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Receptor-Interacting Protein Kinase 3 (RIPK3) mRNA Levels Are Elevated in Blood Mononuclear Cells of Patients with Poor Prognosis of Acute-on-Chronic Hepatitis B Liver Failure

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Necroptosis refers to a programmed form of necrosis, which involves the receptor-interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL). In this study, to investigate the role of necroptosis in the pathogenesis of acute-on-chronic hepatitis B liver failure (ACHBLF), we retrospectively analyzed 122 patients with ACHBLF, 131 patients with chronic hepatitis B (CHB), and 35 healthy controls (HCs). Using quantitative real-time polymerase chain reaction (RT-qPCR), we measured RIPK3 mRNA levels in peripheral blood mononuclear cells (PBMCs). ELISA was performed to measure the serum levels of MLKL, TNF-*α* and caspase-8. We found that RIPK3 mRNA levels were significantly higher in patients with ACHBLF than those with CHB or HCs. RIPK3 mRNA levels in patients with ACHBLF were positively correlated with serum levels of TNF-*α* or MLKL and negatively correlated with caspase-8 levels. Univariate and multivariate analysis revealed that RIPK3 mRNA level was predictive of 3-month mortality of ACHBLF. The area under receiver operating characteristic curve (AUC) of RIPK3 mRNA levels was 0.810 (95% CI: 0.729-0.876), which was higher than that of MELD scores (0.766, 95% CI: 0.681-0.838). The optimal cut-off point of 8.81 was determined for RIPK3 mRNA levels, which showed a sensitivity of 80.7% and a negative predictive value of 80.4%. These results indicate that elevated RIPK3 mRNA levels in PBMCs are associated with poor prognosis of ACHBLF. We thus propose that necroptosis may play an important role in pathogenesis of ACHBLF.

Keywords: acute-on-chronic hepatitis B liver failure; necroptosis; peripheral blood mononuclear cells; prognosis; receptor-interacting protein kinase 3
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Introduction

Acute-on-chronic liver failure (ACLF) refers to a rapid and acute deterioration of liver function in patients with chronic liver diseases (Sarin et al. 2014). In Asian, hepatitis B virus (HBV) infection is the main cause of ACLF and acute-on-chronic hepatitis B liver failure (ACHBLF) accounts for more than 70% of ACLF (Gao et al. 2015). ACHBLF usually progress rapidly and result in multiple organ failure within 4 weeks (Katoonizadeh et al. 2010; Jalan et al. 2012). A previous study showed that the short- and medium-term mortality of ACHBLF was about 50-90%, which resulted in the death of more than 120,000 patients every year (Polson et al. 2005). Although liver transplantation remains the only effective therapy for patients with ACHBLF (Fan et al. 2015), it cannot be widely applied because of the shortage of liver donors (Arulraj and Neuberger 2011). Consequently, new prognostic markers are urgently needed for mortality prediction and severity discrimination in patients with ACHBLF (Laleman et al. 2011).

Necroptosis refers to a programmed form of necrosis, which results in programmed cell death via apoptosis (Linkermann and Green 2014). In the process of necroptosis, tumor necrosis factor-*α* (TNF-*α*) leads to stimulation of its receptor TNFR1. Then, TNFR1 recruits the TNF receptor-associated death domain (TRADD) and signals to receptor-interacting protein kinase 1 (RIPK1). In the absence of caspase-8, RIPK1 recruits RIPK3 and forms the necrosome (Vanden Berghe et al. 2014). The necrosome then activates mixed lineage kinase domain-like protein (MLKL) through phosphorylation, eventually leading to expulsion of cellular contents into the extracellular space (Wang et al. 2014). Necroptosis was shown to play an important role in viral defense and inflammatory diseases (Gunther et al. 2011). Meanwhile, necroptosis was also found to be involved in the pathogenesis and progression of severe liver diseases (Gunther et al. 2016). We therefore

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hypothesized that RIPK3 might participate in the pathogenesis of ACHBLF and serve as its prognostic marker.

In this study, we investigated the role of necroptosis in ACHBLF. Using quantitative real-time polymerase chain reaction (RT-qPCR), we measured RIPK3 mRNA levels in peripheral blood mononuclear cells (PBMCs). ELISA was performed to measure serum levels of TNF-α, MLKL and caspase-8. We thus investigated the role of necroptosis in ACHBLF and evaluated the predictive value of RIPK3 mRNA levels in PBMCs for the prognosis.

Methods

Study design and population

One hundred and twenty-two patients with ACHBLF, 131 patients with CHB and 35 healthy controls (HCs) were retrospectively enrolled from July 2014 to November 2015 at the Department of Hepatology, Qilu Hospital of Shandong University. CHB was diagnosed by a positive hepatitis B surface antigen (HBsAg) for more than 6 months before the enrollment (Lok and McMahon 2009). ACHBLF was defined according to the guideline of Asian Pacific Association for the Study of the Liver (APASL) (Sarin et al. 2009).

Exclusion criteria included co-infection with hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, human immunodeficiency virus, autoimmune liver disease, alcoholic liver disease, pregnancy, and hepatocellular carcinoma.

Written informed consents were signed by all participants under protocols approved by the local Research and Ethics Committee at Qilu Hospital of Shandong University, in accordance with the guidelines of the 1975 Declaration of Helsinki.

Isolation of peripheral blood mononuclear cells (PBMCs)

Venous peripheral blood of 5 ml was drawn from each participant at the first day of diagnosis, using EDTA as an anticoagulant agent. PBMCs were isolated by gradient centrifugation from the blood via Ficoll-Plaque Plus (Healthcare, Uppsala, Sweden). Then, PBMCs were washed three times with phosphate-buffered saline and stored at –20°C until use.

RNA extraction and RT-qPCR

Total RNA of PBMCs was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined by the Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY). RNAs were then converted into cDNAs via first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). The RIPK3 mRNA levels were determined by RT-qPCR, which was performed on the Lightcycler 480 (Roche Diagnostic, Mannheim, Germany) with SYBR Green (Toyobo, Osaka, Japan). β-Actin was used as the endogenous control. Amplification was performed in a total volume of 20 μL, which contained 0.5 mM of cDNA, 0.5 mM of each primer and 10 × SYBR Green. The primers were described as follows: RIPK3 forward: 5′-CAAGATCGTAAACTCGAAGG-3′ and reverse: 5′-CCGTTCTCCATGAATTTAGT-3′ (Geserick et al. 2015); β-actin forward: 5′-ATGGGTCAGAAGGATTCCTATGTG-3′, and reverse: 5′-CTTCATGAGGTAGTCAGTCAGGTC-3′. The reaction of PCR was performed as follows: the initial step was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, and a final step of 72°C for 30 s. The RIPK3 mRNA levels were calculated by the $2(-^{\Delta\Delta}Ct)$ method.

ELISA for serum levels of TNF-α, MLKL and caspase-8

All blood samples were centrifuged at 2,000 rpm for 5 min to obtain the serum. The serum was then stored at –80°C until subsequent experiments. Serum levels of TNF-α (EIAab, Wuhan, China), caspase-8 (eBioscience, San Diego, CA, USA) and MLKL (Lengton Bioscience Co., Shanghai, China) were determined by ELISA. The lower detection limit of the TNF-α, MLKL and Caspase-8 was 3.9 pg/mL, 1 μg/mL and 0.1 ng/mL, respectively.

Clinical and pathological data

The serum biochemical markers included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), prothrombin activity (PTA), international normalized ratio (INR) and creatinine (Cr). Hepatitis B e antigen (HBeAg) was determined using an automatic analyzer (Roche Diagnostics, Basel, Switzerland). The serum HBV DNA level was quantified using a real-time PCR System (Applied Biosystems, Foster city, CA, USA). All markers were measured according to the standard methodologies at the Department of Laboratory Medicine, Qilu Hospital of Shandong University.

Model for end-stage liver diseases (MELD)

MELD score was calculated by the following formula:

MELD score = $9.57 \times \log_e$ [creatinine (mg/dl)] + $3.78 \times \log_e$ [bilirubin (mg/dl)] + $11.2 \times \log_e$ (INR) + 6.43 × (etiology: 0 if cholestatic or alcoholic, 1 otherwise) (Kamath et al. 2001).

Follow-up of patients with ACHBLF

All the patients with ACHBLF were followed up from the first date of diagnosis. They were followed up for at least 3 months for monitoring the outcome (death or survival).

Statistical analysis

Statistical analyses of the data were performed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as median (centile 25; centile 75). Categorical variables were expressed as number (%). Mann-Whitney *U*-test was performed to compare the quantitative variables. Chi-square test was applied to compare the categorical data. Spearman test was applied for correlation analysis. Diagnostic value of RIPK3 mRNA and MELD score in predicting 3-month mortality for ACHBLF patients was assessed by the area under the receiver operating characteristic curve (AUC). From the receiver operating characteristic (ROC) curve coordinates, the optimal cut-off point associated with the maximum of the Youden index was determined. Youden index-based cut-off point was determined. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) was used to assess the diagnostic accuracy. Survival curves of ACHBLF patients were drawn using the Kaplan-Meier method. All statistical analyses were two-sided and P value < 0.05 was considered statistically significant.

Results

Basic characteristic of the study population

From July 2014 to November 2015, 329 participants (139 ACHBLF patients, 155 CHB patients and 35 HCs) were screened at the Department of Hepatology, Qilu Hospital of Shandong University. Seventeen ACHBLF patients were excluded from this study for co-infection with

hepatitis C virus (n = 5), hepatocellular carcinoma (n = 8), alcohol abuse (n = 3) and autoimmune liver disease (n = 1). Twenty-four CHB patients were excluded from this study for co-infection with hepatitis C virus (n = 6), hepatocellular carcinoma (n = 11), alcohol abuse (n = 5) and pregnancy (n = 2), which left 288 participants (122 ACHBLF patients, 131 CHB patients and 35 HCs) (Fig. 1).

Table 1 showed the baseline characteristics of the participants. There was no significant difference between ACHBLF and CHB patients in sex (P = 0.100), age (P = 0.200), HBsAg (P = 0.085), HBeAg (P = 0.310) and $\log_{10}$[HBV DNA] (P = 0.080), respectively. However, significant differences were found between ACHBLF and CHB patients in ALT, AST, TBIL, ALB, PTA, INR and Cr (P < 0.001; respectively). Liver cirrhosis (LC), hepatic encephalopathy (HE) and ascites were found in 65, 39 and 63 patients with ACHBLF. In addition, the 3-month mortality of patients with ACHBLF was 46.72%. After 3-month follow-up, patients with ACHBLF were divided into survivors and non-survivors. The baseline characteristics are shown in Table 2.

RIPK3 mRNA levels in PBMCs

We measured RIPK3 mRNA levels in PBMCs of patients with ACHBLF, CHB and HCs. As shown in Fig. 2a, RIPK3 mRNA levels in patients with ACHBLF [9.64 (3.99, 22.68)] were significantly higher than those with CHB [1.81 (1.02, 3.78); P < 0.001] and HCs [0.78 (0.67, 1.35); P < 0.001]. Meanwhile, RIPK3 mRNA levels in patients with CHB were significantly higher than those in HCs (P < 0.001).

Correlation between RIPK3 mRNA levels and clinical parameters in patients with ACHBLF

Among patients with ACHBLF, we compared the RIPK3 mRNA levels in PBMCs between HBeAg (+) and HBeAg (–) patients. No significant difference was found between them (P = 0.522) (Fig. 2b). We also analyzed the correlations between RIPK3 mRNA levels in PBMCs and clinical parameters in patients with ACHBLF. There was no significant correlation between RIPK3 mRNA levels and $\log_{10}$[HBV DNA] (r = 0.051, P = 0.581), HBsAg (r = 0.127, P = 0.164), or ALB (r = 0.093, P = 0.308) (Fig. 2c, d, f). By contrast, RIPK3 mRNA levels were positively correlated with TBIL (r = 0.377, P < 0.001), Cr (r = 0.214, P < 0.05), INR (r = 0.364, P < 0.001), and MELD scores (r = 0.406, P < 0.001), respectively (Fig. 2e, g, h, i).

Furthermore, we compared RIK3 mRNA levels between patients with LC and those without LC, HE or ascites. Patients with LC, HE or ascites had significantly higher RIPK3 levels than those without (P < 0.05, respectively; Fig. 3).

Serum levels of TNF-α, MLKL and caspase-8

We also measured the serum levels of TNF-*α*, MLKL and caspase-8 (Fig. 4a-c). We found that the TNF-*α* levels in patients with ACHBLF [21.81 (12.51-36.90)] were significantly higher than those with CHB [9.34 (4.83-15.36), P < 0.001] and HCs [7.38 (5.29-8.83), P < 0.001]. There was no significant difference between patients with CHB and HCs (P = 0.058). The MLKL levels were significantly higher in patients with ACHBLF [45.71 (26.03-70.36)] than those with CHB [17.00 (11.21-29.31), P < 0.001] and HCs [11.01 (7.26-13.85), P < 0.001]. Meanwhile, Patients with CHB showed significantly higher MLKL levels than HCs (P < 0.001). In addition, patients with ACHBLF [0.38 (0.22-0.74)] showed decreased levels of caspase-8 than those with CHB [0.78 (0.58-0.97), P < 0.001] and HCs [0.67 (0.55-0.97), P < 0.001]. However, no significant difference was found between patients with CHB and HCs (P = 0.705).

Importantly, RIPK3 mRNA levels in patients with ACHBLF were positively correlated with TNF-*α* levels (r = 0.412, P < 0.001) and MLKL levels (r = 0.625, P < 0.001), respectively. By contrast, RIPK3 mRNA levels were negatively correlated with caspase-8 levels (r = –0.279, P < 0.05) (Fig. 4d-f).

RIPK3 mRNA levels as an independent predictive factor for the outcomes of ACHBLF patients

Univariate and multivariate regression analysis were

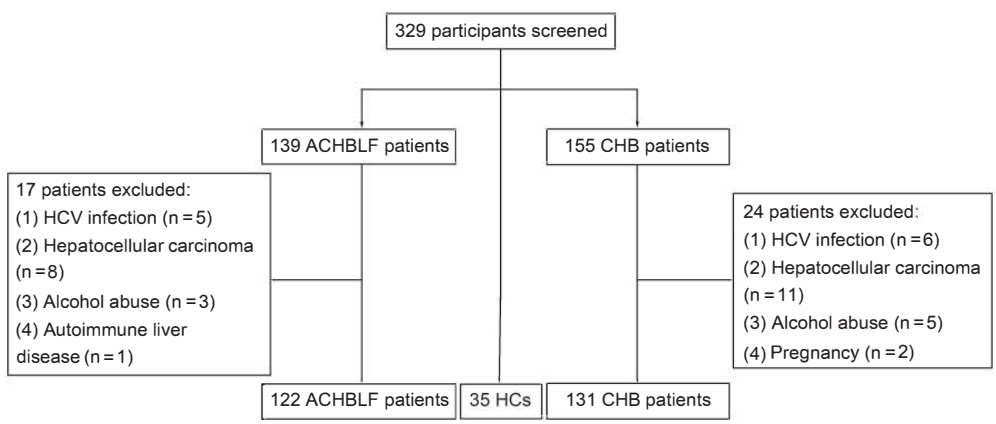

Fig. 1. Flow diagram depicting the participants' selection process.

Table 1. The baseline characteristics of enrolled participants.

Variables	ACHBLF (n = 122)	CHB (n = 131)	HCs (n = 35)	P value
Male (%)	95 (77.9)	90 (68.7)	22 (62.9)	0.100
Age (year)	46.5 (37.8-57.0)	44.0 (39.0-51.0)	28.0 (26.0-31.0)	0.200
HBsAg	3843.0 (951.3-6622.8)	3864.0 (1531.0-5257.0)	NA	0.085
$HBeAg^+$ (%)	72 (59.0)	69 (52.8)	NA	0.310
log_{10} [HBV DNA]	4.5 (3.3-5.8)	4.0 (2.8-6.0)	NA	0.080
ALT (U/L)	155.0 (95.8-382.3)	107.0 (45.0-261.0)	24.0 (19.0-29.0)	< 0.001*
AST (U/L)	125.5 (71.8-243.3)	56.0 (32.0-119.0)	25.0 (21.0-28.0)	< 0.001*
TBIL (μmol/L)	266.7 (177.4-411.9)	16.9 (11.5-27.1)	10.9 (8.4-13.2)	< 0.001*
ALB (g/L)	32.0 (29.3-36.0)	42.0 (39.1-44.8)	46.2 (44.4-47.8)	< 0.001*
Cr (μmol/L)	71.0 (61.0-86.0)	62.0 (54.0-73.0)	57.0 (50.0-61.0)	< 0.001*
INR	2.36 (1.91-3.10)	1.01 (0.97-1.09)	1.04 (1.00-1.09)	< 0.001*
PTA	30.0 (26.0-36.0)	94.0 (82.0-102.0)	98.0 (92.0-105.0)	< 0.001*
LC (%)	65 (53.3)	0	0	-
HE (%)	39 (32.0)	0	0	-
Ascites (%)	63 (51.6)	0	0	-
Mortality	57 (46.7)	0	0	-
MELD score	24.5 (21.1-28.0)	NA	NA	-

P value refers to the difference between patients with ACHBLF and CHB.
*Significant difference (P < 0.05).

performed to investigate the association between clinicopathological parameters and the outcomes of ACHBLF patients (Table 3). Univariate analysis identified that the outcomes of ACHBLF patients were significantly associated with TBIL (OR = 1.007, 95% CI: 1.004-1.010, P < 0.05), INR (OR = 4.269, 95% CI: 2.254-8.086, P < 0.05), PTA (OR = 0.865, 95% CI: 0.811-0.922, P < 0.05), ascites (OR = 2.775, 95% CI: 1.329-5.795, P < 0.05), LC (OR = 2.445, 95% CI: 1.175-5.087, P < 0.05), HE (OR = 4.740, 95% CI: 2.065-10.877, P < 0.05), RIPK3 mRNA levels (OR = 1.106, 95% CI: 1.063-1.151, P < 0.05), or MELD score (OR = 1.207, 95% CI: 1.110-1.312, P < 0.05). Then, multivariate regression analysis was performed with those variables. The RIPK3 mRNA level (OR = 1.082, 95% CI: 1.037-1.129, P < 0.05) was identified as an independent predictive factor for the outcome of ACHBLF patients.

Predictive value of RIPK3 mRNA levels for prognosis of ACHBLF

In this study, the 3-month mortality of ACHBLF patients was 46.7% (57/122). After 3-month follow-up, patients with ACHBLF were then divided into survivors and non-survivors. The 3-month mortality was significantly associated with ALT (P = 0.002), TBIL (P < 0.001), PTA (P < 0.001), INR (P < 0.001), LC (P = 0.016), HE (P < 0.001), ascites (P = 0.006) or MELD scores (P < 0.001). No significant association was found between 3-month mortality and gender (P = 0.480), age (P = 0.210), HBsAg (P = 0.633), HBeAg (P = 0.616), Log_{10} [HBV DNA] (P = 0.460), AST (P = 0.277), ALB (P = 0.226) or Cr (P = 0.125) (Table 2). Meanwhile, The RIPK3 mRNA levels in survivors were significantly higher than those in non-survivors (P < 0.001) (Fig. 5a).

Fig. 5b showed Kaplan-Meier survival analysis for ACHBLF patients with RIPK3 mRNA levels above 8.81 or not. Compared with patients with RIPK3 mRNA levels

Table 2. The baseline characteristics of survivors and non-survivors in patients with ACHBLF.

Variables	Survivors (n = 65)	Non-survivors (n = 57)	P value
Male (%)	49 (75.4)	46 (80.7)	0.480
Age (year)	46.0 (37.0-52.5)	47.0 (38.0-59.5)	0.210
HBsAg	3918.0 (1501.5-6302.0)	3276.0 (799.5-6868.0)	0.633
HBeAg+ (%)	37 (56.92)	35 (61.4)	0.616
$\log_{10}$[HBV DNA]	4.5 (3.5-5.9)	4.5 (3.0-5.8)	0.460
ALT (U/L)	117.0 (77.5-241.0)	227.0 (107.9-484.0)	0.002*
AST (U/L)	125.0 (69.5-219.0)	135.0 (74.0-282.5)	0.277
TBIL (μmol/L)	202.6 (152.3-294.8)	368.6 (249.8-479.2)	< 0.001*
ALB (g/L)	32.7 (30.2-35.8)	31.5 (27.7-36.0)	0.226
Cr (μmol/L)	67.0 (60.5-80.0)	75.0 (62.0-90.0)	0.125
INR	2.1 (1.7-2.7)	3.0 (2.3-3.3)	< 0.001*
PTA	35.0 (29.5-38.5)	27.0 (23.0-29.0)	< 0.001*
LC (%)	28 (43.1)	37 (64.9)	0.016*
HE (%)	11 (16.9)	28 (49.1)	< 0.001*
Ascites (%)	26 (40.0)	37 (64.9)	0.006*
MELD score	22.1 (19.7-25.3)	27.3 (24.0-31.6)	< 0.001**

*Significant difference ($P < 0.05$).

≤ 8.81, survival rate in those with RIPK3 mRNA levels > 8.81 were significantly lower (log rank analysis, $P < 0.001$). The mean survival time for ACHBLF patients with RIPK3 mRNA levels ≤ 8.81 or > 8.81 was 78.750 ± 3.232 days or 38.820 ± 4.395 days. Then, we performed the receiver operating characteristic (ROC) curve to evaluate the predictive value of RIPK3 mRNA levels for predicting 3-month mortality in patients with ACHBLF. As shown in Fig. 5c, the AUC of RIPK3 mRNA levels (0.810, 95% CI: 0.729-0.876) was higher than that of MELD scores (0.766, 95% CI: 0.681-0.838). With a cut-off point of 8.81, RIPK3 mRNA levels had a sensitivity of 80.7%, a specificity of 69.2%, a positive predictive value of 69.7%, and a negative predictive value of 80.4%.

Discussion

ACHBLF is characterized by its high morbidity and mortality. Accurate and effective noninvasive markers to predict the prognosis of ACHBLF are urgently needed (Wlodzimirow et al. 2013; Zheng et al. 2013). In this study, we found that RIPK3 mRNA levels were significantly higher in patients with ACHBLF than those with CHB or HCs. Meanwhile, the RIPK3 expression in ACHBLF patients was positively correlated with serum TBIL, INR and MELD score, which were often used to predict liver injury. A previous study already demonstrated that TBIL and INR were positively associated with mortality in patients with ACLF (Wlodzimirow et al. 2013). Meanwhile, MELD score was widely accepted as a model to evaluate short-term mortality in patients with end-stage liver diseases (Kamath et al. 2001; Northup et al. 2015).

In this study, we found that RIPK3 mRNA levels in PBMCs were associated with poor prognosis of ACHBLF. Therefore, it might serve as a prognostic marker in further clinical work. Meanwhile, patients with LC, HE or ascites had significantly higher RIPK3 mRNA levels than those without. A previous study also indicated that cirrhosis, HE as well as ascites were associated with adverse outcomes in patients with ACHBLF (Seto et al. 2012).

It was reported that necroptosis might participate in the pathogenesis of acute liver failure (ALF) and was associated with its prognosis (Rastogi et al. 2011). TNF-α, MLKL and caspase-8 were essential for the process of necroptosis (Holler et al. 2000). TNF-α is a cytokine involved in systemic inflammation. In fulminant liver failure, the serum level of TNF-α was significantly increased correlated with serious complications, such as infection and HE (de la Mata et al. 1990; Zhao et al. 2015). In the process of necroptosis, MLKL was phosphorylated and insert into the plasma membranes and organelles, which resulted in the expulsion of cellular contents into the extracellular space. Caspase-8 could negatively regulate the RIPK3 dependent necroptosis and participate in the pathogenesis of viral infection and inflammation (Remijsen et al. 2014). In this study, we found that serum TNF-α, MLKL levels were significantly elevated in ACHBLF. Serum caspase-8

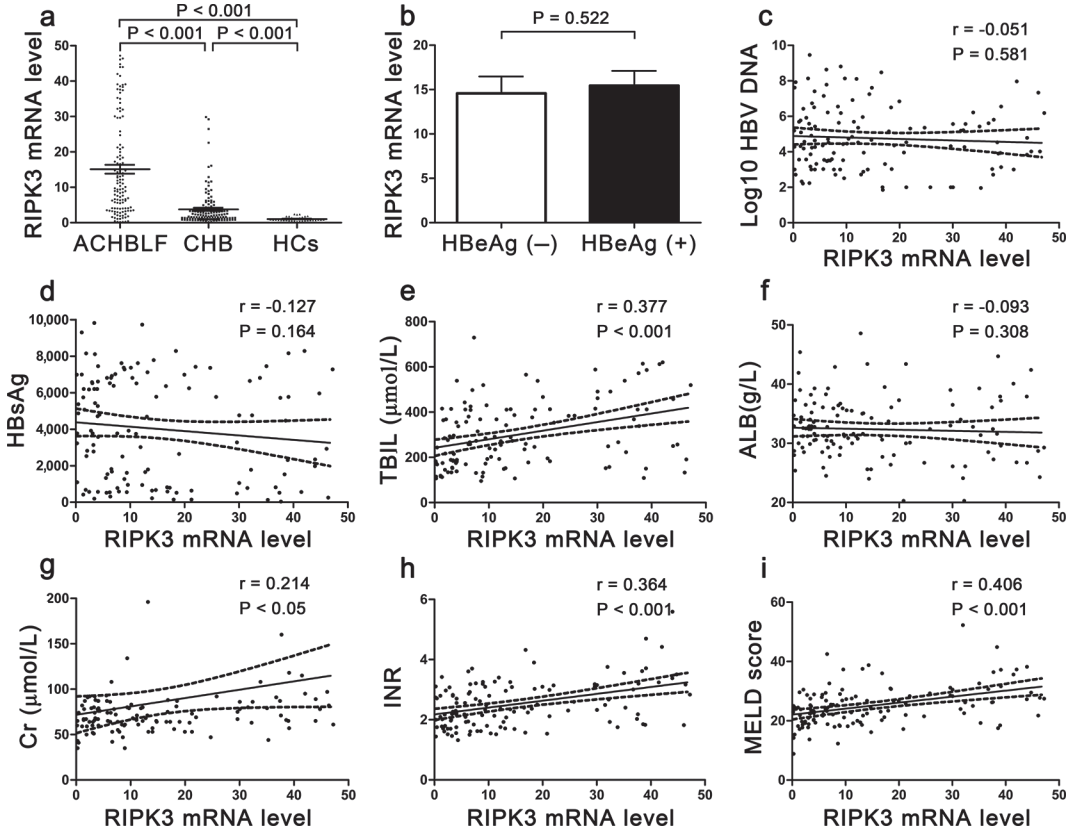

Fig. 2. The association of RIPK3 mRNA levels in PBMCs and clinical parameters.
a. The comparison among RIPK3 mRNA levels in patients with ACHBLF (n = 122), CHB (n = 131) and HCs (n = 35).
b. No significant differences (P = 0.522) were found between RIPK3 mRNA levels in HBeAg(+) group (n = 72) and HBeAg (–) group (n = 50).
c. No significant correlation (P = 0.581) was found between RIPK3 mRNA level and $\log_{10}$ [HBV DNA]. The solid line refers to the best-fit line, and the broken lines refer to the 95% confidence bands of the best-fit line.
d. No significant correlation (P = 0.164) was found between RIPK3 mRNA level and HBsAg.
e. Significant correlation (P < 0.001) was found between RIPK3 mRNA level and TBIL.
f. No significant correlation (P = 0.308) was found between RIPK3 mRNA level and ALB.
g. Significant correlation (P < 0.05) was found between RIPK3 mRNA level and Cr.
h. Significant correlation (P < 0.001) was found between RIPK3 mRNA level and INR.
i. Significant correlation (P < 0.001) was found between RIPK3 mRNA level and MELD score.

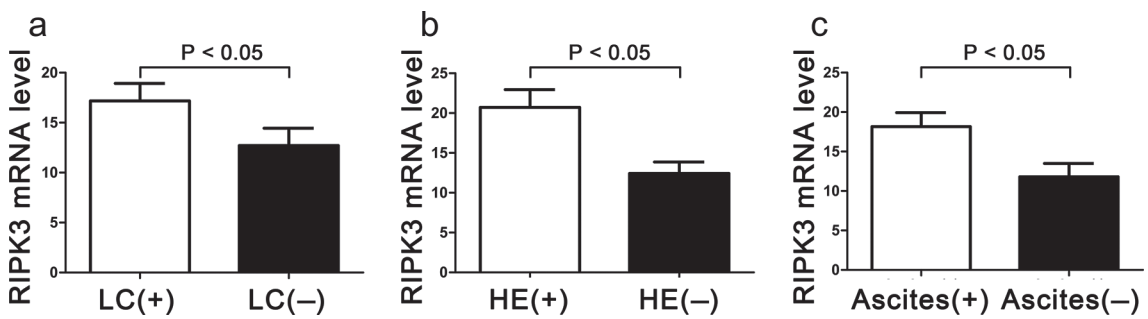

Fig. 3. The correlations between RIPK3 mRNA levels and complications of ACHBLF.
a. RIPK3 mRNA levels were significantly higher (P < 0.05) in patients with LC (n = 65) than those without (n = 57).
b. RIPK3 mRNA levels were significantly higher (P < 0.05) in patients with hepatic encephalopathy (HE) (n = 39) than those without (n = 83).
c. RIPK3 mRNA levels wre significantly higher (P < 0.05) in patients with ascites (n = 63) than those without (n = 59).

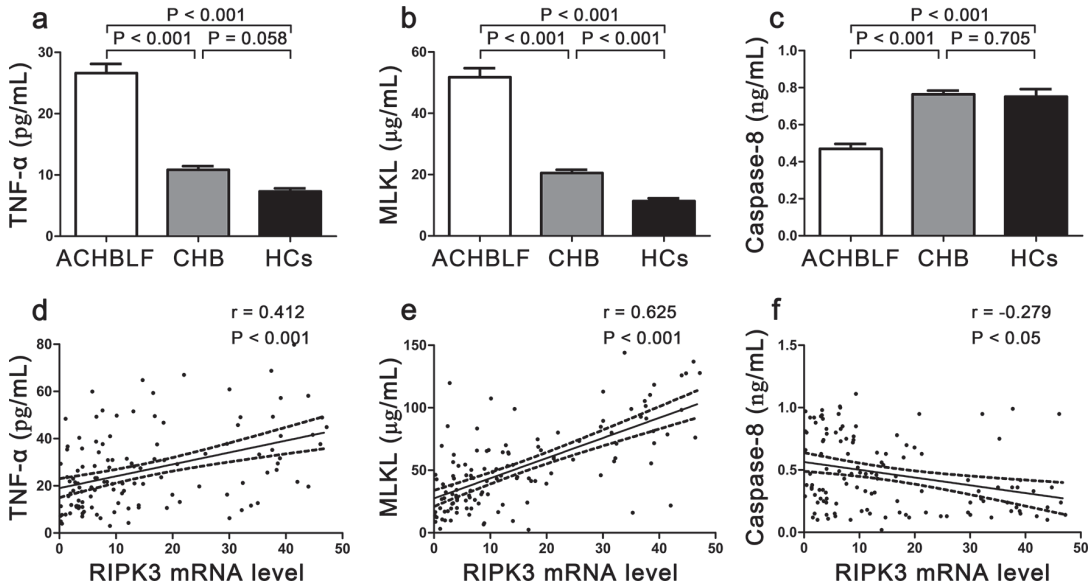

Fig. 4. The relationships between RIPK3 and RIPK3-associated cytokines.
a. The comparison among TNF-*α* levels in patients with ACHBLF, CHB and HCs.
b. The comparison among MLKL levels in patients with ACHBLF, CHB and HCs.
c. The comparison among Caspase-8 levels in patients with ACHBLF, CHB and HCs.
d. Significant correlation (P < 0.001) were found between RIPK3 mRNA levels and TNF-*α* levels.
e. Significant correlation (P < 0.001) were found between RIPK3 mRNA levels and MLKL levels.
f. Significant correlation (P < 0.05) was found between RIPK3 mRNA levels and Caspase-8 levels.

Table 3. Univariate and multivariate logistic analysis of clinical parameters for predicting the outcome of ACHBLF.

	Univariate			Multivariate		
	OR	95% CI	P	OR	95% CI	P
Male	0.732	0.308-1.742	0.481			
Age (year)	1.018	0.991-1.045	0.189			
HBsAg	1.000	1.000-1.000	0.545			
HBeAg	1.204	0.583-2.485	0.616			
$\log_{10}$[HBV DNA]	0.914	0.747-1.119	0.385			
ALT(U/L)	1.001	1.000-1.002	0.079			
AST(U/L)	1.001	1.000-1.003	0.074			
TBIL(μmol/L)	1.007	1.004-1.010	< 0.05*	1.003	0.999-1.007	0.171
ALB(g/L)	0.975	0.914-1.040	0.444			
Cr(μmol/L)	1.007	0.998-1.017	0.140			
INR	4.269	2.254-8.086	< 0.05*	1.679	0.524-5.376	0.383
PTA	0.865	0.811-0.922	< 0.05*	0.925	0.846-1.012	0.088
LC	2.445	1.175-5.087	< 0.05*	1.247	0.448-3.472	0.672
HE	4.740	2.065-10.877	< 0.05*	2.199	0.738-6.553	0.157
Ascites	2.775	1.329-5.795	< 0.05*	1.260	0.425-3.741	0.677
RIPK3 mRNA	1.106	1.063-1.151	< 0.05*	1.082	1.037-1.129	< 0.05*
MELD score	1.207	1.110-1.312	< 0.05*	0.992	0.873-1.127	0.905

*Significant difference (P < 0.05).

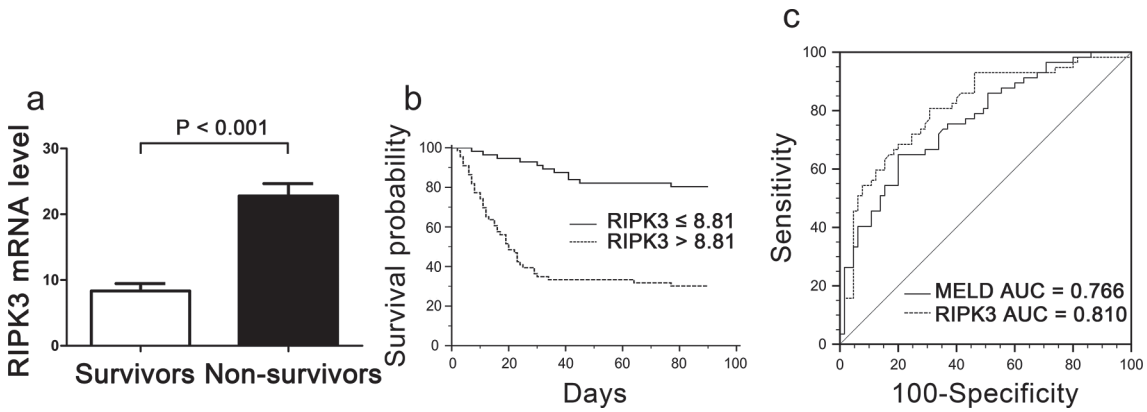

Fig. 5. ROC curve and the Kaplan–Meier graph of RIPK3 mRNA level in predicting 3-month mortality of patients with ACHBLF.

a. RIPK3 mRNA levels were significantly higher ($P < 0.001$) in non-survivors ($n = 57$) than survivors ($n = 65$).

b. Kaplan-Meier graph showing survival probability at the end of 3-month follow-up in ACHBLF patients with RIPK3 mRNA levels > 8.81 and ≤ 8.81.

c. ROC curves of RIPK3 mRNA level and MELD score in predicting 3-month mortality of ACHBLF patients.

level was significantly decreased in ACHBLF. Meanwhile, RIPK3 mRNA levels in patients with ACHBLF were positively correlated with TNF-α, MLKL levels and negatively correlated with caspase-8 levels. We, therefore, demonstrated that necroptosis might play an important role in the pathogenesis of ACHBLF.

Because of the coagulopathy, liver biopsies were hard to be performed in patients with ACHBLF. PBMCs were easy to be obtained from venous peripheral blood and suitable to be used as non-invasive markers in clinical work. Therefore, we evaluated the RIPK3 mRNA levels in PBMCs in this study. We demonstrated the potential predictive value of RIPK3 mRNA levels in PBMCs for prognosis of ACHBLF. However, the RIPK3 mRNA levels in PBMCs might not reflect the expression levels in hepatocytes. The expression of RIPK3 in hepatocytes and its potential role in the necroptosis of ACHBLF still need to be analyzed in further studies.

There were also several limitations in this study. Firstly, the molecular mechanism that how RIPK3 was involved in the development and progression of ACHBLF remained unclear and might be investigated in our further study. Secondly, the enrolled participants were from a single unit and data from large-scale, multi-center cohort might be more valuable. Therefore, these findings still need further validation prior to its clinical usage.

In conclusion, this study revealed that elevated RIPK3 mRNA levels in PBMCs were associated with poor prognosis of ACHBLF. We thus propose that necroptosis may play an important role in the pathogenesis of ACHBLF.

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Conflict of Interest

The authors declare no conflict of interest.

References

Arulraj, R. & Neuberger, J. (2011) Liver transplantation: filling the gap between supply and demand. *Clin. Med.* (*Lond*), **11**, 194-198.

de la Mata, M., Meager, A., Rolando, N., Daniels, H.M., Nouri-Aria, K.T., Goka, A.K., Eddleston, A.L., Alexander, G.J. & Williams, R. (1990) Tumour necrosis factor production in fulminant hepatic failure: relation to aetiology and superimposed microbial infection. *Clin. Exp. Immunol.*, **82**, 479-484.

Fan, Y.C., Wang, N., Sun, Y.Y., Xiao, X.Y. & Wang, K. (2015) TIPE2 mRNA level in PBMCs serves as a novel biomarker for predicting short-term mortality of acute-on-chronic hepatitis B liver failure: a prospective single-center study. *Medicine* (*Baltimore*), **94**, e1638.

Gao, S., Sun, F.K., Fan, Y.C., Shi, C.H., Zhang, Z.H., Wang, L.Y. & Wang, K. (2015) Aberrant GSTP1 promoter methylation predicts short-term prognosis in acute-on-chronic hepatitis B liver failure. *Aliment. Pharmacol. Ther.*, **42**, 319-329.

Geserick, P., Wang, J., Schilling, R., Horn, S., Harris, P.A., Bertin, J., Gough, P.J., Feoktistova, M. & Leverkus, M. (2015) Absence of RIPK3 predicts necroptosis resistance in malignant melanoma. *Cell Death Dis.*, **6**, e1884.

Gunther, C., He, G.W., Kremer, A.E., Murphy, J.M., Petrie, E.J., Amann, K., Vandenabeele, P., Linkermann, A., Poremba, C., Schleicher, U., Dewitz, C., Krautwald, S., Neurath, M.F., Becker, C. & Wirtz, S. (2016) The pseudokinase MLKL mediates programmed hepatocellular necrosis independently of RIPK3 during hepatitis. *J. Clin. Invest.*, **126**, 4346-4360.

Gunther, C., Martini, E., Wittkopf, N., Amann, K., Weigmann, B., Neumann, H., Waldner, M.J., Hedrick, S.M., Tenzer, S., Neurath, M.F. & Becker, C. (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. *Nature*, **477**, 335-339.

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.L., Schneider, P., Seed, B. & Tschopp, J. (2000) Fas triggers an alternative, caspase-8-independent

cell death pathway using the kinase RIP as effector molecule. *Nat. Immunol.*, **1**, 489-495.

Jalan, R., Gines, P., Olson, J.C., Mookerjee, R.P., Moreau, R., Garcia-Tsao, G., Arroyo, V. & Kamath, P.S. (2012) Acute-on chronic liver failure. *J. Hepatol.*, **57**, 1336-1348.

Kamath, P.S., Wiesner, R.H., Malinchoc, M., Kremers, W., Therneau, T.M., Kosberg, C.L., D'Amico, G., Dickson, E.R. & Kim, W.R. (2001) A model to predict survival in patients with end-stage liver disease. *Hepatology*, **33**, 464-470.

Katoonizadeh, A., Laleman, W., Verslype, C., Wilmer, A., Maleux, G., Roskams, T. & Nevens, F. (2010) Early features of acute-on-chronic alcoholic liver failure: a prospective cohort study. *Gut*, **59**, 1561-1569.

Laleman, W., Verbeke, L., Meersseman, P., Wauters, J., van Pelt, J., Cassiman, D., Wilmer, A., Verslype, C. & Nevens, F. (2011) Acute-on-chronic liver failure: current concepts on definition, pathogenesis, clinical manifestations and potential therapeutic interventions. *Expert Rev. Gastroenterol. Hepatol.*, **5**, 523-537; quiz 537.

Linkermann, A. & Green, D.R. (2014) Necroptosis. *N. Engl. J. Med.*, **370**, 455-465.

Lok, A.S. & McMahon, B.J. (2009) Chronic hepatitis B: update 2009. *Hepatology*, **50**, 661-662.

Northup, P.G., Intagliata, N.M., Shah, N.L., Pelletier, S.J., Berg, C.L. & Argo, C.K. (2015) Excess mortality on the liver transplant waiting list: unintended policy consequences and Model for End-Stage Liver Disease (MELD) inflation. *Hepatology*, **61**, 285-291.

Polson, J. & Lee, W.M.; American Association for the Study of Liver Disease (2005) AASLD position paper: the management of acute liver failure. *Hepatology*, **41**, 1179-1197.

Rastogi, A., Kumar, A., Sakhuja, P., Bihari, C., Gondal, R., Hissar, S., Garg, H. & Sarin, S.K. (2011) Liver histology as predictor of outcome in patients with acute-on-chronic liver failure (ACLF). *Virchows Arch.*, **459**, 121-127.

Remijsen, Q., Goossens, V., Grootjans, S., Van den Haute, C., Vanlangenakker, N., Dondelinger, Y., Roelandt, R., Bruggeman, I., Goncalves, A., Bertrand, M.J., Baekelandt, V., Takahashi, N., Berghe, T.V. & Vandenabeele, P. (2014) Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. *Cell Death Dis.*, **5**, e1004.

Sarin, S.K., Kedarisetty, C.K., Abbas, Z., Amarapurkar, D., Bihari, C., Chan, A.C., Chawla, Y.K., Dokmeci, A.K., Garg, H., Ghazinyan, H., Hamid, S., Kim, D.J., Komolmit, P., Lata, S., Lee, G.H., et al. (2014) Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL) 2014. *Hepatol. Int.*, **8**, 453-471.

Sarin, S.K., Kumar, A., Almeida, J.A., Chawla, Y.K., Fan, S.T., Garg, H., de Silva, H.J., Hamid, S.S., Jalan, R., Komolmit, P., Lau, G.K., Liu, Q., Madan, K., Mohamed, R., Ning, Q., et al. (2009) Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the study of the liver (APASL). *Hepatol. Int.*, **3**, 269-282.

Seto, W.K., Lai, C.L. & Yuen, M.F. (2012) Acute-on-chronic liver failure in chronic hepatitis B. *J. Gastroenterol. Hepatol.*, **27**, 662-669.

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H. & Vandenabeele, P. (2014) Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat. Rev. Mol. Cell Biol.*, **15**, 135-147.

Wang, H., Sun, L., Su, L., Rizo, J., Liu, L., Wang, L.F., Wang, F.S. & Wang, X. (2014) Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol. Cell*, **54**, 133-146.

Wlodzimirow, K.A., Eslami, S., Abu-Hanna, A., Nieuwoudt, M. & Chamuleau, R.A. (2013) A systematic review on prognostic indicators of acute on chronic liver failure and their predictive value for mortality. *Liver Int*, **33**, 40-52.

Zhao, Z.H., Fan, Y.C., Zhao, Q., Dou, C.Y., Ji, X.F., Zhao, J., Gao, S., Li, X.Y. & Wang, K. (2015) Promoter methylation status and expression of PPAR-gamma gene are associated with prognosis of acute-on-chronic hepatitis B liver failure. *Clin. Epigenetics*, **7**, 115.

Zheng, Y.B., Huang, Z.L., Wu, Z.B., Zhang, M., Gu, Y.R., Su, Y.J., Lin, C.S., Zhu, R.H., Lin, B.L. & Gao, Z.L. (2013) Dynamic changes of clinical features that predict the prognosis of acute-on-chronic hepatitis B liver failure: a retrospective cohort study. *Int. J. Med. Sci.*, **10**, 1658-1664.