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Branched DNA-based Alu quantitative assay for cell-free plasma DNA levels in patients with sepsis or systemic inflammatory response syndrome

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ABSTRACT

Cell-free circulating DNA (cf-DNA) can be detected by various of laboratory techniques. We described a branched DNA–based Alu assay for measuring cf-DNA in septic patients. Compared to healthy controls and systemic inflammatory response syndrome (SIRS) patients, serum cf-DNA levels were significantly higher in septic patients (1426.54 ± 863.79 vs 692.02 ± 703.06 and 69.66 ± 24.66 ng/mL). The areas under the receiver operating characteristic curve of cf-DNA for normal vs sepsis and SIRS vs sepsis were 0.955 (0.884-1.025), and 0.856 (0.749-0.929), respectively. There was a positive correlation between cf-DNA and interleukin 6 or procalcitonin or Acute Physiology and Chronic Health Evaluation II. The cf-DNA concentration was higher in intensive care unit nonsurviving patients compared to surviving patients (2183.33 ± 615.26 vs 972.46 ± 648.36 ng/mL; $P < .05$). Branched DNA–based Alu assays are feasible and useful to quantify serum cf-DNA levels. Increased cf-DNA levels in septic patients might complement C-reactive protein and procalcitonin in a multiple marker format. Cell-free circulating DNA might be a new marker in discrimination of sepsis and SIRS.

1. Introduction

Systemic inflammatory response syndrome (SIRS) is an inflammatory condition that affects the entire body. It is frequently a response to a wide spectrum of conditions, including burns, trauma, infection, and surgery [1]. The current criteria for SIRS diagnosis are based on any combination of fever from hypothermia, tachycardia, tachypnea, leukocytosis, or leukopenia. Sepsis is syndrome of infection complicated by organ failure [1]. Despite improvements in diagnostic and therapeutic techniques, sepsis remains a major cause of death in the intensive care unit (ICU), and delayed diagnoses and intervention are usually associated with high mortality [2,3]. Most parameters for SIRS diagnosis are either nonspecific or insensitive for a sepsis diagnosis. One study reported that early diagnosis of sepsis and appropriate use of antibodies could effectively reduce patient mortality [3]. Furthermore, avoiding unnecessary antimicrobial therapy may also reduce the cost of care.

To date, a variety of indicators have been studied for the diagnosis of sepsis, including acute phase proteins (C-reactive protein [CRP]), cytokines (interleukin 6 [IL-6], interleukin 8, tumor necrosis factor α), and procalcitonin (PCT). However, the accuracy and specificity of each of these indicators are still in question for the diagnosis of sepsis [4-7]. In addition, none can strictly differentiate sepsis from noninfectious SIRS [8]. Thus, it is imperative to find a novel marker that can diagnose sepsis with high specificity and is easy to measure, with low cost and rapid application.

Circulating nucleic acids in human peripheral blood were identified in 1984 by Mandel and Metais [9,10]. Increased cell-free circulating DNA (cf-DNA) has been reported in various diseases, including trauma, cancer, stroke, and myocardial infarction [11-15]. Although the exact mechanism is poorly understood, available evidence shows that cell lysis, necrosis, apoptosis, and active release might be related to circulating DNA production [16-19]. Apoptosis plays an important role in the pathophysiology of sepsis, and cf-DNA has been found in the plasma of septic patients [20]. Therefore, it is important to investigate whether cf-DNA increases in SIRS and septic patients and to determine its role in the diagnosis and differential diagnosis of sepsis.

In the present study, novel branched DNA (bDNA)–based Alu assays were used to examine the serum cf-DNA in healthy subjects, SIRS patients, and septic patients in an effort to evaluate the role of cf-DNA in the diagnosis and differential diagnosis of sepsis.

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Table 1
Clinical characteristics of septic patients

Variable	Sepsis (n = 24)
Age (y)	58.3 ± 12.7
Sex (M/F)	15/9
Primary diagnosis	
Major surgery	10
Intestinal obstruction	5
Intestinal perforation	3
Mesentric vein thrombosis	2
Abdominal or pelvic abscess	1
Multiple trauma	3
Site of infection	
Abdomen	10
Thorax	6
Blood	4
Others	4
Pathogen types	
Gram-negative infection	13
Gram-positive infection	8
Fungi	3
APACHE II	18.2 ± 6.6
Survivors	15
Nonsurvivors	9
Septic shock	9

2. Materials and methods

2.1. Participants and sample preparation

Sixty-seven consecutive patients who presented to the Medical ICU of SongJiang Central Hospital (Shanghai, China) over a 1-year period participated in the study. Samples were taken within 6 hours after the onset of clinical syndromes without treatment. For the SIRS group, patients had to fulfill at least 2 criteria of SIRS but have no evidence of organ dysfunction or sepsis. For the sepsis group, patients had to fulfill both the criteria for SIRS and have microbiological evidence of local infection. Bacteria, fungi, or parasites were cultured from the blood of all sepsis patients. Systemic inflammatory response syndrome patients had no symptoms of local infection, and no microorganisms were cultured from their blood. The causes of sepsis included major surgery, multiple traumas, intestinal perforation, and intestinal obstruction. Age-matched healthy volunteers (n = 73) were recruited as healthy controls.

For serum separation, blood samples were collected into clot activation additive-containing tubules, centrifuged at 1600*g* for 10 minutes, followed by 16000*g* for 1 minute. The supernatant (serum) was collected into a clear tube and stored at −80°C until use. The institution ethics committee approved this study, and informed written consent was obtained from each patient.

2.2. Cell-free circulating DNA quantification

Serum (20 μL) was added to a working probe set (80 μL) working solution that contained lysis buffer, proteinase K, and target gene probes (Panomics, Santa Clara, CA). This mixture was transferred to a 96-well plate that was incubated overnight (12-18 hours) at 55°C. After incubation, plates were washed 3 times with 300 μL of washing buffer (Panomics). Next, 100 μL of preamplified working solution, 100 μL of amplified working solution, and 100 μL of label probe working solution were sequentially added to each well (Panomics). After each addition, the plate was hybridized for 1 hour at 55°C, followed by another 3 washes. Finally, 100 μL of substrate was added to each well, followed by incubation at room temperature for 5 minutes. Luminescent signals were detected by Perkin Elmer Victor 3 (Perkin Elmer, Newark, NJ). The cf-DNA concentration was calculated according to the standard curve simultaneously performed in the same plate.

2.3. Procalcitonin and IL-6 quantification

Procalcitonin and IL-6 concentrations were evaluated by electrochemical luminescence on a Roche COBAS-e601. This is an automated heterogeneous sandwich immunoassay, with a total assay time of 18 minutes. A PCT concentration greater than 46 pg/mL is considered positive, whereas an IL-6 concentration higher than 7 pg/mL is considered positive.

2.4. Statistical analyses

Quantitative data are described as the mean ± SD. Statistical analyses were performed using SPSS 13.0 for Microsoft Windows (SPSS, Chicago, IL), except that MedCalcR4.20.011 (Frank Schoonjans, Mariakerke, Belgium) was used to compare the receiver operating characteristic (ROC) curve. For nonparametric data, Mann-Whitney *U* tests were performed for comparisons between 2 groups. Correlations between 2 quantitative variables are expressed by Spearman correlation coefficient. Receiver operating characteristic curves were applied to assess the predictive value of cf-DNA, and the area under curve (AUC) was calculated and compared. Confidence intervals for areas under ROC curves were calculated using nonparametric assumptions. A 2-sided $P < .05$ was considered statistically significant.

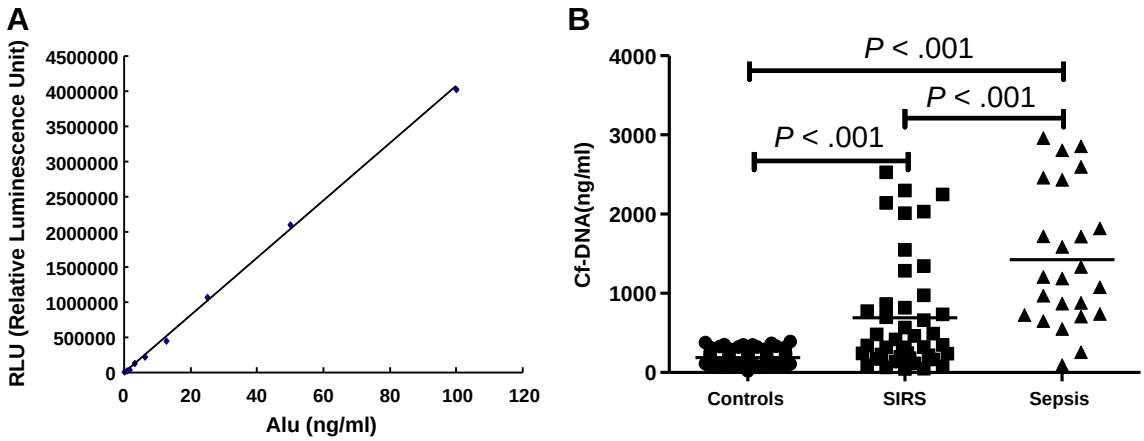

Fig. 1. Cell-free circulating DNA concentration in control subjects, SIRS patients, and sepsis patients. A, Cell-free circulating DNA concentration standard curve. A typical calibration curve with a correlation coefficient of 0.991. B, Cell-free circulating DNA concentration in control subjects, SIRS patients, and sepsis patients. The cf-DNA levels were 69.66 ± 24.66 in controls (n = 48), 692.02 ± 703.06 in SIRS patients (n = 43), and 1426.54 ± 863.79 ng/mL in sepsis patients (n = 24). Nonparametric Mann-Whitney *U* tests showed significant differences in serum cf-DNA between any two of the groups ($P = .000$).

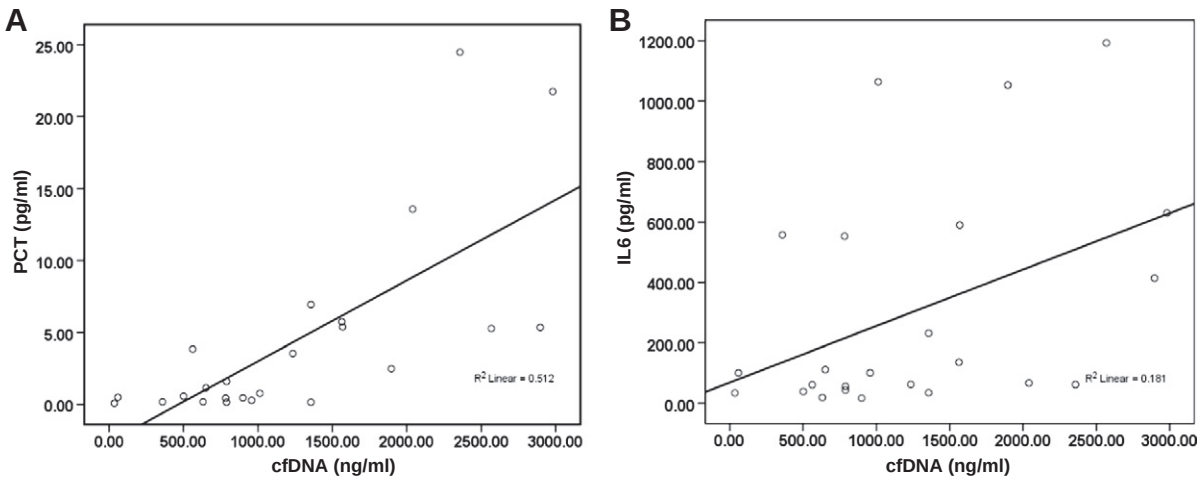

Fig. 2. Correlations between serum levels of cf-DNA and PCT or IL-6 in 24 specimens from septic patients. A, PCT vs cf-DNA ($r = 0.717$; $P < .001$). B, IL-6 vs cf-DNA ($r = 0.481$; $P = .017$).

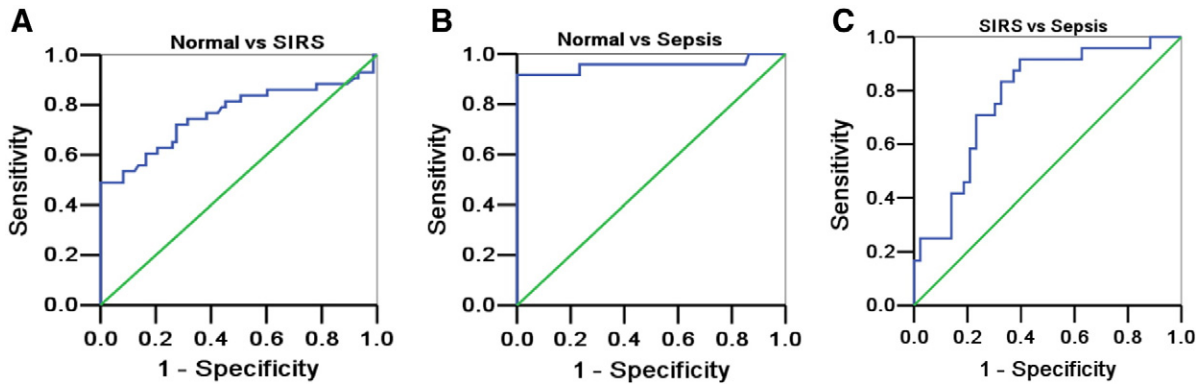

Fig. 3. Receiver operating characteristic curves of cf-DNA. A, Normal vs SIRS (AUC, 0.763; $P = .000$). B, Normal vs sepsis (AUC, 0.955; $P = .000$). C, SIRS vs sepsis (AUC, 0.856; $P = .000$).

3. Results

3.1. General characteristics of the patients

The characteristics of the patients are shown in Table 1. The leading causes of sepsis were major surgery, intestinal obstruction, intestinal perforation, and multiple traumas. Abdomen, thorax, and blood were the primary sites of infection for both gram-negative and gram-positive infections. All sepsis patients were admitted to the ICU.

The 43 SIRS patients had a mean age of 55.4 ± 10.6 years. There were 26 males and 17 females, and organ dysfunction and sepsis were not found. We recruited 73 healthy volunteers (45 males and 28 females) with a mean age of 52.6 ± 12.9 years.

3.2. Detection with bDNA-based Alu assays

The sensitivity, specificity, accuracy, and reproducibility of Alu-based assays have been described in previous studies [21]. In this study, serial 2-fold dilutions of standard human genomic DNA were used. Standard curves showed the linear correlation ($R^2 = 0.991$) was out of range (0-100 ng/mL) of the cf-DNA concentration, and the formula for the regression line was $y = 2 \times 10^{-5}x$ (Fig. 1A).

3.3. Serum cf-DNA levels

The bDNA-based Alu assays revealed that serum cf-DNA levels were significantly increased in septic patients (1426.54 ± 863.79 ng/mL) and SIRS patients (692.02 ± 703.06 ng/mL) compared to healthy controls (69.66 ± 24.66 ng/mL; $P < .05$). Compared to SIRS patients, the serum cf-DNA of septic patients was also significantly elevated ($P < .05$) (Fig. 1B).

3.4. Correlation of cf-DNA with PCT and IL-6

Serum cf-DNA levels were elevated in all sepsis patients. The correlation of cf-DNA concentration with PCT or IL-6 was PCT vs cf-DNA ($r = 0.717$; $P < .001$) and IL-6 vs cf-DNA ($r = 0.481$; $P = .017$) (Fig. 2).

Table 2
Diagnostic performance of cf-DNA in SIRS and sepsis

Groups	Area	SE	*P*	95% CI		Cutoff (ng/mL)	Sensitivity (%)	Specificity (%)
				Lower bound	Upper bound			
Normal vs SIRS	0.763	0.052	.000	0.661	0.865	228.5	72.1	72.6
Normal vs sepsis	0.955	0.036	.000	0.884	1.025	385	91.7	88.6
SIRS vs sepsis	0.856	0.048	.000	0.749	0.929	493	94.1	70.6

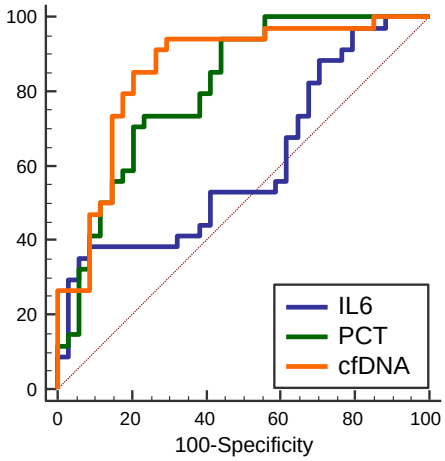

Fig. 4. Receiver operating characteristic curves of PCT, IL-6, and cf-DNA in differential diagnosis between SIRS and sepsis.

3.5. Role of cf-DNA in the diagnosis and differential diagnosis of sepsis

Receiver operating characteristic curves were generated to evaluate the sensitivity and specificity of cf-DNA in the diagnosis of SIRS and sepsis. The AUC of cf-DNA was 0.763 (95% confidence interval [CI], 0.611-0.865) for SIRS (Fig. 3A) and 0.955 (95% CI, 0.884-1.025) for sepsis (Fig. 3B). At a cutoff value of 228.5 ng/mL, cf-DNA gave a sensitivity of 72.1% and a specificity of 72.6% for SIRS. The optimal cutoff value of cf-DNA was 385 ng/mL in the prediction of sepsis, which had a sensitivity of 91.7% and a specificity of 88.6% (Table 2).

Furthermore, ROC curves were also used to distinguish SIRS from sepsis. The AUC of cf-DNA was 0.856 (95% CI, 0.749-0.929) for distinguishing SIRS from sepsis (Fig. 3C). With a cutoff value of 493 ng/mL, cf-DNA yielded a sensitivity of 94.1% and a specificity of 70.6% (Table 2).

3.6. Comparisons with other clinical parameters in differential diagnosis between SIRS and Sepsis

To compare cf-DNA with other clinical indicators used in the differential diagnosis of sepsis and SIRS, the serum levels of PCT and IL-6 were measured in all blood samples. As shown in Fig. 4 and Table 3, cf-DNA is the best index with a sensitivity of 94.12% and a specificity of 70.59% at the cutoff value of 493 ng/mL for diagnosing sepsis. The AUC of PCT was 0.807 (95% CI, 0.693-0.893) for distinguishing SIRS from sepsis. With a cutoff value of 0.125 ng/mL, PCT yielded a sensitivity of 92.12% and a specificity of 55.88%. Interleukin 6 is an acute inflammatory molecule with a sensitivity of 35.29% and a specificity of 88.24% at the cutoff value of 242.6 pg/mL for the diagnosis of sepsis. The AUC of IL-6 was 0.606 (95% CI, 0.480-0.723) for distinguishing SIRS from sepsis. The prediction sensitivity, positive predictive value, and negative predictive value for cf-DNA, IL-6, and PCT are listed in Table 3. Therefore, our data suggest that serum cf-DNA level is comparable to, or better than, the 2 clinically used indicators. Cell-free circulating DNA could be a new marker in discrimination of sepsis and SIRS.

3.7. Serum cf-DNA and disease severity

The Acute Physiology and Chronic Health Evaluation (APACHE) II score represents the severity of sepsis. We evaluated the correlation of cf-DNA with the severity of sepsis (APACHE II score). Our results demonstrate that serum cf-DNA levels significantly correlated with the APACHE II score ($r = 0.496$; $P = .014$) (Fig. 5A). In addition, serum cf-DNA levels were significantly higher on admission in ICU patients who did not survive compared to patients who survived (2183.33 ± 615.26 vs 972.46 ± 648.36 ng/mL; $P < .05$) (Fig. 5B).

4. Discussion

Because sepsis remains an important cause of mortality in the ICU, early and rapid diagnosis and treatment are essential. The classic markers for sepsis diagnosis include fever and leukocyte count. Although these are easy to measure, they are insensitive and nonspecific [22]. Currently, there is controversy regarding the role of new markers, such as PCT and CRP, in the diagnosis of sepsis [23,24]. Microbiological culture is the best way to diagnose sepsis; however, it is time consuming and typically takes 24 to 48 hours. Thus, it is imperative to find better markers for the diagnosis of sepsis and the differential diagnosis of sepsis and SIRS.

Cell-free circulating DNA can be detected by various methods, but nearly all methods require sample preparation. Alu sequences are small interspersed elements that account for greater than 10% of the human genome. Whole blood is rich in Alu sequences. Assaying Alu sequences represents a sensitive new method for the measurement of blood cf-DNA. In the present study, novel bDNA-based Alu assays were performed to directly quantify serum cf-DNA levels. This method avoids the DNA extraction and purification and requires only 20 μL of serum for 1 test. In addition, it has high sensitivity and reproducibility with a dynamic range of 0 to 400 ng/mL human genomic DNA [21].

The results of this study show that serum cf-DNA could complement CRP and PCT measurements for the diagnosis of sepsis. First, serum cf-DNA concentration was significantly increased in septic patients compared to healthy controls (1426.54 ± 863.79 vs 69.66 ± 24.66 ng/mL; $P < .05$). Receiver operating characteristic curve analyses revealed that serum cf-DNA at a cutoff value of 385 ng/mL yielded a sensitivity of 91.7% and a specificity of 88.6% for the diagnosis of sepsis. Second, although we observed increased serum cf-DNA in both septic and SIRS patients, the extent of their elevation was markedly different. The average cf-DNA was 1426.54 ± 863.79 ng/mL in sepsis patients and 692.02 ± 703.06 ng/mL in SIRS patients. At a cutoff value of 522 ng/mL, serum cf-DNA yielded a sensitivity of 91.7% and a specificity of 60.5% for a differential diagnosis between sepsis and SIRS. In addition, serum cf-DNA levels positively correlated with previous indicators such as CRP and PCT and yielded comparable or better sensitivity and specificity compared to these indicators. Serum cf-DNA also positively correlated with the APACHE II score, the most commonly used index to evaluate sepsis severity ($r =$

Table 3
Diagnostic performance of PCT, IL-6 and cf-DNA in differential diagnosis between SIRS and sepsis

Discrimination sepsis from SIRS	cf-DNA (ng/mL)	IL-6 (pg/mL)	PCT (ng/mL)
Optical cutoff points	493	242.6	0.125
AUC ± SEM (95% CI)	0.856 ± 0.048 (0.749-0.929)	0.606 ± 0.072 (0.480-0.723)	0.807 ± 0.053 (0.693-0.893)
Sensitivity, % (95% CI)	94.12% (80.3-99.3)	35.29 (19.7-53.5)	92.12 (80.3-99.3)
Specificity, % (95% CI)	70.59% (52.5-84.9)	88.24 (72.5-96.7)	55.88 (37.9-72.8)
Positive likelihood ratio (95% CI)	3.2 (1.9-5.4)	3.00 (1.1-8.4)	2.13 (1.4-3.1)
Negative likelihood ratio (95% CI)	0.083 (0.02-0.3)	0.73 (0.6-1.0)	0.11 (0.03-0.4)
Positive prediction value, % (95% CI)	76.2 (60.5-87.9)	75.0 (47.6-92.7)	68.1 (52.9-80.9)
Negative prediction value, % (95% CI)	92.3 (74.9-99.1)	57.7 (43.1-71.3)	90.5 (69.6-98.9)

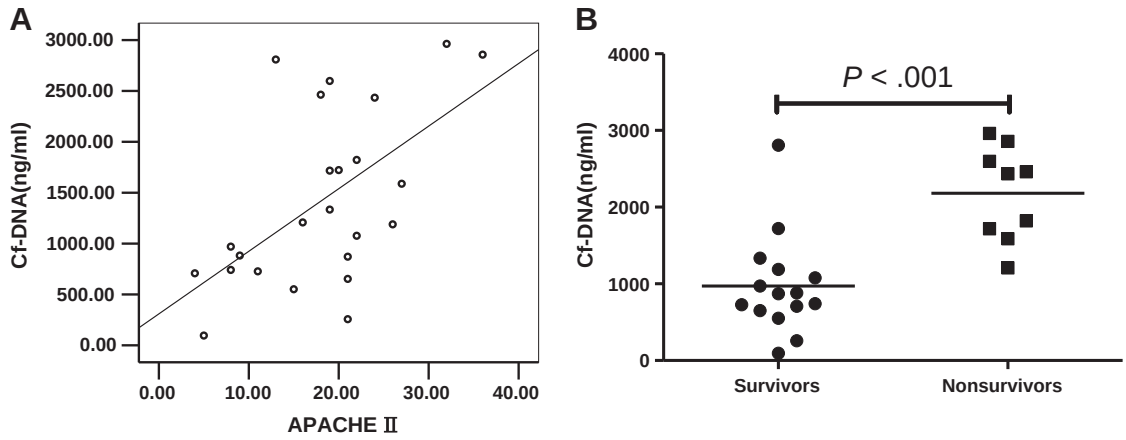

Fig. 5. Serum cf-DNA and disease severity. A, Correlation between cf-DNA and APACHE II scores in septic patients. Cell-free circulating DNA levels significantly correlated with APACHE II scores in septic patients ($r = 0.496$; $P = .014$). B, Cell-free circulating DNA concentrations in septic patients who survived or died in the ICU. Serum cf-DNA was 972.46 ± 648.36 in surviving patients and 2183.33 ± 615.26 ng/mL in nonsurviving patients. Mann-Whitney *U* tests indicated significant differences between the 2 groups ($P = .000$).

0.496; $P = .014$). We also found that nonsurviving patients had higher cf-DNA concentrations compared to surviving patients, which is consistent with previous reports [25,26]. Because the sample size was relatively small in this study, the number of deaths was not sufficient to evaluate the independent effect of serum cf-DNA level on mortality. In addition, this study was not designed to evaluate the predictive power of serum cf-DNA for sepsis. However, the serum cf-DNA concentrations in this study were in agreement with previous reports, suggesting that the novel bDNA-based Alu assay is a potentially feasible and useful method to detect cf-DNA levels in clinical settings. Because cf-DNA levels are elevated in septic patients compared to healthy controls, it could be useful for the diagnosis and differential diagnosis of sepsis.

Notably, although the serum cf-DNA levels significantly differed between SIRS and septic patients, there was still overlap in the serum cf-DNA levels between them. We speculate that high cf-DNA levels in some SIRS patients might contribute to early infection by certain pathogens at an undetectable level, representing the transition from SIRS to sepsis. Similarly, low cf-DNA levels in some septic patients might be related to the transition from sepsis to recovery. Therefore, serum cf-DNA might be valuable in predicting therapeutic outcomes. Future studies with larger sample sizes will be conducted with side-by-side assessments with other indicators to validate this hypothesis.

The origin of circulating DNA is still unclear. It appears that circulating DNA in cancer patients is derived from cancer cells. It has been proposed that circulating DNA may be a result of cellular necrosis and/or apoptosis [27,28]. However, recent studies reveal that the cf-DNA levels do not always correspond to the amount of circulating cancer cells, suggesting that circulating DNA may be actively secreted by cancer cells as well as other cells under stress [29]. Hehlgans and Pfeffer [30] found that, in addition to necrosis and apoptosis, other cellular stresses may be potential mechanisms of cf-DNA secretion. Clearly, more studies in this field are necessary.

The exact mechanism of serum DNA clearance also remains unclear. It has been suggested that the liver and kidney have important roles in the clearance of circulating DNA. In mice, nucleotides are predominately metabolized in the liver [31]. Botezatu et al [32] found that approximately 0.5% to 2% of circulating DNA crossed the kidney barrier and was excreted in the urine. Therefore, high circulating DNA levels may contribute to poor renal or liver function or to increased cellular damage/secretion, which thus questions circulating DNA as a biomarker.

Although many studies have demonstrated that the serum cf-DNA level is a promising biomarker for several diseases, our current data are preliminary. Thus, further studies are required to determine the specific role of circulating DNA in sepsis, including the correlation between cf-DNA and organ injury and the mechanisms of cf-DNA release. These data should provide important information regarding the diagnosis of sepsis.

In conclusion, our findings demonstrate that the bDNA-based Alu assay is a novel and feasible method to quantify human cf-DNA. Serum cf-DNA provides comparable or better sensitivity and specificity compared with previous indicators and might complement CRP, PCT, and IL-6 in a multiple marker format. However, there were only 24 septic patients in this study. Larger studies are needed in the future to evaluate the diagnostic value of serum cf-DNA in sepsis and SIRS.

Declaration of interest

The authors declare no conflicts of interest.

Acknowledgments

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