Plasma cell-free DNA for predicting outcomes of patients with HBV-related acute-on-chronic liver failure: a pilot study

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Abstract: Objective Cell-free DNA (cfDNA) was shown to be a prognostic marker for diverse pathological states in the Intensive Care Unit, but little is known of the role of cfDNA in HBV-related acute-on-chronic liver failure (ACLF). We hypothesize that cfDNA can also be a promising prognostic as well as a diagnostic marker in patients with HBV-related ACLF. Methods Thirty-eight patients with HBV-related ACLF admitted in the Intensive Care Unit were enrolled in the study. The patients were divided, according to the improvement of liver function at discharge, into favorable prognosis group (group 1, n=17) and poor prognosis group (group 2, n=21). Plasma samples were collected from each patient at hospitalization and at discharge to measure cfDNA by real-time quantitative PCR. MELD score was calculated at the same time points. Results The average level of cfDNA of group 1 was lower than that of group 2 both at the time of hospitalization (P<0.044) and at discharge (P<0.001). There was no difference in MELD score between the two groups at hospitalization. Significant correlations were found of cfDNA levels with the MELD score, TBIL, CRE and INR both at hospitalization (γ<0.001; γ<0.001; γ=0.36; γ<0.001, respectively) and at discharge (γ>0.85; P<0.001; γ=0.81; P<0.001; γ=0.65; P<0.001; γ=0.63; P<0.001, respectively). The ROC curve showed that cfDNA level at discharge was optimal in diagnosing ACLF with an area under curve (AUC) value of 0.96, followed by ΔcfDNA (AUC value of 0.923) and cfDNA level at hospitalization (AUC value of 0.67). The MELD scores had an AUC value of only 0.545 at the time of hospitalization. Conclusion cfDNA may serve as a promising prognostic and diagnostic marker for predicting in-hospital prognosis of HBV-related ACLF within 2 to 8 weeks.

Key words: cell-free DNA; hepatitis B virus; acute-on-chronic liver failure; prognosis

INTRODUCTION

Cell-free nucleic acid was first described in 1948 by Mandel and Métais. Only two decades ago, however, did people begin to realize the importance of its relationship to various diseases, including fetal diseases, pregnancy-associated disorders, trauma, sepsis, autoimmune diseases, and malignancies. Cell-free DNA (cfDNA) levels in malignancy have been widely investigated in past years. The changes in cfDNA levels were reported to correlate with tumor burden and disease progression, and the baseline levels of cfDNA could predict the prognosis of the tumors. Similar prognostic abilities of cfDNA were also tested in other diseases including trauma, chest pain, and sepsis, and the results indicated that elevated levels of cfDNA were usually associated with a poor clinical outcome.

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Hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) is the major cause of death in patients with chronic HBV infection, with varied manifestations and a high mortality. The pathogenesis of HBV-related ACLF involves a wide range of inflammatory disorders and immune responses that lead to acute episodes of liver injury, often with multiple organ failure (MOF).

Even though acute liver injuries are potentially reversible, liver transplantation is still the only effective therapy for ACLF. Accurate evaluation of the severity and prognosis of the disease is of utmost importance for making further therapeutic decisions. The Model for End-stage Liver Disease (MELD) is a widely used scoring system and allows reliable prediction of 3 to 6 months survival in patients with liver failure and assists in decision-making of liver transplantation. However, MELD score has limitations in predicting the short-term in-hospital prognosis, especially in ACLF patients in the intensive care unit (ICU), who present not only with liver failure, but also with symptoms of extrahepatic organ dysfunction. Thus we hypothesize that cfDNA level, which has been proven to be linked with the prognosis and severity of many diseases, may also be a potential prognostic marker in ACLF patients.

PATIENTS AND METHODS

Patients and sample collection

From May 2009 to January 2012, patients with HBV-related ACLF admitted to the 302 Hospital of PLA were screened for this study. The patients were diagnosed for chronic HBV infection by history and by laboratory examinations. Only those having a course of HBV infection longer than 6 months were selected. The diagnostic criteria of ACLF were adopted as defined by
APASL: acute hepatic insult with previously diagnosed or undiagnosed chronic liver diseases manifesting jaundice (serum bilirubin ≥5 mg/L [85 mmol/L]) and coagulopathy (international normalized ratio [INR] ≥1.5 or prothrombin activity <40%), and complicated within 4 weeks by ascites and/or encephalopathy [12]. Patients were excluded if they were co-infected with Hepatitis A, C, D, and E virus, cytomegalovirus, Epstein-Barr virus, or HIV. Patients who were hospitalized for less than 14 days were also excluded.

Upon hospitalization, all the patients received similar treatment protocols: Entecavir for antiviral therapy if HBV DNA was positive; antibiotics for confirmed or highly suspected infection; appropriate nutrition support based on the patient’s condition.

This study was approved by the Ethics Committee of the hospital with written informed consent from the patients. Paired blood samples of peripheral venous blood were collected from each patient at the time of hospitalization and at discharge. Blood samples were centrifuged for 10 min at 2500 r/min, and the plasma was transferred into a 1.5 ml Eppendorf tube and centrifuged at 14 000 r/min for another 10 min. The upper portion of plasma was removed into a new 1.5 ml Eppendorf tube and stored at −80 °C for later use.

**DNA extraction and cfDNA quantification**

The plasma samples (200 μl) were used for DNA extraction using PureLink™ Viral RNA/DNA Kits (Invitrogen). cfDNA was detected using a real-time quantitative PCR system (TaqMan) for the β-globin gene, which is considered to be a housekeeping gene used for quantification of cfDNA [8, 9, 11, 14]. PCR amplification was carried out using the primer pair β-globin-F (5’-GTGCACCTGACTCTGAGGAGA-3’), the β-globin-R primer (5’-CCCTGATACCAACCTGCCC-3’) and a dual-labeled fluorescent TaqMan probe [5’-(FAM)AAG GTG GTG GAT GAA GTT GGT GG (TAMRA)-3’], as previously reported [9]. A 1:10 serial dilution of the plasmid sample was used to generate standard curves, and the levels of cfDNA were described as copies/μl [13].

**Clinical data collection and definition of prognosis**

Upon hospitalization, the patients underwent routine tests of liver function, renal function, complete blood count, haemostasis, serology (HBsAg, HBsAg/anti-HBe), and HBV DNA levels. The indexes were monitored closely according to the patients’ conditions. MELD scores were calculated for all the patients according to the formula published [16].

Currently there are no consensus criteria for the prognosis of patients with ACLF undergoing treatment. In this study, the prognosis of the patients who were alive when discharged was defined as follows: compared to the baseline level (at the time of hospitalization), a decrease of ≥50% of total bilirubin (TBIL), and/or an increase to >40% of PA were defined as improvement; an increase of TBIL, with or without decreased PA, was defined as deterioration; other variations were defined as stability.

**Statistical analysis**

The data with normal distribution such as age, albumin (ALB), international normalized ratio (INR), white blood cell (WBC) count, MELD score, and cfDNA levels were expressed as Mean ± SD, and compared between the two groups using Student’s t-test. Comparison of the patients’ cfDNA at hospitalization and at discharge was undertaken with paired t-test. Parameters with non-normal distribution, such as TBIL, ALT, and the length of hospitalization were expressed as median (range), and compared using Mann-Whitney U test between the two groups. Pearson correlation analysis was used to calculate the correlation coefficients. The prognostic and diagnostic performance of cfDNA was analyzed by a receiver-operating characteristic curve (ROC). A P value less than 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Demographic and clinical characteristics of the patients**

Thirty-eight patients were enrolled in the study. Seventeen of the patients showed improvement at discharge, 2 were in stable condition, 13 showed deterioration, and 6 died. The patients were thus divided into group 1 with improvement (good prognosis) and group 2 with deterioration or death (poor prognosis). Because of the uncertain outcome of the patients in stable condition at discharge, 2 patients were excluded. The demographic and clinical characteristics of the two groups at hospitalization and discharge/death were listed in Tab.1, Tab.2, and Tab.3. There were no significant differences in age, gender, liver function test (TBIL, ALB, ALT, INR), HBV DNA level, or MELD score between the two groups at the time of hospitalization, but significant differences were found in TBIL, INR, MELD score, WBC, and cfDNA levels upon discharge. The 2 groups had similar systemic inflammatory response syndrome (SIRS) diagnosis during hospitalization but with significant difference in hospitalization length.

**cfDNA levels in the two groups**

The average level of cfDNA of group 1 was significantly lower than that of group 2 at the time of hospitalization (P=0.044) and also at discharge (P<0.001) (Fig.1). The lower cfDNA level in group 1 at hospitalization indicated the potential of cfDNA as a prognostic marker for short-term outcome (about 2 weeks to 8 weeks) in patients with HBV-related ACLF as an alternative to MELD score. In group 1, the cfDNA level tended to decrease in 14 out of the 17 patients (82.3%), whereas 16 of 19 patients (84.2%) in group 2 showed increased levels of cfDNA during hospitalization (Tab.3). The cfDNA levels at hospitalization and at discharge, compared using paired-sample t test, differed significantly in both group 1 (P=0.001) and group 2 (P<
Tab.1 Demographic and clinical characteristics of the patients at the time of hospitalization

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=17)</th>
<th>Group 2 (n=19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>44.4±8.9</td>
<td>48.1±10.7</td>
<td>0.272 (1=1.12)</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>16:1</td>
<td>14:5</td>
<td>0.182 (χ^2=2.697)</td>
</tr>
<tr>
<td>T.BIL (umol/L)</td>
<td>309.6 (147.0, 471.0)</td>
<td>343.7 (121.5, 502.9)</td>
<td>0.975 (U=160)</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>28.9±3.9</td>
<td>27.8±4.3</td>
<td>0.456 (1=0.75)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>172(41-2642)</td>
<td>81 (10-2131)</td>
<td>0.100 (U=109.5)</td>
</tr>
<tr>
<td>INR</td>
<td>2.00±0.47</td>
<td>2.06±0.39</td>
<td>0.706 (1=0.38)</td>
</tr>
<tr>
<td>MELD score</td>
<td>65±11</td>
<td>54±10</td>
<td>0.830 (1=0.41)</td>
</tr>
<tr>
<td>WBC (×10^9)</td>
<td>10.3±7.6</td>
<td>7.3±3.7</td>
<td>0.141 (1=1.51)</td>
</tr>
<tr>
<td>cfDNA (log value of copies/ul)</td>
<td>3.23±0.33</td>
<td>3.44±0.23</td>
<td>0.044 (1=2.09)</td>
</tr>
<tr>
<td>HBV DNA (log value)</td>
<td>3.87±3.36</td>
<td>4.51±2.60</td>
<td>0.447 (1=0.769)</td>
</tr>
</tbody>
</table>

Tab.2 Liver function tests, MELD score and cfDNA levels of the patients at discharge

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=17)</th>
<th>Group 2 (n=19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.BIL (umol/L)</td>
<td>80.0 (42.0, 215.3)</td>
<td>438.0 (162.0, 731.2)</td>
<td>&lt;0.001 (U=4)</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>28.8±5.8</td>
<td>27.1±2.9</td>
<td>0.266 (1=1.13)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>45 (15-386)</td>
<td>51 (20-337)</td>
<td>0.285 (U=127.0)</td>
</tr>
<tr>
<td>INR</td>
<td>1.51±0.46</td>
<td>2.47±0.71</td>
<td>&lt;0.001 (1=0.78)</td>
</tr>
<tr>
<td>MELD score</td>
<td>16.5±4.4</td>
<td>34.0±6.9</td>
<td>&lt;0.001 (1=0.03)</td>
</tr>
<tr>
<td>WBC (×10^9)</td>
<td>4.9±2.4</td>
<td>10.3±6.9</td>
<td>0.004 (1=3.08)</td>
</tr>
<tr>
<td>cfDNA (log value of copies/ul)</td>
<td>2.87±0.23</td>
<td>3.67±0.31</td>
<td>&lt;0.001 (1=0.83)</td>
</tr>
<tr>
<td>SIRS* (yes: no)</td>
<td>11: 6</td>
<td>10: 9</td>
<td>0.516 (χ^2=0.538)</td>
</tr>
<tr>
<td>SBP* (yes: no)</td>
<td>10: 7</td>
<td>11: 8</td>
<td>0.999 (χ^2=0.003)</td>
</tr>
<tr>
<td>HE* (yes: no)</td>
<td>4: 13</td>
<td>6: 13</td>
<td>0.717 (χ^2=0.290)</td>
</tr>
<tr>
<td>Hospitalized time (days)</td>
<td>40 (21, 74)</td>
<td>25 (14, 42)</td>
<td>&lt;0.001 (U=43.5)</td>
</tr>
</tbody>
</table>

DISCUSSION

As a potential prognostic and diagnostic marker, cfDNA has been investigated by several studies for evaluating prognosis of patients admitted to the ICU for a diverse variety of conditions. Consistent results were found in these studies that the level of cfDNA was increased in patients who died compared to survivors. Compared to the existing scoring systems for predicting clinical outcomes of patients in ICU, such as the Acute Physiology and Chronic Health Evaluation (APACHE) II and Sequential Organ Failure Assessment (SOFA) score, cfDNA showed a similar prognostic power, and according to Wijeratne et al, cfDNA has an advantage in predicating the need for ventilation. More interestingly, Saukkonen et al found that the maximum cfDNA level was independently associated with hospital mortality.

In this pilot study we found that the average cfDNA level in group 1 was lower than that in group 2, suggesting the potential of cfDNA as a prognostic marker of short-term outcome (~2 weeks to 8 weeks) in patients with HBV-related ACLF. The changes in cfDNA during hospitalization were also important in predicating the prognosis given the difference in the levels of cfDNA between the two groups at discharge and the distinctive trends of variations in both groups (Fig.1).

Our results demonstrated that cfDNA level at the time of hospitalization was the best indicators of ACLF with an AUC value of 0.960, followed by ΔcfDNA (AUC value of 0.893) and cfDNA level at discharge (AUC value of 0.667). cfDNA allowed more accurate diagnosis than MELD scores, which had an AUC value of 0.545 at the time of hospitalization (data not shown). These
results suggest that cfDNA level serves as a better predictor than MELD score for diagnosis and short-term prognosis in ACLF patients.

It is not clear, however, whether cfDNA is superior to MELD score in predicting the prognosis of HBV-related ACLF in a longer period of time. Previous study showed a better performance of cfDNA in predicting mortality and 6-month morbidity in stroke patients than the conventional approaches[13]. cfDNA also proved useful in predicting mortality in patients with lung and renal cancers by survival analysis during the follow-up for 10 to 28 months[12, 21].

The mechanism by which cfDNA is released from cells into the circulation remains unclear, and is presumed to be related to cell apoptosis and necrosis[22] and active secretion from living cells[23]. The source of
cfDNA varies in different diseases. In patients with cancer, for instance, cfDNA is mainly derived from tumor cells, as tumor-specific gene mutations were found in cfDNA\(^{11}\). In SIRS and sepsis, the inflammatory cells are likely sources of cfDNA. In ACLF, complex hepatic inflammation, apoptosis and necrosis of liver cells, cholestasis, and fibrosis\(^{12}\) may all contribute to cfDNA. In ACLF with MOF, the mechanism of cfDNA release is more complicated. Our results showed that the levels of cfDNA were correlated with the severity of ACLF, suggesting that cfDNA release was largely a result of liver dysfunction. The correlation between cfDNA levels and WBC count (at discharge) suggests the contribution of extrahepatic inflammatory responses to cfDNA release. We found no difference in the incidences of SIRS, SBP and HE during hospitalization between the two groups. In addition, a similar correlation between cfDNA and leukocytes was described in a study of patients with hepatitis C-related hepatocellular carcinoma\(^{23}\).

There are other two limitations of this study. First, the small number of patients enrolled did not allow reliable statistic analysis of the patients with different outcomes (improvement, stabilization, deterioration or death). Second, the intervals of sample collection were not consistent between the two groups given the differences in the hospitalization length. The reason for the differences in hospitalization length is that most patients referred to our hospital (which is at the highest level in the hepatology treatment in China) were from other cities and did not have health insurance. For economic reasons, most of the patients chose their local health services when the disease became deteriorated into an end-stage condition.

In conclusion, the preliminary data of this pilot study demonstrate that cfDNA level may serve as a promising prognostic and diagnostic marker for HBV-related ACLF, with superior performance to MELD score in predicting in-hospital prognosis within 2 to 8 weeks. A large-scale, longer follow-up study is needed to further examine the value of cfDNA as a prognostic factor of HBV-related ACLF.

**Fig.1** Changes in cfDNA levels in Group 1 and Group 2 during hospitalization and at discharge.

**Fig.2** Correlation between cfDNA levels and MELD score at hospitalization (A) and at discharge (B).

**Fig.3** Receiver-operating characteristic curves for cfDNA level at hospitalization (CAH), cfDNA level at discharge (CAD), and ΔcfDNA to predict or diagnose ACLF outcome.

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REFERENCES


