13	12/8	χ^2=0.083	0.773‡
HBV-DNA (+/−)	25/11	13/7	χ^2=0.116	0.733‡
MELD score	21.1 (19.12–23.01)	19.22 (18.36–20.43)	Z=−2.454	0.014†
Encephalopathy	11/25	8/12	χ^2=0.512	0.561‡
Ascites	27/9	13/7	χ^2=0.630	0.540‡
HRS	9/27	4/16	χ^2=0.180	0.752‡
SBP	23/13	9/11	χ^2=1.873	0.260‡
mRNA	0.58 (0.33–0.75)	1.06 (0.81–1.33)	Z=−4.814	0.000†

Abbreviations: ACHBLF, acute-on-chronic hepatitis B liver failure; ALB, albumin; ALT, alanine aminotransferase; CHB, chronic hepatitis B; CHE, cholinesterase; Cr, creatinine; HC, healthy control; HRS, hepatorenal syndrome; HBeAg, hepatitis B e antigen; MELD, model for end-stage liver disease; mRNA, messenger RNA; pre-ALB, pre-albumin; PTA, prothrombin activity; SBP, spontaneous bacterial peritonitis; Tβ4, thymosin β4; TBIL, total bilirubin. Data are expressed as median (IQR) or N. †Mann–Whitney U-test; ‡χ^2 test.

Table 3. Results of multivariate logistic regression analysis of the relationships of clinicopathological parameters with Tβ4 promoter methylation in participants with ACHBLF

Parameter	Coefficient	OR	95% CI	p-value
TBIL (μmol/L)	0.009	1.009	1.000 to 1.019	0.048
ALB (g/L)	−0.202	0.817	0.560 to 1.192	0.294
PTA (%)	−0.399	0.671	0.475 to 0.948	0.024
HBeAg (+/−)	0.571	1.77	0.182 to 17.232	0.623
HBV-DNA (+/−)	1.695	5.444	0.459 to 64.526	0.179
MELD score	0.196	1.217	0.806 to 1.836	0.351
Encephalopathy	0.364	1.439	0.453 to 4.569	0.537
Ascites	−0.432	0.649	0.195 to 2.159	0.481
HRS	−0.785	0.456	0.149 to 1.395	0.169
SBP	−0.333	0.717	0.185 to 2.780	0.631

Abbreviations: ALB, albumin; HBeAg, hepatitis B e antigen; HRS, hepatorenal syndrome; MELD, model for end-stage liver disease; PTA, prothrombin activity; SBP, spontaneous bacterial peritonitis; TBIL, total bilirubin.

demethylation.[45] Previous studies have suggested that glucocorticoids affect DNA, and in particular can cause gene demethylation.[30] Hamm et al.[29] demonstrated that the tumor microenvironment induces changes in the Tβ4 DNA methylation pattern. Given the role of Tβ4 in hepatocytes, we speculated that glucocorticoids may have a direct effect on Tβ4 methylation status in patients with ACHBLF.

In the present study, we determined the effect of glucocorticoid on Tβ4 in patients with ACHBLF, and found that Tβ4 mRNA expression in PBMCs gradually increased with treatment, which was accompanied by an improvement in liver function and a reduction in the frequency of promoter methylation, as well as demethylation. In addition, the change in serum Tβ4 concentration was consistent with that in mRNA expression. This implies that glucocorticoid therapy may upregulate Tβ4 mRNA expression in PBMCs and alter the Tβ4 promoter methylation status. To further characterize the relationship between methylation status and glucocorticoid treatment, the patients with ACHBLF were allocated to a highly methylated group and a less methylated group. On days 7 and 28 of glucocorticoid therapy, the Tβ4 mRNA expression was higher and the liver function was superior in patients from the low methylation group than in patients in the high methylation group. In addition, 90 d after the start of glucocorticoid treatment, the survival ratio of the low methylation group was significantly higher than that of the high methylation group. The results indicate that there is a more favorable response to glucocorticoid treatment in patients with ACHBLF who display low Tβ4 promoter methylation.

Although there are almost 20 scores to predict the severity of Acute-on-chronic liver failure (ACLF), the MELD score is still a widely used tool due to its feasibility in the real world.[50,51] Coagulation and TBIL were the main laboratory indicators of MELD score,[51] and they are also the main reference indicators for

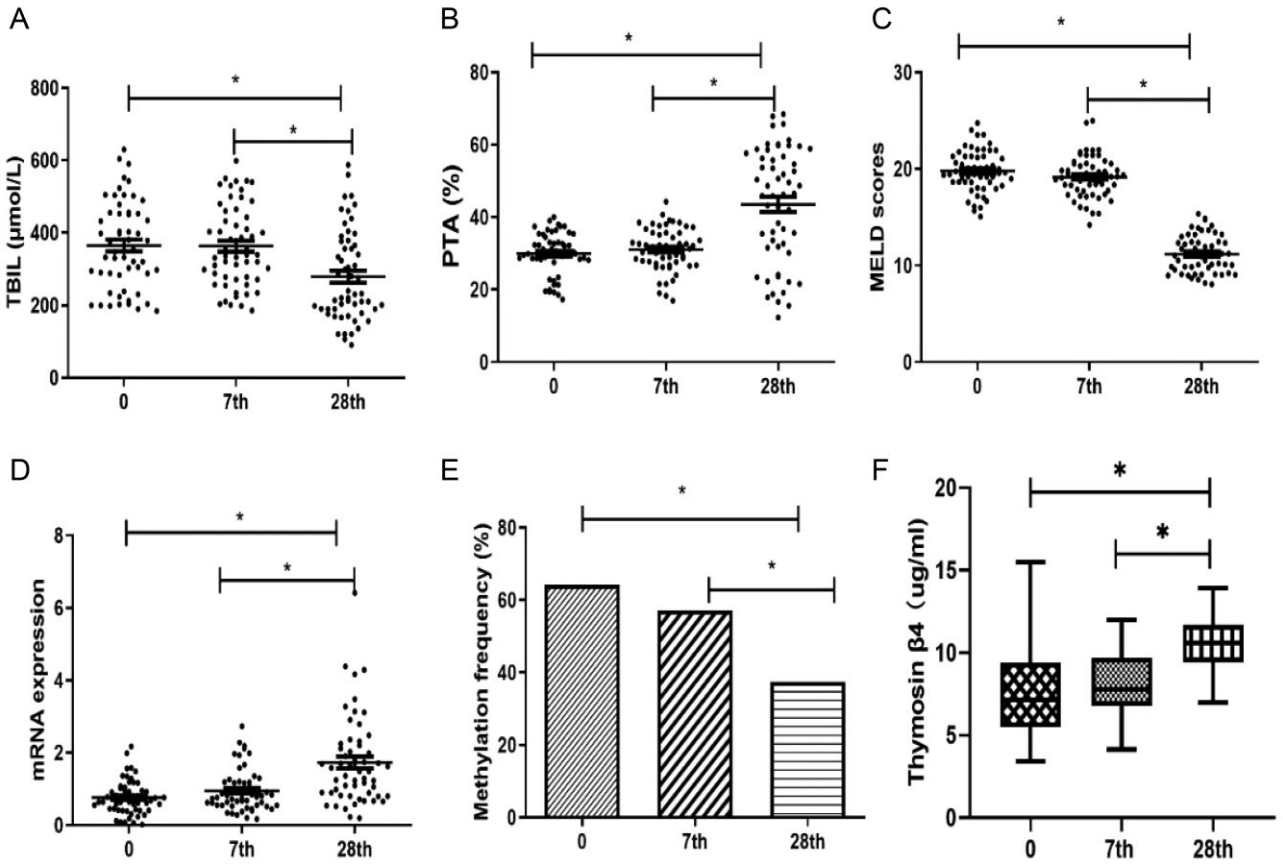

Figure 3. Effect of glucocorticoid treatment on liver function and the thymosin $\beta 4$ (T$\beta 4$) promoter methylation status and mRNA expression in peripheral blood mononuclear cells (PBMCs) of participants with acute-on-chronic hepatitis B liver failure (ACHBLF). (A–C) Liver function of the participants after 0, 7 and 28 d of glucocorticoid therapy. (D and E) T$\beta 4$ mRNA expression and methylation frequency in PBMCs at each time point. (F) Plasma T$\beta 4$ concentration of patients at each time point. *p<0.05.

the diagnosis and staging of ACHBLF.[35,46] However, there are approximately 15–20% of patients whose survival cannot be accurately predicted.[46,51] It has been reported that T$\beta 4$ level is a novel and reliable indicator of the prognosis of patients with ACHBLF.[52] Nevertheless, studies about T$\beta 4$ methylation and prognosis of liver failure are rarely reported. Our preliminary results suggest that T$\beta 4$ methylation status was associated with the 3-mo mortality of patients with ACHBLF. ROC curve analysis further revealed that the predictive value of T$\beta 4$ methylation was higher than that of the MELD score for the 3-mo mortality of patients with ACHBLF. Therefore, it is considered that T$\beta 4$ methylation was superior to the MELD score for predicting the prognosis of ACHBLF.

There are several limitations to the present study. First, it was relatively small, but the number of participants satisfied the basic need for statistical analysis. Therefore, the patients should be followed for longer and a multicenter cohort study should be performed to confirm our findings in future. Second, the use of glucocorticoids remains controversial because of the potential adverse effects, which include poor coagulation, hemorrhage and hepatic encephalopathy. A number of studies have indicated that glucocorticoids can effectively inhibit the inflammatory response. Hence, the use of glucocorticoids for patients with ACHBLF may be appropriate. Third, the coagulopathy and consequent high risk of hemorrhage in the present participants discouraged us from performing liver biopsy, and therefore we could not analyze liver histology. However, the alterations of T$\beta 4$ level in PBMCs might reflect its changes in the liver tissue. Therefore, future studies should investigate the effects of altering T$\beta 4$ expression and promoter methylation in animal models and cells in vitro. Moreover, we did not sequence the T$\beta 4$ genes of the patients, but this would provide more comprehensive information than MSP.

In conclusion, the present findings indicate that T$\beta 4$ methylation might represent a biomarker of the severity of ACHBLF and that glucocorticoids affect the methylation status of T$\beta 4$ in patients with ACHBLF. Therefore, the methylation status of the T$\beta 4$ promoter could be used to screen patients with ACHBLF for their potential responsiveness to glucocorticoid therapy in order to target the treatment more effectively. The relationship between the frequency of T$\beta 4$ promoter methylation and survival suggests that the aberrant methylation of T$\beta 4$ might also be used as a marker of the prognosis of patients with ACHBLF. However, further investigation into the relationship between aberrant methylation of the T$\beta 4$ promoter and the prognosis of patients with ACHBLF is required.

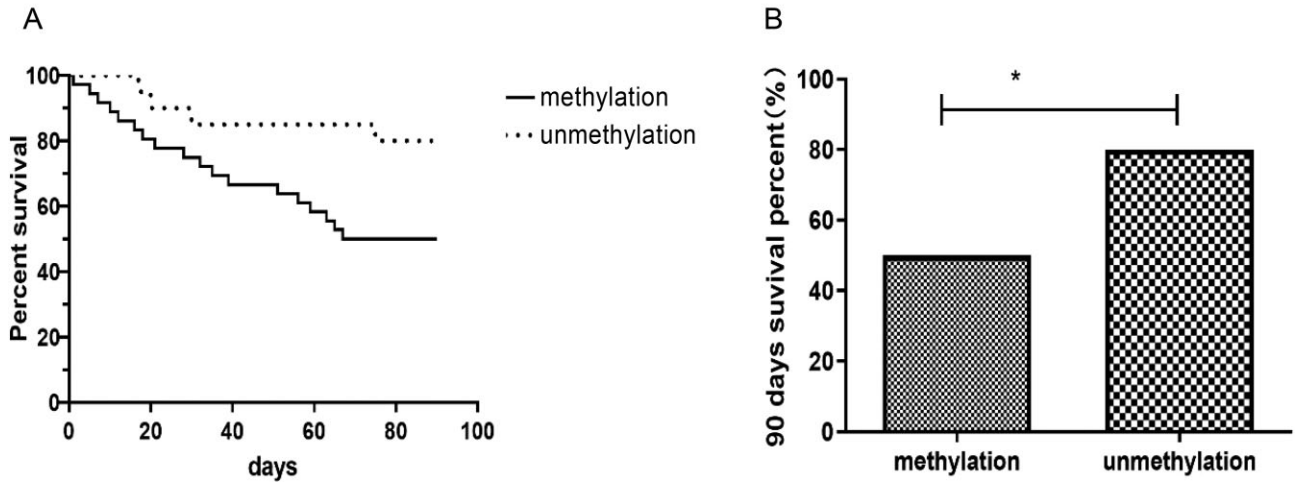

Figure 4. (A) Kaplan–Meier survival curves for the high and low methylation groups. The log-rank test was used to compare these. (B) 90-d survival of the two groups, compared using the χ^2 test. *p<0.05.

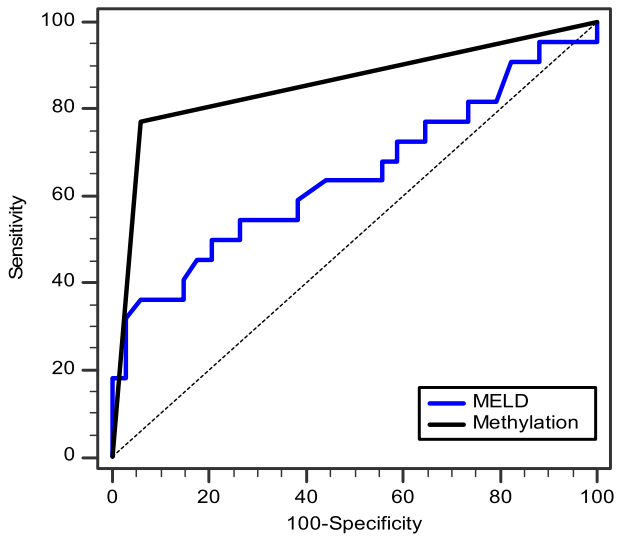

Figure 5. ROC curve for Tβ4 methylation and the MELD score for predicting the 3-mo mortality of patients with ACHBLF.

Supplementary data

Supplementary data are available at *International Health* online.

Authors' contributions: HW: conceptualization, data collection, methodology, investigation, statistical analysis and writing (original draft, review and editing); YQ: data collection, investigation, resources and formal analysis; J-WW: methodology, formal analysis and investigation; YF: resources, formal analysis and investigation; Y-CF: formal analysis, methodology and writing (review and editing); H-HL: data collection and methodology; and KW: supervision, conceptualization and project administration.

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Ethical approval: Ethical approval was obtained from the Ethics Committee of Qilu Hospital of Shandong University and the study was conducted according to the principles of the Declaration of Helsinki (Hellmann and others, 2014).

Data availability: Data available on request from the authors.

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