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Original Article

Elevated level of circulating histones in acute-on-chronic liver failure patients and mice with ConA-induced acute liver injury

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Abstract: Background: Hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) is characterized by a systematic inflammatory response and immunity disorder. Histones are extracellular damage-associated molecular pattern (DAMP) molecules that modulate the inflammatory response in a wide range of diseases; however, their clinical significance in HBV-related ACLF remains elusive. Objective: We aimed to elucidate the relationship between the expression of circulating histones and disease severity in ACLF patients. Method: We measured the histone mRNA expression in the peripheral blood mononuclear cells (PBMCs) of ACLF patients. We also measured serum histones and other inflammatory cytokines in both ACLF patients and ConA-induced liver injury/failure mice. Results: Circulating histone levels were significantly higher in ACLF patients than chronic hepatitis B patients and healthy volunteers ($P<0.05$ and $P<0.001$, respectively). No significant difference in HIST2H4A mRNA expression was observed between groups. Serum histone levels closely correlated with serological markers and cytokine levels, including total bilirubin (TBIL), international normalized ratio (INR), model for end-stage liver disease (MELD), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6). A negative correlation was found between circulating histones and the prognosis of ACLF patients. Conclusion: Excessive circulating histones may play a crucial role in the systemic inflammation associated with ACLF and represent a potential therapeutic target.

Keywords: Circulating histones, ACLF, acute liver injury

Introduction

Acute-on-chronic liver failure (ACLF) is defined by severe acute deterioration of liver function due to chronic liver diseases [1] and is associated with an extremely high mortality rate [2]. According to the Asian Pacific Association for the Study of the Liver (APASL) consensus, the acute and severe hepatic derangements manifested in ACLF result from variable insults. In most Asian countries, a hepatitis B virus (HBV) infection accounts for 70% of all ACLF cases [3]. Numerous clinical studies and basic research on HBV-related ACLF have been conducted; however, the pathophysiology and exact mechanisms of HBV-related ACLF remains unclear, and a truly effective treatment has not been achieved.

Although the pathophysiology of chronic hepatitis B (CHB)-related ACLF remains poorly understood, an increased systemic inflammatory response mainly mediated by cytokines is thought to play a pivotal role. In patients with a continuous HBV infection, an over-reaction of the immune system mediated by cytotoxic T-lymphocytes leads to the significant, persistent, and unregulated necrosis of hepatocytes and the overwhelming release of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), high mobility group box 1 (HMGB1), and interleukin 18 (IL-18). These pro-inflammatory cytokines may perpetuate liver damage and extend the inflammatory cascade, which contributes to disease activity, the decompensation of liver function, and ultimately liver failure [4].

Histones are intracellular nucleosome components and a new class of extracellular damage-associated molecular pattern (DAMP) molecules that modulate chromatin remodeling and the inflammatory response. Inside the nucleus, histones and DNA compose a complex known as the nucleosome, which constitutes the basic repeating subunit of chromatin [5-7]. Outside the cell, the release of nuclear DAMPs, including histones, HMGB1, and circulating DNA, has been implicated in many inflammatory diseases [8]. The functions of extracellular histones and nucleosomes have been intensely studied in various inflammatory models [9-11]. Additionally, histones contribute to death from inflammation-based injuries and chemical-induced liver injuries [12, 13]. However, the expression and clinical role of circulating histones on ACLF remains obscure. To explore this clinical issue, we assessed circulating histones in HBV-related ACLF patients and mice with ConA-induced acute liver injury/failure, the correlation between circulating histones and important serological markers, and the diagnostic and prognostic value of circulating histones.

Patients and methods

Study subjects

Sixty-four confirmed HBV-related ACLF and CHB patients who were attending the outpatient department or admitted to the infection department from September 2015 to January 2016 at the Xiangya Hospital of Central South University (Changsha, Hunan) were enrolled in this study. Thirty sex- and age-matched healthy individuals who had no indication of viral hepatitis infection or other liver diseases were recruited as healthy controls. Informed written consent was obtained from patients and healthy volunteers. The study was approved by the Medical Ethics Committee of Xiangya hospital at Central South University.

The diagnostic criteria for CHB used in this study followed the Asian-Pacific consensus statement on the management of chronic hepatitis B [14-16]. Briefly, CHB patients were diagnosed based on histopathological manifestation and/or consistent laboratory results and an ultrasonographic diagnosis.

HBV-related ACLF, according to the consensus recommendations of the APASL [17], was defined as an acute hepatic insult complicated by ascites and/or encephalopathy within 4 weeks in a patient with previously diagnosed or undiagnosed chronic liver disease. No serious complications occurred in any ACLF patient when admitted, e.g., hepatic encephalopathy (degree III or higher), hepatorenal syndrome, severe infection, or other refractory complications. The exclusion criteria were co-infection with hepatitis C virus, hepatitis D virus, hepatitis E virus and/or human immunodeficiency virus, autoimmune liver disease, or hepatocellular carcinoma.

The subjects were divided into three groups: ACLF, chronic hepatitis B (CHB), and healthy volunteers (HV). The ACLF group was divided into survivor and non-survivor groups based on the mortality of ACLF patients within two months.

Sample collection

Serum samples collected from healthy volunteers and patients within 24 h after admission were centrifuged at 3000 g for 15 min within 1-2 h after venipuncture. Subsequently, serum samples were aliquoted and stored with 10 mM EDTA at -80°C [18].

Histones, cytokine ELISAs, and determination of blood parameters

Serum samples were used to measure the concentration of circulating histones, TNF-α, and interleukin 6 (IL-6). We used the serum levels of nucleosomes to reflect the released histone concentration [19]. The serum concentration of histones, TNF-α, and IL-6 were assayed using the Cell Death Detection-ELISA^{plus} Kit from Roche Diagnostics (Mannheim, Germany) (No. 1774 425) and Human TNF-α/IL-6 Immunoassay Valukine™ ELISA Kit from R&D (San Diego, CA, USA), respectively. Histone concentrations were calculated by establishing a standard curve with cow nucleosome antigen full-length protein (ab136595, Abcam, USA) as a standard. Baseline laboratory results were obtained at admission for all patients, including total bilirubin (TBIL), alanine aminotransferase (ALT), albumin (ALB), international normalized ratio (INR), and prothrombin time activity (PTA), using standard clinical chemistry methods and automated analyzers. Additionally, the model for end-stage liver disease (MELD) score was calculated.

Table 1. Primers used for qRT-PCR

Gene	Primer sequence (5' to 3')
β-actin Forward	5'-CCTGGCACCCAGCACAAT-3'
β-actin Reverse	5'-GGGCCGGACTCGTCATAC-3'
HIST2H4A Forward	5'-ACATTCAGGGCATCACCAAGCC-3'
HIST2H4A Reverse	5'-TCTCCAGGAACACCTTCAGCAC-3'

The ΔΔCT method was used to calculate relative histone gene expression.

Table 2. Clinicopathological information of patients and healthy volunteers

	ACLF	CHB	HV
Gender (M/F)	27/6	24/7	25/5
Age in years	44.2±11.7	40.3±10.2	39.5±5.4
TBIL (umol/L)	403.5 (335.5, 514.1)[a,b]	60.5 (17.9, 105.2)[c]	8.9 (6.3, 12.4)
ALT (U/L)	437.7 (130.6, 808.4)[b]	321.4 (64.9, 742.0)[c]	36.3 (27.8, 42.8)
ALB (g/L)	32.6 (31.3, 34.8)[a,b]	41 (36.9, 44.7)[c]	46.7 (43.6, 47.8)
PTA (%)	29.4 (23.9, 39.0)[a,b]	96.7 (72.3, 108.7)[d]	106.1 (97.3, 111.6)
INR	2.1 (1.9, 2.4)[a,b]	1.2 (1.0, 1.3)[d]	1.0 (0.9, 1.1)

[a]P<0.0001 vs CHB group. [b]P<0.0001 vs HV group. [c]P<0.0001 vs HV group. [d]P<0.05 vs HV group. ACLF = acute-on-chronic liver failure; CHB = chronic hepatitis B; HV = healthy volunteers; M = male; F = female; TBIL = total bilirubin; ALT = alanine transaminase; ALB = albumin; PTA = prothrombin time activity; INR = international normalized ratio.

RNA extraction from PBMCs followed by qRT-PCR

Whole blood was collected from patients and healthy donors. PBMCs were isolated by density-gradient centrifugation using a Lymphocytes separation medium from Tianjin Hao Yang Biological Manufacture Co., Ltd. Total RNA was extracted from PBMCs using an Omega total RNA Kit (R6934-02). The relative histone mRNA expression was measured by qRT-PCR, and cDNA was transcribed from mRNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The qRT-PCR was subsequently performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s, and 95°C for 15 s. Reactions were completed in triplicate with human β-actin as an internal control.

Xu et al. reported that histone H4 was cytotoxic and a tissue injury mediator [20, 21]. Histone proteins consist of massive clusters; therefore, we used the mRNA expression of HIST2H4A as a representative in the PBMCs of CHB patients. The primers used for qRT-PCR are listed in **Table 1**.

ConA-induced liver injury in mice

Twelve male C57BL/6 mice aged 6 to 8 weeks-old with a weight of 18-22 g were obtained from the Experimental Animal Center of Xiangya Medical College at Central South University. All mice were treated in accordance with the strict guidelines of the National Institution of Health for the experimental care and use of animals [22]. Our experimental procedures were approved by the Medical Ethics Committee of Xiangya hospital at Central South University.

Mice were randomly distributed into two groups: ConA and control. Mice were intravenously injected via the tail with 20 mg/kg of ConA (Sigma Chemical Co. St. Louis, MO, USA) or pyrogen-free saline as a control [23]. All animals were euthanized 24 h after injection and then serum samples were collected.

Serum concentrations of ALT and aspartate aminotransferase (AST) were examined using an automated analyzer. Liver sections were fixed with a 10% neutral formalin solution, washed multiple times with PBS times, embedded in paraffin, and cut into several sections with a thickness of 5 μm. Liver sections were stained with hematoxylin and eosin (H&E) using standard protocols and analyzed by light microscopy [12].

Statistical analysis

The quantitative data were expressed as dispersion (standard deviation & IQR) according to their normality. Kruskal-Wallis and Mann-Whitney U tests were used to compare data between groups. A receiver operating curve (ROC) was generated, and the area under the curve (AUC),

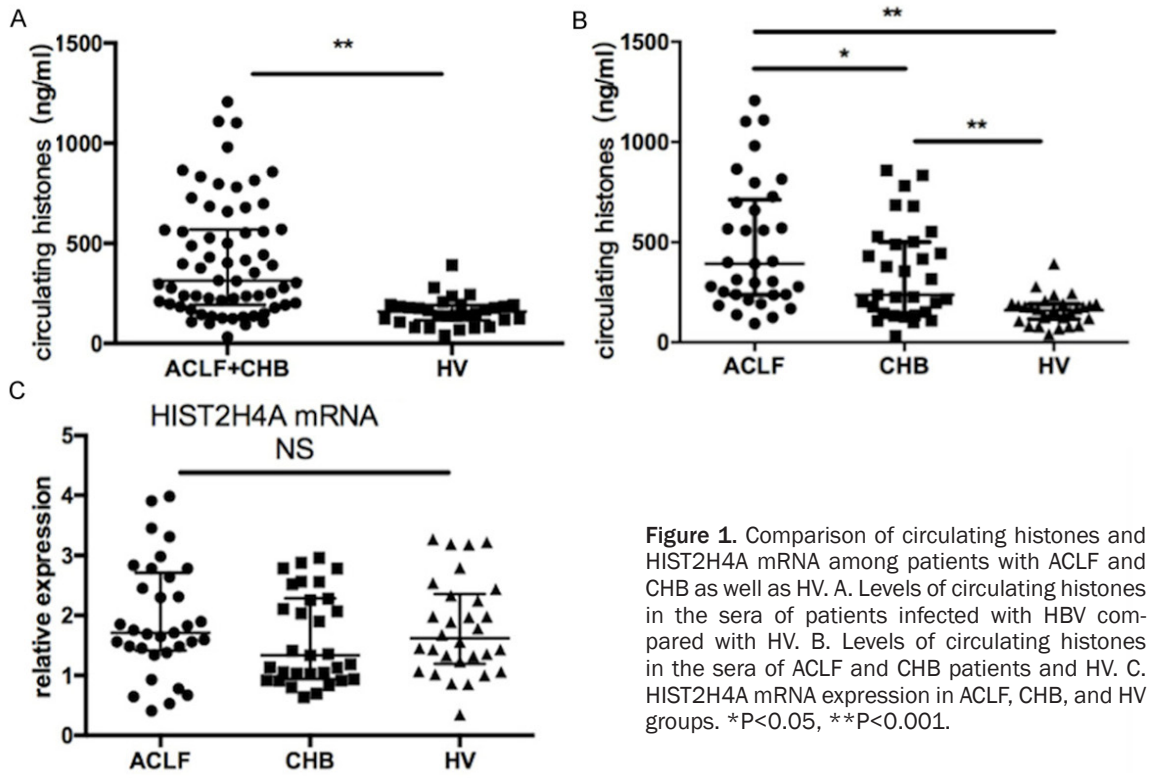


Figure 1. Comparison of circulating histones and HIST2H4A mRNA among patients with ACLF and CHB as well as HV. A. Levels of circulating histones in the sera of patients infected with HBV compared with HV. B. Levels of circulating histones in the sera of ACLF and CHB patients and HV. C. HIST2H4A mRNA expression in ACLF, CHB, and HV groups. *P<0.05, **P<0.001.

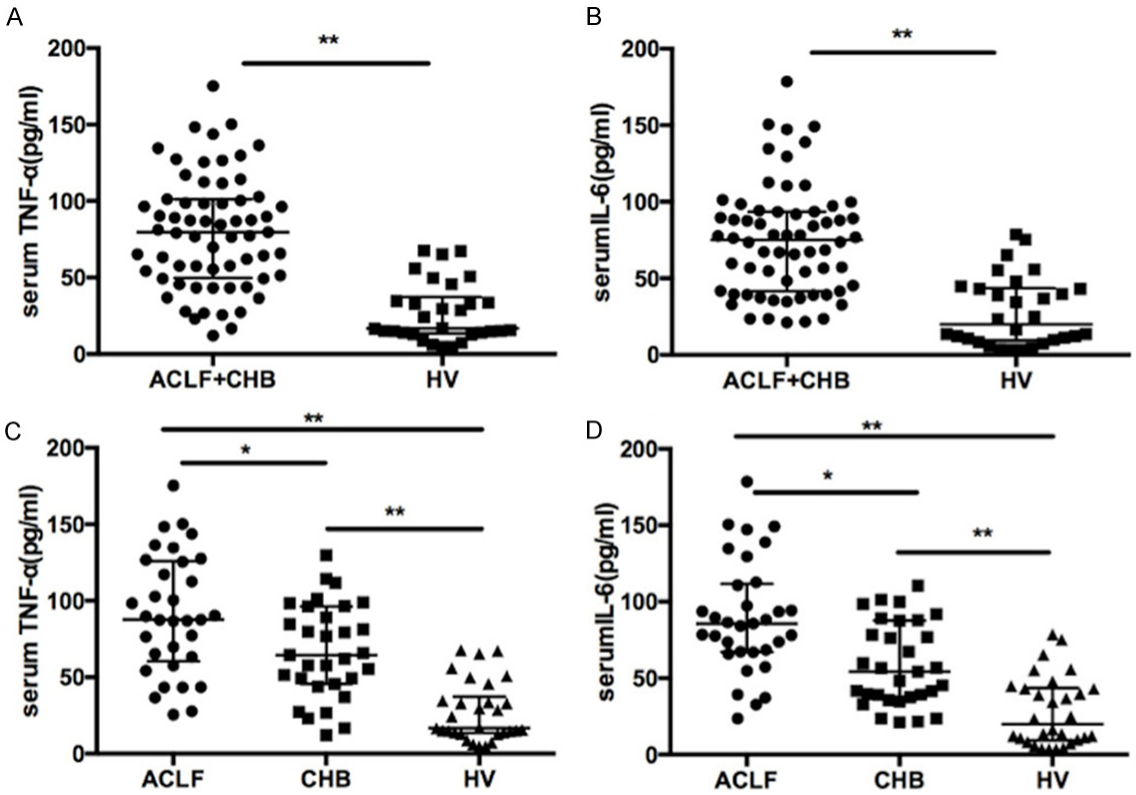


Figure 2. Comparisons of serum TNF-α and IL-6 among the ACLF, CHB, and HV groups. A. Serum TNF-α in patients with HBV compared with HV. B. Serum IL-6 in patients with HBV compared with HV. C. Serum TNF-α in ACLF and CHB patients and HV. D. Serum IL-6 in ACLF and CHB patients and HV. *P<0.05, **P<0.001.

Table 3. Biochemical parameters compared between survivors and non-survivors in ACLF patients

	Survivors (n=11)	Non-survivors (n=22)
TBIL (umol/L)	367.4 (350.2, 474.2)	423.9 (304.2, 511.4)
ALT (U/L)	675.1 (138.0, 947.4)	353.1 (117.8, 689.5)
ALB (g/L)	32.4 (30.9, 34.5)	32.7 (31.7, 34.8)
PTA (%)	38.1 (30.4, 42.0)*	26.6 (22.4, 34.7)
INR	1.9 (1.7, 2.1)*	2.2 (1.9, 2.9)
MELD	28.7 (27.2, 29.8)	28.3 (27.3, 30.5)

*P<0.05 vs non-survivors. TBIL = total bilirubin; ALT = alanine transaminase; ALB = albumin; PTA = prothrombin time activity; INR = international

sensitivity, and specificity were calculated. Patient survival rates were calculated using the Kaplan-Meier method, and statistically significant differences in survival were identified using the log-rank test. A Spearman's rank correlation was used to assess correlations between variables. A *p*-value less than 0.05 was considered statistically significant. SPSS 21.0 was used to complete the statistical analyses.

Results

Demographics

The baseline clinical characteristics of the ACLF, CHB, and HV groups are summarized in **Table 2**. Of the 64 ACLF and CHB patients, males drastically outnumbered females with a ratio of 3.9:1. However, there were no significant differences in age among groups. Notably, TBIL (P<0.0001) and INR (P<0.0001) levels were significantly higher in ACLF patients when compared with CHB patients (**Table 2**).

Circulating histone levels were increased in ACLF patients

The median serum histone levels for ACLF, CHB, and HV groups were 391.5 [237.7-697.8] ng/ml, 236.5 [147.3-488.0] ng/ml, and 158.8 [120.2-189.5] ng/ml, respectively. The serum histone levels were increased in HBV-related hepatitis patients when compared with healthy volunteers (P<0.001, **Figure 1A**). Notably, the level of circulating histones was significantly higher in ACLF patients when compared with the CHB group (P<0.05, **Figure 1B**).

Next, we explored if histone expression was altered at the transcription level. Using β-actin as a housekeeping gene, the relative expression of HIST2H4A in PBMCs was evaluated. The HIST2H4A gene expression in healthy controls was set as one. However, there were no significant differences between the three groups (P= 0.2997) (**Figure 1C**).

Serum TNF-α and IL-6 levels were elevated in ACLF patients

The median circulating TNF-α levels in ACLF, CHB, and HV groups were 102.8 [86.9, 126.5] pg/ml, 64.4 [47.4, 92.7] pg/ml, and 15.5 [13.8, 29.4] pg/ml, respectively, and the median circulating IL-6 values were 89.3 [73.8, 112.5] pg/ml, 59.7 [41.6, 88.5] pg/ml, and 10.3 [7.5, 22.8] pg/ml, respectively. The serum levels of TNF-α and IL-6 were increased in HBV-related hepatitis patients when compared with healthy controls (P<0.001, **Figure 2A** and **2B**). Notably, the levels of TNF-α and IL-6 were significantly higher in ACLF patients when compared with the CHB group (all P<0.05, **Figure 2C** and **2D**).

The levels of circulating histones and specific serological markers were significantly different between survivors and non-survivors of ACLF

The mortality of ACLF patients was 22/33 (66.67%). Biochemical parameters were compared between surviving and non-surviving ACLF patients (**Table 3**). The PTA (P<0.05) was significantly lower and the INR (P<0.05) was significantly higher in non-survivors when compared with survivors. However, no difference was found between these two groups for TBIL, ALT, and MELD scores. We observed a significant difference in the circulating levels of serum histones (**Figure 3A**, P<0.05) between these two groups, whereas the serum levels of TNF-α and IL-6 were not significantly different.

Circulating histone levels correlated with the prognosis of ACLF patients

Plotting the ROC for the circulating histone levels of ACLF patients revealed that circulating histones successfully predicted mortality within two months (AUC=0.7459, 95% CI=0.5722-0.9195, P<0.05). A histone concentration of 236.6 ng/ml yielded a sensitivity of 86.36% and specificity of 54.55% (**Figure 4**).

According to the cut-off value of 236.6 ng/ml, we divided ACLF patients into two groups: high

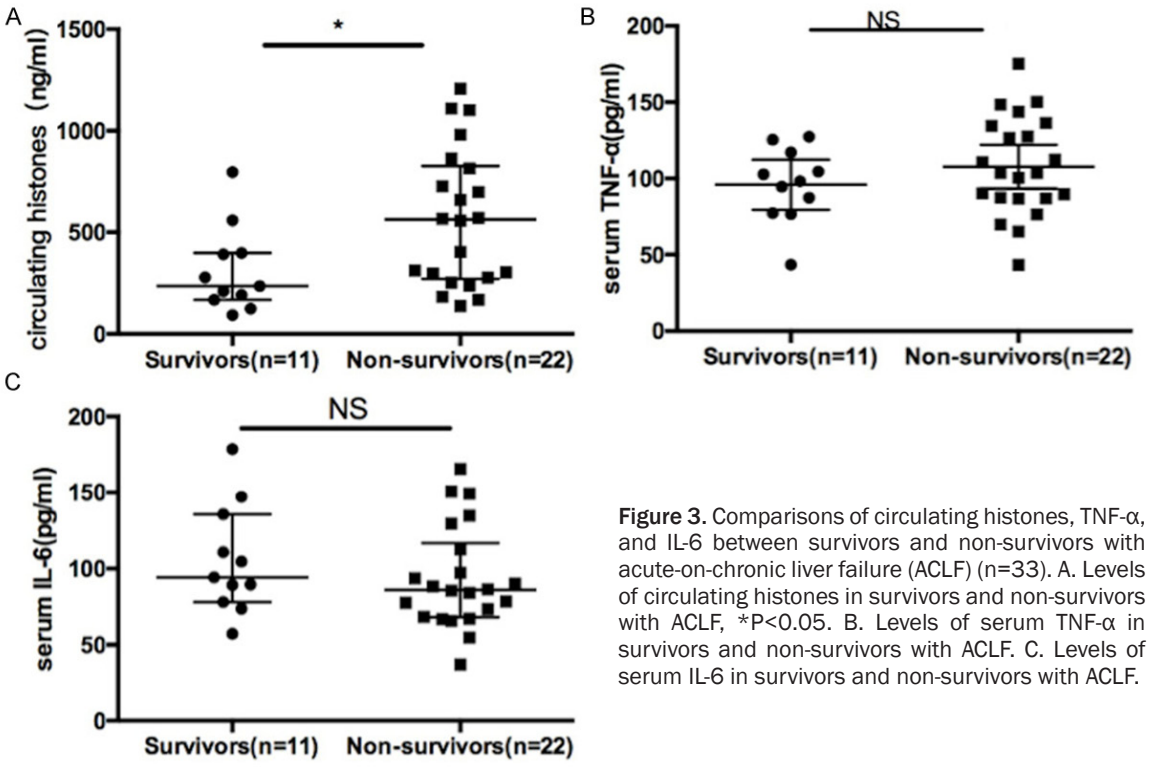


Figure 3. Comparisons of circulating histones, TNF-α, and IL-6 between survivors and non-survivors with acute-on-chronic liver failure (ACLF) (n=33). A. Levels of circulating histones in survivors and non-survivors with ACLF, *P<0.05. B. Levels of serum TNF-α in survivors and non-survivors with ACLF. C. Levels of serum IL-6 in survivors and non-survivors with ACLF.

and low histone expression. The association between circulating histone levels and prognosis of ACLF patients was evaluated using a Kaplan-Meier analysis and log-rank tests. The Kaplan-Meier survival analysis indicated that high histone levels in ACLF patients was significantly associated with a worse overall survival within two months (χ^2=4.632, P=0.0314) (**Figure 5**).

Circulating histone levels correlated with other serological markers

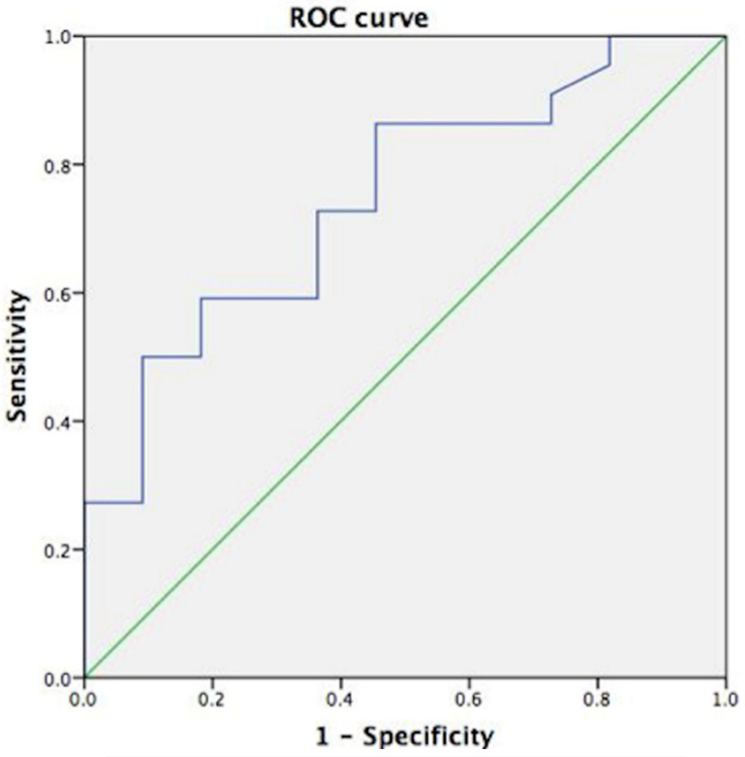
Circulating histone levels in ACLF patients positively correlated with serum TBIL and INR values (r=0.3668, 0.4332, all P<0.05) and negatively correlated with PTA levels (r=-0.4195, P<0.05). However, no correlation was found between circulating histone levels and ALT. A positive correlation was also observed between MELD scores and circulating histone levels in ACLF patients (r=0.3862, P<0.05). We completed a correlation analysis of individual TNF-α and IL-6 values and serum histone levels in ACLF patients. Serum TNF-α and IL-6 levels positively correlated with histone values (r=0.3488, 0.4001, all P<0.001) (**Figure 6A-F**).

Circulating histone levels were increased in mice with ConA-induced acute liver injury

A murine model of severe liver injury induced by ConA was established, as evidenced by a typical histopathological appearance (**Figure 7B**) and elevated serum ALT and AST levels (**Figure 7C**). Following the ConA challenge, the level of circulating histones were significantly elevated when compared with the control group (P<0.01, **Figure 7D**). The median (interquartile range) serum histone levels were 1115.1679 [1097.7849-1140.0701] ng/ml for the liver injury/failure group and 288.0140 [237.1865-311.0492] ng/ml for healthy mice. Circulating histone levels in ConA-induced mice and healthy mice positively correlated with serum ALT and AST values (r=0.8042, 0.7692, all P<0.005).

Discussion

The pathophysiology of HBV-related ACLF remains poorly understood. Various clinical trials and animal experiments have shown that ACLF initiates excessive and enduring systemic inflammation mediated by cytokines, e.g., TNF-



Circulating histones (ng/ml)	Sensitivity	Specificity
236.6	86.36%	54.55%

Figure 4. ROC curve of circulating histones in ACLF patients.

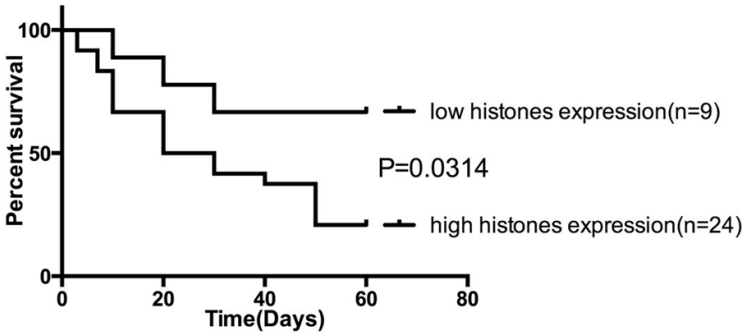


Figure 5. Kaplan-Meier curves revealed an association of higher circulating histone levels with a shorter overall survival (P=0.0314).

α, IL-2, IL-6, IL-10 [24-26]. Accumulating evidence revealed that extracellular histones fuel inflammation by promoting downstream inflammatory responses that lead to the production of TNF-α, IL-6, IL-1β, IL-10, and IL-12p70 [12, 13]. Increased circulating histones were implicated in patients and experimental animals suffering from sepsis [20], hand, foot, and mouth disease (HFMD) [19], septic shock [27], and trauma [11]; furthermore, the histone levels were associated with disease severity. Hence, we investigated the expression of circulating histones in HBV-induced ACLF patients and acute liver injury mice.

Histones are found in nucleosome fragments of the extracellular space complexed with DNA [20]. The measurement of nucleosome levels, which include all histone proteins, can be used as a relative quantification of histones [28, 29]. Here we showed that serum histone levels in healthy volunteers were slightly elevated. This is in accordance with previous studies indicating that small amounts of histones are released under physiological conditions [30-34]. Several previous studies reported significantly elevated circulating histone levels in various murine models of liver injury [12, 21, 35]. We observed that circulating histone levels were markedly increased in ACLF and CHB patients when compared with healthy controls. Notably, histone levels were higher in the ACLF group when compared with the CHB group. The same trend was observed in mice with ConA-induced acute liver failure. In accordance with the increase in circulating histones, serum cytokines, including TNF-α and IL-6, were elevated in ACLF patients.

Interestingly, there was no significant difference in HIST2H4A mRNA levels in the PBMCs of chronic hepatitis B patients when compared with healthy volunteers. We did not observe a significant difference in H3F3A mRNA and H3F3B mRNA levels among the three groups (data not shown). There are three possible reasons for this finding: 1) the elevated circulating

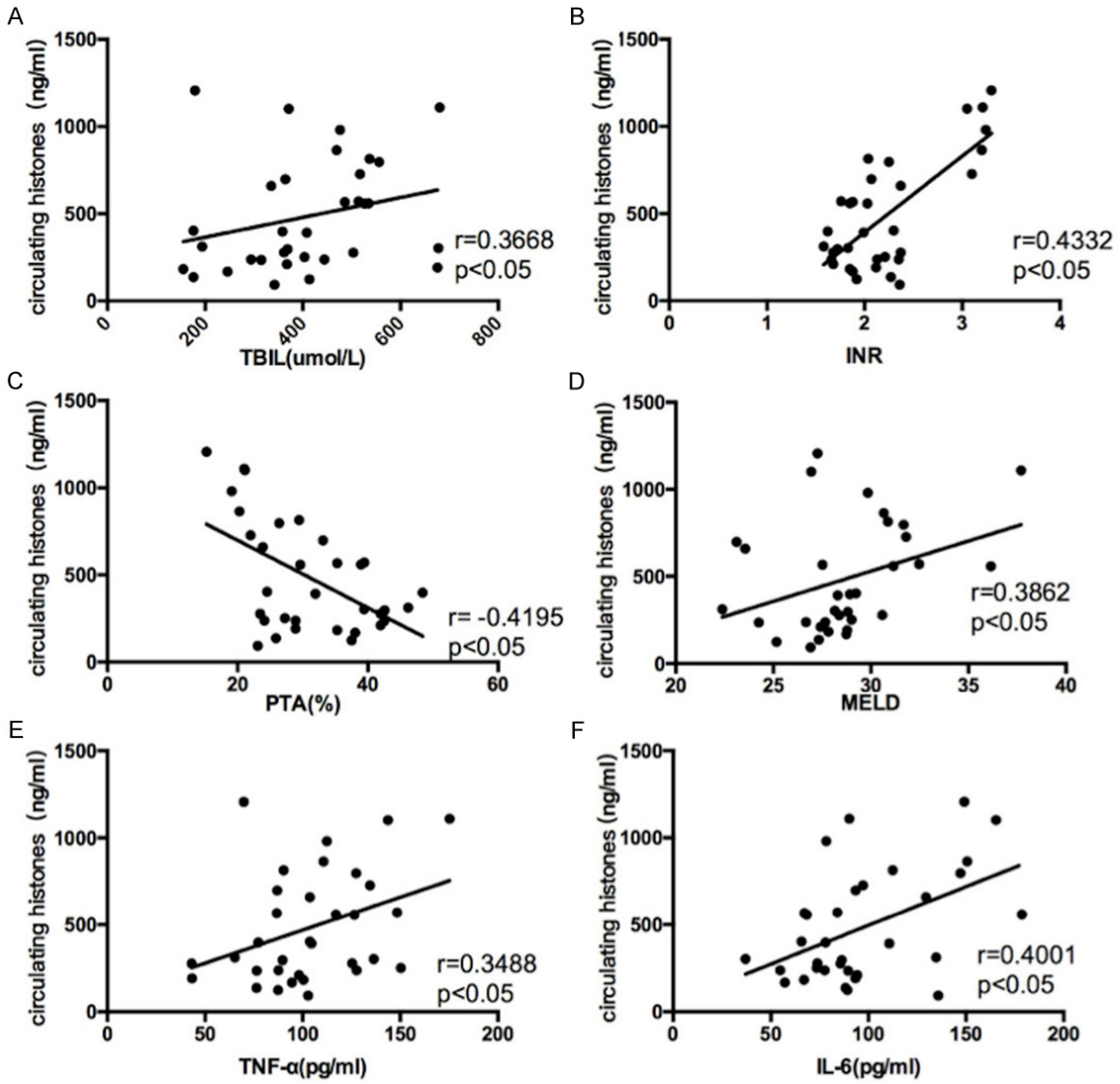


Figure 6. Correlation between circulating histone levels and other serological markers. Significant correlations were found between circulating histones and (A) total bilirubin (TBIL), (B) international normalized ratio (INR), (C) prothrombin time activity (PTA), (D) model for end-stage liver disease (MELD) score, (E) serum level of TNF-α level, and (F) serum levels of IL-6 in patients with HBV-related acute-on-chronic liver failure.

histones mainly come from intracellular histones released passively or secreted actively into the extracellular environment in the stress state but not from newly synthesized histones; 2) the extracellular histones mainly originated from necrosis/injury hepatocytes but not from PBMCs; or 3) considering the abundance in histones subtypes, HIST2H4A as well as H3F3A and H3F3B may not represent the mRNA levels of other subtypes. The mRNA of other histones subtypes should be detected in future studies. Therefore, further investigation is needed to elucidate the method and origin of histone release.

Under clinical conditions, the judgment of the severity of liver injury/failure is often based on several parameters, including ALT, TBIL, PTA, and INR [36]. We found that serum histone levels positively correlated with TBIL and INR and negatively correlated with PTA. Likewise, we observed that serum histone levels positively correlated with ALT and AST in our mice model. The serum samples of patients were collected at a relatively early stage of disease onset when all patients did not present with serious complications, suggesting that circulating histones may be an early predictive indicator of disease severity.

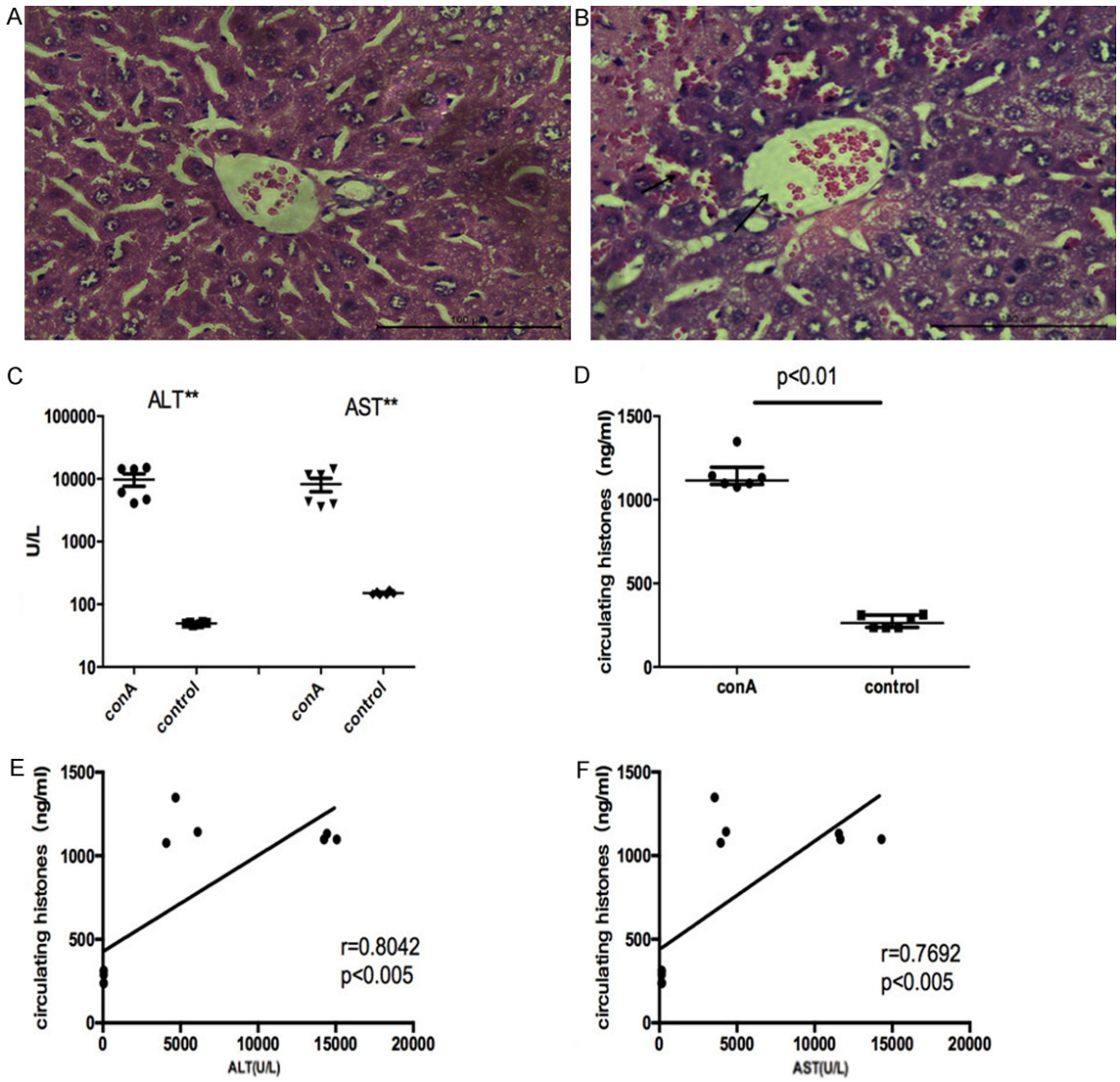


Figure 7. Histopathological manifestations of liver injury, increased circulating ALT, AST and histone levels were observed in ConA-induced acute liver injury mice. A. Liver from mice in the control group killed 24 h after saline injection. No edema and necrosis of hepatocytes and inflammatory cells infiltration. B. Liver from mice in the conA group killed 24 h after conA (20 mg/kg) injection. Structural disorder, severe hepatocyte necrosis or apoptosis, central vein enlargement (as indicated by the arrow) were observed. C. Both serum ALT and AST levels were strikingly higher in mice treated with ConA than healthy mice. **p<0.001. D. The concentrations of histone in ConA treated group were strikingly higher than the control mice (p<0.01). E. Correlation between the circulating histone levels and ALT. F. Correlation between the circulating histone levels and AST.

To date, the mortality of patients with HBV-related ACLF without liver transplantation remains high, and accurately determining prognosis remains a significant challenge in clinical practice. Consisting of three biochemical parameters, TBIL, INR, and creatinine, the MELD score model has been widely used to predict mortality in patients with end-stage liver disease [37]. However, in this study, no difference was found between survivors and non-survivors using the MELD score or TNF-α and IL-6 levels. These results highlight the limitation of these parameters for predicting the prognosis of ACLF patients. However, the serum histone level, tested at a relatively early stage of ACLF, was significantly higher in non-survivors when compared with survivors and showed a significant predictive value for mortality within two months with good sensitivity and specificity. Moreover, the Kaplan-Meier curve showed that

the group with high circulating histones had a shorter overall survival than the group with low circulating histones. Thus, a higher histone level may indicate a poor prognosis in ACLF patients.

Mounting evidence suggests that a massive release of inflammatory cytokines plays a vital role in the pathogenicity of liver injury/failure [38]. Similarly, inflammatory cytokines were elevated in the plasma of ACLF patients in our study. Xu et al. reported that exogenous histones increased the concentrations of pro-inflammatory cytokines, including TNF-α, IL-6, IL-12p70, IL-10, and IFN-α, in mouse models of sterile inflammation [12, 13]. Likewise, histones caused systemic inflammation and induced cytokine releasein murine models of ischemia-reperfusion injury of the liver or kidney [11, 39]. We speculate that a massive necrosis/injury of hepatocytes in ACLF patients may lead to the excessive release of histones into the extracellular environment; thus, the elevated circulating histones at an early stage of ACLF may be responsible for the increased concentrations of cytokines in ACLF patients. Moreover, the level of circulating histones was closely associated with disease severity and prognosis in HBV-related ACLF patients. Therefore, blocking or neutralizing increased circulating histones is a potential therapeutic strategy for ACLF.

Briefly, the present investigation demonstrated that the serum concentration of circulating histones at a relatively early stage of liver injury could distinguish between severe liver failure/injury and moderate liver injury. Circulating histones may be a useful biomarker of disease severity in chronic hepatitis B patients and prognostic indicator and therapeutic target for ACLF.

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Disclosure of conflict of interest

None.

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