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MAJOR ARTICLE

High Levels of Circulating Cell-free DNA Are Associated With a Poor Prognosis in Patients With Severe Fever With Thrombocytopenia Syndrome

Yue Zhang,[1,2,a] Rui Song,[3,a] Yi Shen,[4,a] Yongxiang Zhao,[4] Zhenghua Zhao,[5] Tianli Fan,[6] Xiaoyu Yang,[1,2] Lin Wang,[3] Wei Zhang,[3] Chong Chen,[3] Di Tian,[3] Ying Wang,[3] Jing Wen,[3] Ziruo Ge,[3] Xiaoli Yu,[4] Li Liu,[5] Yang Feng,[5] Jianping Duan,[6] Yanli Ma,[6] Xingwang Li,[3] Hui Zeng,[1,2] Zhihai Chen,[3,b] and Liuluan Zhu[1,2,b]

[1]Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, [2]Beijing Key Laboratory of Emerging Infectious Diseases, and [3]Center of Infectious Disease, Beijing Ditan Hospital, Capital Medical University, [4]Department of Infectious Diseases, Dandong Infectious Disease Hospital, [5]Department of Infectious Diseases, Taian City Central Hospital, and [6]Department of Infectious Disease, Qing Dao No. 6 People's Hospital, China

Background. The extensive geographical distribution and high mortality rate of severe fever with thrombocytopenia syndrome (SFTS) have made it an important threat to public health. Neutrophil extracellular traps (NETs) can be activated by a variety of pathogens and are associated with thrombocytopenia in viral infections. We aimed to identify NET production and its predictive value for disease progression and prognosis in patients with SFTS.

Methods. A prospective study was performed with a multicenter cohort of patients with SFTS (n = 112) to quantify serum NET levels. Three markers of NETs—namely, cell-free DNA (cfDNA), myeloperoxidase-DNA complexes, and lactoferrin-DNA complexes—were measured with PicoGreen double-stranded DNA assays and enzyme-linked immunosorbent assays. Receiver operating characteristic curves and multivariate regression analyses were performed to calculate the predictive value of cfDNA levels.

Results. SFTS was characterized by pronounced NET formation. The serum levels of NETs changed dynamically during disease progression, with an inverse pattern of the trends of platelet and neutrophil levels. High cfDNA levels were strongly associated with multiple pathological processes, including coagulopathy, myocardial damage, liver dysfunction, and the development of encephalopathy. A high level of cfDNA (>711.7 ng/mL) at the time of the initial diagnosis predicted severe illness in patients with SFTS (odds ratio, 8.285 [95% confidence interval, 2.049–33.503]; $P = .003$).

Conclusions. This study has a high degree of clinical impact for identification of cfDNA as a useful predictive biomarker of clinical outcomes of SFTS.

Keywords. severe fever with thrombocytopenia syndrome; prognosis; neutrophil extracellular traps; cell-free DNA.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by the SFTS virus (SFTSV), a recently identified phlebovirus in the Bunyaviridae family [1, 2]. SFTS was first discovered in China in 2009 [3] and was subsequently reported in Japan [4] and South Korea [5]. Thereafter, more SFTSV-like viruses with close genetic relationships with SFTSV were further identified in the United States, India, and Australia [2, 6–8]. Because of their extensive geographical distribution and high case fatality rate (6%–30%) [1, 5], SFTSV and SFTSV-like infections have become a potential threat to public health.

SFTSV and SFTSV-like viruses can cause similar, if not identical, clinical symptoms, including abrupt onset of high fever, respiratory symptoms, or gastrointestinal symptoms. Severe cases might develop hemorrhaging, encephalopathy, disseminated intravascular coagulation, and multiorgan failure [2, 9–14]. Laboratory abnormalities include progressive thrombocytopenia and leukopenia and elevated serum levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) [11, 15]. Although previous studies revealed that the impairment of host immune response as well as inflammatory cytokine storm played important roles in the disease progression [2, 16–18], the mechanisms of disease pathogenesis are still not quite clear. Understanding the cellular and molecular mechanisms underlying the pathogenesis of SFTS is critical for developing novel effective treatments. The identification of predictive biomarkers for fatality is also key for early intervention and optimal clinical management.

Neutropenia has been observed in several clinical studies of patients with SFTS, especially in severe cases [9, 13]. Increasing numbers of studies have demonstrated that a variety of viruses can induce a unique type of cell death in neutrophils, termed

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[a]Y. Zhang, R. S., and Y. S. contributed equally to this work.

[b]L. Z., and Z. C. are co-senior authors and contributed equally to this work.

Correspondence: L. Zhu, Beijing Key Laboratory of Emerging Infectious Diseases, Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China (zhuliuluan@ccmu.edu.cn).

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NETosis [19–24]. During this process, neutrophils release neutrophil extracellular traps (NETs), which are composed of uncoiled chromatin DNA, histones and granule proteins, such as myeloperoxidase (MPO), neutrophil elastase, and lactoferrin (LF) [25]. NETs generally prevent viral infection in the acute stage; however, excessive production or ineffective clearance of NETs may cause tissue damage [26]. A strong correlation between the acute stage serum levels of NETs and poor outcomes has been observed in patients with influenza H1N1 or H7N9 infections [24].

Recently, an animal study showed that poxvirus-activated platelets interact with neutrophils and induce the release of NETs, which results in thrombocytopenia and neutropenia [19]. This phenomenon is very similar to the hallmark symptoms of SFTS. In addition, SFTSV has been demonstrated to bind to platelets [27]. Thus, we wondered whether NETs might be involved in the development of SFTS. In this study, we examined circulating NETs in a large cohort of patients with SFTS (n = 112) and explored the predictive value of cell-free DNA (cfDNA) for disease severity in patients with SFTS.

MATERIALS AND METHODS

Patients and Serum Samples

From October 2014 to September 2017, 112 patients with SFTSV infections were enrolled from 4 hospitals in northern China (Beijing Ditan Hospital Capital Medical University, Dandong Hospital for Infectious Disease, Tai'an Central Hospital, and No. 6 Hospital of Qingdao). Forty age- and sex-matched healthy donors were enrolled as controls. This study was approved by the local Ethics Committee of the Beijing Ditan Hospital, Capital Medical University (number 2014-003). Written informed consent was obtained from each patient or healthy control prior to their enrollment. All the patients received detailed physical examination on admission to hospital, including routine blood examination, biochemical examination, etiological examination, and neurologic evaluation.

The presence of SFTSV was confirmed by reverse-transcription polymerase chain reaction or serology detecting immunoglobulin M or immunoglobulin G antibodies against SFTSV. Encephalopathy was defined as altered consciousness including lethargy, irritability, or a change in personality, with the behavior persisting for >24 hours [28]. According to previous reports, cases that met any one of the following criteria were classified as severe: (1) presenting with severe neurological symptoms; (2) platelet count $<50 \times 10^9$ cells/L; (3) AST, alanine aminotransferase (ALT), LDH, or CK >5 times the upper limit of normal (ULN); (4) multiple organ failure; (5) severe bleeding; (6) severe secondary infection; or (7) death [9, 13]. Patients who discontinued therapy or had been discharged from the hospital were followed for 28 days after disease onset.

The patient blood samples were collected in ethylenediaminetetraacetic acid at the indicated time points. The blood was processed within 2 hours to centrifuge at 3000 rpm for 10 minutes at 20°C, and the serum was stored at −80°C and thawed at the time of assays.

Measurement of cfDNA

The Quant-iT PicoGreen double-stranded DNA (dsDNA) assay (Thermo Fisher Scientific, Waltham, Massachusetts) was used to quantify the cfDNA levels in the serum according to the manufacturer's instructions. In brief, we prepared a 5-point standard from 1 ng/mL to 1 μg/mL by serial dilutions. Standards or samples (50 μL) were loaded into black 96-well plates followed by adding 50 μL of the working solution of the Quant-iT PicoGreen reagent to each sample, then incubated for 5 minutes at room temperature, protected from light. The fluorescence (emission at 485 nm wavelength) intensity was quantified with a fluorescence reader (Varioskan Flash, Thermo Fisher Scientific).

MPO-DNA Enzyme-linked Immunosorbent Assay

A capture enzyme-linked immunosorbent assay (ELISA) based on MPO associated with DNA was used to quantify the NET formation as previously described [24]. Anti-MPO antibody was coated on a 96-well plate at 1:500 (Millipore Sigma, Burlington, Massachusetts) for 12 hours, and a 20-μL serum sample was mixed with 80 μL incubation buffer containing 5% anti-DNA horse radish peroxidase (Cell Death Detection ELISA Kit, Roche, Basel, Switzerland) and incubated for 2 hours at room temperature. The chromogenic substrate 3-ethylbenzothiazoline-6-sulphonic acid was added, followed by measurement of the absorption at 405 nm. To reduce the variation between different test batches, we used one serum sample as a reference. The optical density (OD) values of the samples in each experiment were calibrated as the OD index.

LF-DNA Complex Quantification

The concentration of the LF-DNA complex was measured in the serum of healthy donors and SFTS patients by detecting the concentration of LF-bound DNA. In brief, a capture antibody against LF was coated on a 96-well flat-bottom plate at 1:200 (Abcam, Cambridge, Massachusetts), and the amount of LF-bound DNA was quantified using the Quant-iT PicoGreen dsDNA assay described above.

Statistical Analysis

All statistical analyses were performed with the SPSS 25.0 statistical package (IBM, Armonk, New York). Values are presented as the mean ± standard deviation for data that were normally distributed, or median and interquartile range for data that were not normally distributed. The Kolmogorov-Smirnov test was used to inspect the normality

and homogeneity of variance of all the data. *P* values were derived from one-way *t* tests to determine differences among several groups with normally distributed data, and the Mann-Whitney nonparametric test was used for the other data. Correlations were analyzed by means of Spearman or Pearson correlation analyses. Outcome comparisons were analyzed by the χ^2 test, and the results are presented as *P* values and 95% confidence intervals (CIs). Binary logistic analyses were performed to identify the factors associated with the severity of SFTS. Odds ratios (ORs) and 95% CIs were used to measure the strength of the association. For all comparisons, $P < .05$ was considered statistically significant.

RESULTS

Dynamic Changes in Serum NET Levels During the Progression of SFTS

According to the typical clinical features, the course of SFTSV infection has the following 3 distinct phases: the acute stage (1–7 days postinfection), the progressing stage (8–14 days postinfection), and the convalescent stage (>14 days postinfection). Consistent with previous studies [3], the patients with SFTS in this study developed thrombocytopenia and neutropenia (Figure 1A). We assessed the serum lev-els of 3 markers for NETs—namely, cfDNA, MPO-DNA complexes, and LF-DNA complexes—which were significantly elevated in the patients with sepsis as previously reported (Supplementary Figure 1). Interestingly, we observed a

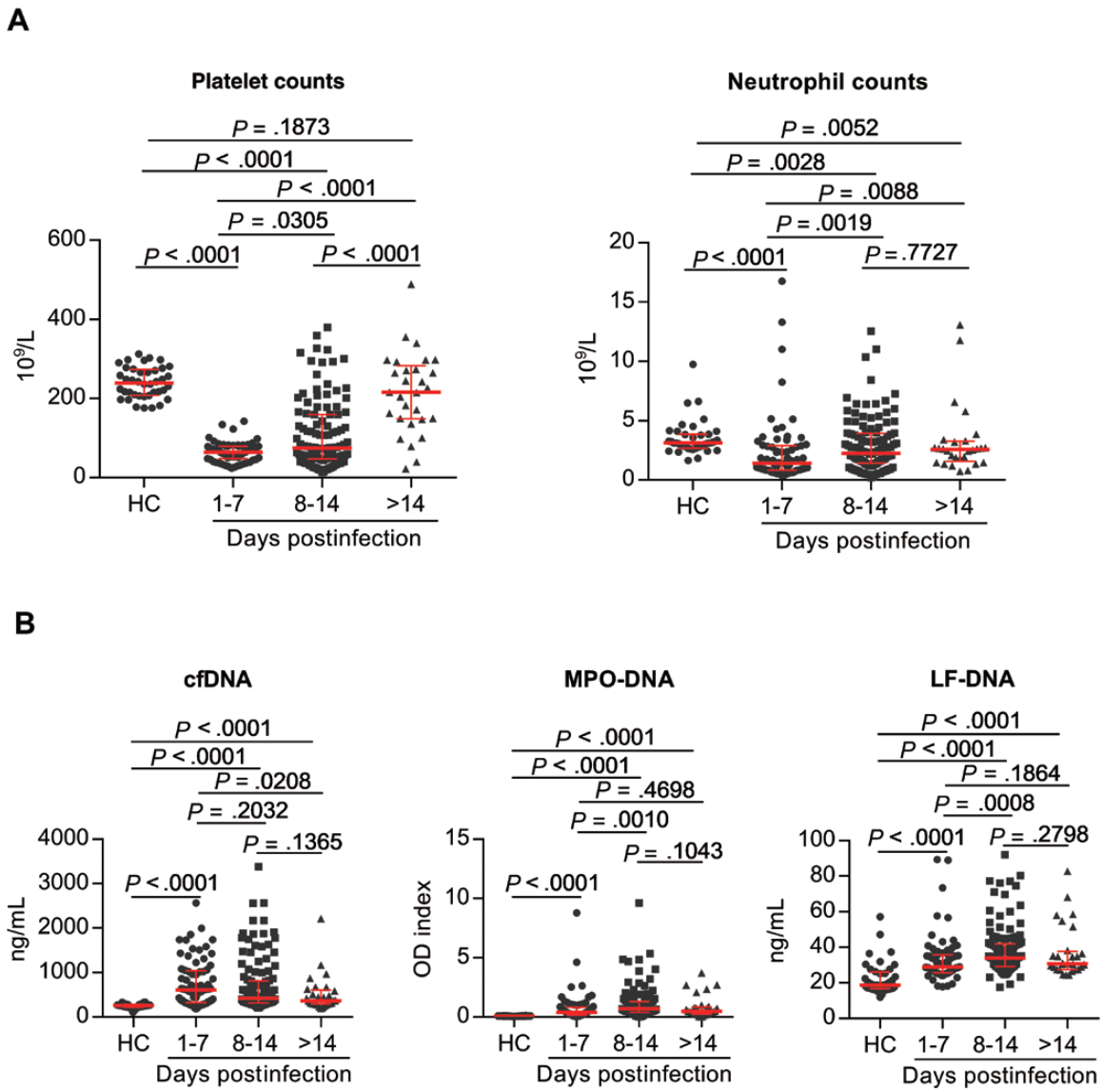

Figure 1. Platelet and neutrophil counts and neutrophil extracellular trap levels in the peripheral blood of patients. *A*, Platelet and neutrophil counts in HCs and patients with severe fever with thrombocytopenia syndrome (SFTS) during disease progression. HC, n = 40; SFTS, n = 112; 208 data points from patients were analyzed during hospitalization. *B*, Serum levels of cfDNA, MPO–DNA complexes (OD index), and LF–DNA complexes were detected during disease progression. HC, n = 40; SFTS, n = 112; 224 data points from patients were analyzed during hospitalization. Data are presented as median with interquartile range. *P* values were obtained by the Mann-Whitney test. Abbreviations: cfDNA, cell-free DNA; HCs, healthy controls; LF–DNA, lactoferrin–DNA; MPO–DNA, myeloperoxidase–DNA; OD, optical density.

Table 1. Comparison of Cell-free DNA Levels in Patients With Severe Fever With Thrombocytopenia Syndrome With Normal and Abnormal Values in Laboratory Parameters

Variable	cfDNA, ng/mL, Median (IQR)	*P* Value
Coagulation parameters		
Platelet count (×10^9/L)		
<100	576.3 (343.2–1074)	**< .0001**
≥100	348.6 (296.4–570.5)	Reference
APTT(s)		
24–36	452.8 (358.1–763)	Reference
>36	773.2 (390.7–1481)	**.0224**
Myocardial enzyme parameters		
CK (U/L)		
≤2 × ULN	439.6 (313.7–855.5)	Reference
>2 × ULN	716.3 (383.5–1339)	**.0095**
CK-MB (U/L)		
≤2 × ULN	442.9 (313.1–838.6)	Reference
>2 × ULN	644.5 (365.3–1039)	**.0386**
α-HBDH (U/L)		
≤2 × ULN	371 (301.6–615.6)	Reference
>2 × ULN	711.7 (427.1–1188)	**< .0001**
LDH (U/L)		
≤2 × ULN	386.6 (304.9–691.5)	Reference
>2 × ULN	723.8 (420–1415)	**.0001**
Hepatic function parameters		
ALT (U/L)		
≤2 × ULN	390.2 (306.5–771.7)	Reference
>2 × ULN	547.4 (340.9–1016)	**.0248**
AST (U/L)		
≤2 × ULN	396.7 (315.1–638.9)	Reference
>2 × ULN	616.8 (330.4–1311)	**.0019**
Total bilirubin (μmol/L)		
≤21	434.7 (313.3–878.7)	Reference
>21	584.4 (380.6–1383)	**.0351**
Direct bilirubin (μmol/L)		
≤6.8	398.6 (398.6–780.9)	Reference
>6.8	613 (375.8–1311)	**.0059**
Albumin (g/L)		
<40	479.5 (321–1002)	**.0013**
40–55	320 (255–348.6)	Reference
ALP (U/L)		
≤100	425.7 (315.1–740.7)	Reference
>100	886.6 (472.7–1644)	**.0006**
ALB/GLB		
<1.5	697.9 (438–1106)	**.0013**
1.5–2.5	394.7 (288.2–605.2)	Reference
GGT (U/L)		
≤45	398.6 (310.1–829.4)	Reference
>45	552.1 (328.4–1033)	.1291
Renal function parameters		
BUN (mmol/L)		
<2.6	345.9 (283.2–705.3)	.2413
2.6–7.5	439.6 (313.7–924.7)	Reference
>7.5	750.9 (459–1116)	**.0374**
Serum creatinine (μmol/L)		
<41	505.9 (352.4–1384)	.0873
41–73	392.5 (301.5–827)	Reference
>73	618.7 (369.9–1044)	.0615
Electrolyte disturbance		
Na^+ (mmol/L)		
<137	611 (320.2–1119)	**.0009**
137–147	369 (294.2–570.5)	Reference
>147	456.9 (435.3–715.9)	**.1560**
Ca^{2+} (mmol/L)		
<2.11	610.3 (347.8–1161)	**< .0001**
2.11–2.52	341 (278.2–468.4)	Reference

P values are derived from Mann-Whitney nonparametric test. Statistically significant data are shown in bold.

Abbreviations: α-HBDH, α-hydroxybutyrate dehydrogenase; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca^{2+}, Calcium^{2+}; cfDNA, cell-free DNA; CK, creatine kinase; CK-MB, creatine kinase MB fraction; GGT, γ-glutamyl transpeptidase; GLB, globulin; LDH, lactate dehydrogenase; Na^+, Sodium$^+$; ULN, upper limit of normal.

pattern that was the inverse of the trend seen for platelets and neutrophils; the levels of all tested NET parameters were higher in SFTS patients in the acute stage than in the healthy controls, reaching plateaus in the progressing stage. In the convalescent stage, the values were still markedly higher than those of the healthy controls (Figure 1B).

Correlations Between cfDNA Levels and Tissue and Organ Damage

Next, we subdivided the patients into 2 or 3 groups according to the normal ranges of laboratory parameters or 2 times the ULN. Compared with patients with normal levels, patients with abnormal platelet counts, activated partial thromboplastin time (APTT), and levels of CK, CK-MB fraction (CK-MB), α-hydroxybutyrate dehydrogenase (α-HBDH), LDH, AST, ALT, total bilirubin and direct bilirubin, albumin, blood urea nitrogen, sodium, and calcium had significantly elevated cfDNA levels (Table 1). Consistently, cfDNA levels were negatively correlated with platelet counts, serum albumin, and calcium concentrations, and positively correlated with APTT and the levels of CK, α-HBDH, LDH, and AST (Supplementary Table 1). Thus, the increased production of cfDNA is associated with multiple organ injury in patients with SFTS.

Encephalopathy is the primary severe clinical manifestation that is strongly associated with death in patients with SFTS [29]. We also observed significantly higher levels of cfDNA in patients who suffered encephalopathy than in patients without this symptom (Figure 2A). Compared with patients who survived, patients who died from SFTS had significantly higher serum cfDNA levels (Figure 2B).

Elevated Levels of cfDNA in Severe Cases

We performed a comprehensive analysis to evaluate the predictive value of cfDNA for severe illness in SFTS patients.

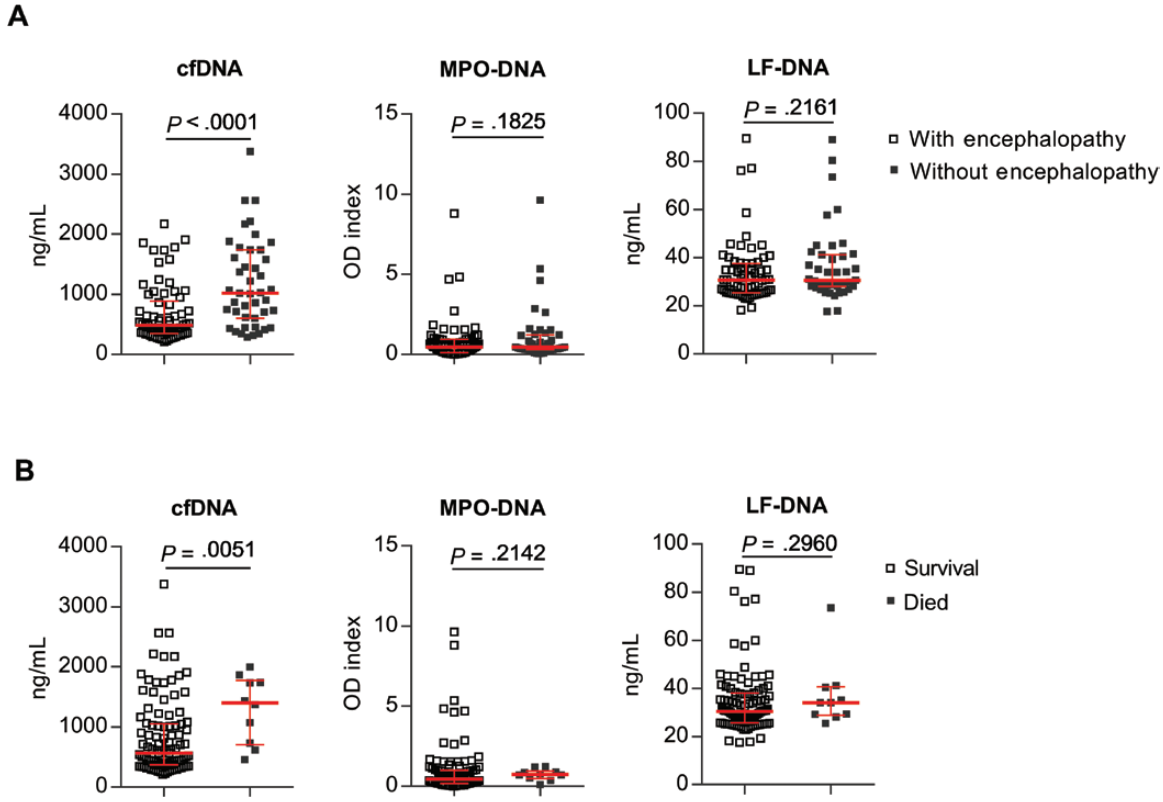

Figure 2. Associations between neutrophil extracellular trap levels and disease prognosis. *A*, Serum levels of cfDNA, MPO–DNA complexes (OD index), and LF–DNA complexes in patients with (n = 43) and without (n = 69) encephalopathy. *B*, Serum levels of cfDNA, MPO-DNA complexes, and LF-DNA complexes in living (n = 102) and deceased (n = 10) patients. Data are presented as median with interquartile range. *P* values were obtained by the Mann-Whitney test. Abbreviations: cfDNA, cell-free DNA; LF–DNA, lactoferrin–DNA; MPO–DNA, myeloperoxidase–DNA; OD, optical density.

According to a previous report on SFTS, 42 patients were classified as having severe SFTS, and the other 70 patients were defined as having mild/moderate SFTS. The demographic characteristics of the mild/moderate vs severe cases are summarized in Table 2. The fatality rate was 8.93% (95% CI, 4.92%–15.67%) in all patients and 23.81% (95% CI, 13.48%–38.53%) in patients with severe SFTS. Compared to patients with mild/moderate SFTS, those with severe SFTS had significantly higher levels of cfDNA, MPO-DNA, and LF-DNA at the time of their initial diagnosis (Figure 3A).

Predictive Value of cfDNA for the Prognosis of SFTS

To identify whether the cfDNA level can predict the severity of SFTS, we further divided all the patients into the following 2 cohorts: The evaluation cohort consisted of 55 patients who were hospitalized from October 2014 to June 2016, and the validation cohort consisted of 57 patients who were hospitalized from July 2016 to September 2017. There were no differences in age, death rate, or any laboratory values between the 2 cohorts (Supplementary Table 2). Receiver operating characteristic curve analysis of the evaluation cohort identified an optimal cutoff value of 711.7 ng/mL for cfDNA. The sensitivity and specificity of this cfDNA level for predicting severe illness were 72.0% (95% CI, 50.61%–87.93%) and 75.0% (58.80%–87.31%), respectively (Figure 3B).

Subsequently, we analyzed the validation cohort using 711.7 ng/mL as the cfDNA cutoff value. Consistent with the values in the evaluation cohort, the sensitivity and specificity of cfDNA for predicting severe illness were 82.35% (95% CI, 56.57%–96.20%) and 73.33% (54.11%–87.72%), respectively (Figure 3B). Moreover, we found that patients with cfDNA levels >711.7 ng/mL had a fatality rate of 16.0% (95% CI, 8.3%–28.5%), which was significantly higher than the fatality rate in patients with lower cfDNA levels (3.2% [95% CI, .6%–11.0%]; *P* = .0407; Figure 3C).

In univariate analysis, we identified 14 variables (age, encephalopathy, platelet count, leukocyte count, and levels of CK, CK-MB, α-HBDH, LDH, AST, direct bilirubin, albumin, albumin/globulin, calcium, and cfDNA) that were significantly different between patients with severe and mild/moderate SFTS (Table 2). Importantly, after adjusting for the effects of confounding factors in multivariate analysis, cfDNA (>711.7 ng/mL; OR, 8.285 [95% CI, 2.049–33.503]; *P* = .003) and 3 other variables—namely, LDH level, encephalopathy, and leukocyte count—were independent risk factors for severe illness (Table 3).

Table 2. Characteristics of Patients With Severe Fever With Thrombocytopenia Syndrome (Mild/Moderate or Severe)

Characteristic	Patients With SFTS		
	Mild/Moderate	Severe	*P* Value
Patients, No.	70	42	
Age, y, mean ± SD	57.8 ± 10.9	63.5 ± 11.2	**.0056**[a]
Male sex, No. (%)	37 (52.9)	20 (47.6)	.6968[b]
Previous or preexisting conditions, No. (%)			
Respiratory disease	1 (1.4)	4 (9.5)	.0647[b]
Hypertension	7 (10.0)	7 (16.7)	.3788[b]
Diabetes	4 (5.7)	4 (9.5)	.4704[b]
Heart disease	3 (4.3)	4 (9.5)	.4218[b]
Autoimmune disease	0	0	
Liver disease	0	2 (4.8)	.1385[b]
Gestation	0	0	
Time from symptom onset to hospital admission, d	5 (4–6)	5 (4–7)	.3111[b]
Hospitalization period, d	11 (9–15)	11 (6–18)	.8047[b]
General symptoms, No. (%)			
Nausea	33 (47.1)	16 (38.1)	.4322[b]
Diarrhea	3 (4.3)	4 (9.5)	.4218[b]
Hemorrhagic signs	2 (2.9)	1 (2.4)	> .9999[b]
Encephalopathy	13 (18.6)	30 (71.4)	**< .0001**[b]
No. of deaths (%)	0	10 (23.8)	**< .0001**[b]
Laboratory values			
Platelet count, $\times 10^9$/L	79 (56–136.5)	52 (37.1–93.3)	**.0030**[b]
Leukocyte count, $\times 10^9$/L	3.3 (2.3–5.0)	5.2 (3.9–6.2)	**.0004**[b]
APTT,s	35.7 (30.3–40.2)	40.0 (30.8–49.5)	.2267[b]
CK, U/L	192 (55.7–668.5)	495 (250.5–1200)	**.0012**[b]
CK-MB, U/L	18.4 (13–32.1)	25.1 (15.6–43.5)	**.0244**[b]
α-HBDH, U/L	289.5 (238.8–367.5)	772 (406–1005)	**< .0001**[b]
LDH, U/L	357.5 (282–464.3)	987 (460.5–1340)	**< .0001**[b]
ALT, U/L	72 (50–102)	86 (54–190)	.0577[b]
AST, U/L	74 (52.5–137)	164.5 (57–421.5)	**.0071**[b]
TBIL, μmol/L	10.8 (8.1–16.7)	12.4 (9.4–19)	.1069[b]
DBIL, μmol/L	4.2 (3.3–5.7)	5.6 (3.8–9.3)	**.0227**[b]
ALB, g/L	35.2 (32.7–37.9)	30.6 (28.7–33.2)	**< .0001**[b]
ALP, U/L	71 (56–81)	71 (54.5–95.8)	.4237[b]
ALB/GLB	1.5 (1.3–1.6)	1.1 (1.0–1.2)	**< .0001**[b]
GGT, U/L	50 (33–74)	47.5 (26.8–124.3)	.6560[b]
BUN, mmol/L	3.8 (2.9–4.9)	4.2 (3.0–6.9)	.3393[b]
sCr, μmol/L	63 (53–71)	59.5 (48.8–72)	.4761[b]
Na^+, mmol/L	136.9 (134–140)	136.1 (133–139.2)	.3434[b]
Ca^{2+}, mmol/L	2.1 (1.9–2.2)	2 (1.9–2.1)	**.0170**[b]
cfDNA, ng/mL	396.5 (294.7–638.9)	957.2 (612.1–1738)	**< .0001**[b]

Data were collected at initial diagnosis in each patient. Values are presented as median (interquartile range) unless otherwise indicated. Statistically significant data are shown in bold.

Abbreviations: α-HBDH, α-hydroxybutyrate dehydrogenase; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca^{2+}, Calcium^{2+}; cfDNA, cell-free DNA; DBIL, direct bilirubin; CK, creatine kinase; CK-MB, creatine kinase MB fraction; GGT, γ-glutamyl transpeptidase; GLB, globulin; LDH, lactate dehydrogenase; Na^+, Sodium$^+$; sCr, serum creatinine; SD, standard deviation; SFTS, severe fever with thrombocytopenia syndrome; TBIL, total bilirubin.

[a] *t* test.

[b] Nonparametric test.

Taken together, these results demonstrated that the cfDNA level at the time of initial diagnosis is a predictive marker for severe illness in patients with SFTS.

DISCUSSION

In the present study, we measured NET levels in SFTS patients from a region with a high prevalence of SFTSV infection in northern China. In our cohort, the age distribution of patients, clinical symptoms, and case fatality rate were consistent with those reported in previous studies. We observed a rapid increase in NET levels in the serum of SFTS patients and dynamic changes during 3 phases of the clinical course. High NET levels were strongly associated with disease severity and multiple organ dysfunction. Specifically, as a marker of NETs, high cfDNA levels in the serum were closely related to multiple pathological lesions

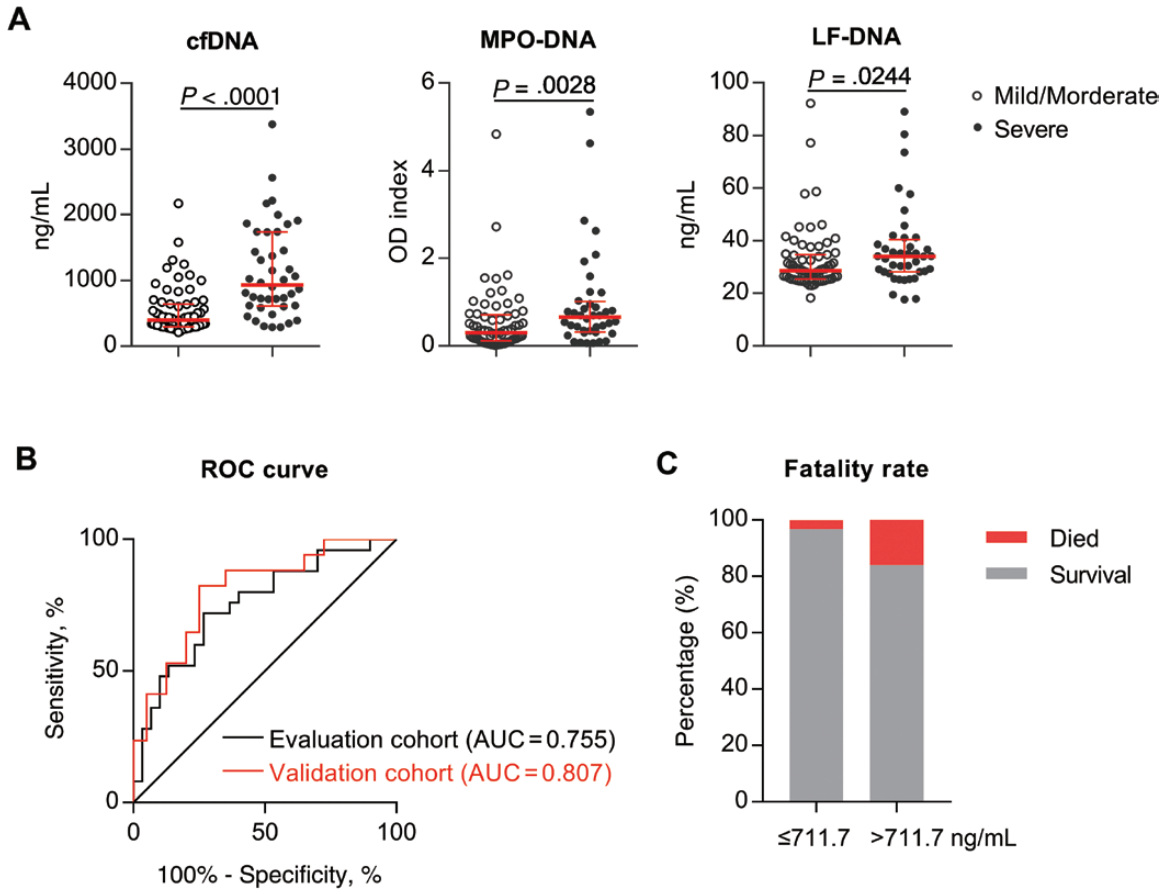

Figure 3. A high level of cfDNA predicted severe illness in patients with severe fever with thrombocytopenia syndrome (SFTS). *A*, Serum levels of cfDNA, MPO–DNA complexes (OD index), and LF–DNA complexes in patients with mild/moderate (n = 72) and severe (n = 40) SFTS. *B*, ROC curve and AUC in the evaluation cohort and validation cohort. The evaluation cohort consisted of 30 patients with mild/moderate SFTS and 25 patients with severe SFTS. The validation cohort consisted of 40 patients with mild/moderate SFTS and 17 patients with severe SFTS. *C*, Comparison between the fatality rates of patients with cfDNA levels >711.7 ng/mL (n = 42) and patients with lower cfDNA levels (n = 70). Data were collected at the time of initial diagnosis in each patient. Data are presented as median with interquartile range. *P* values were obtained by the Mann-Whitney test. Abbreviations: AUC, area under the receiver operating characteristic curve; cfDNA, cell-free DNA; LF–DNA, lactoferrin–DNA; MPO–DNA, myeloperoxidase–DNA; OD, optical density; ROC, receiver operating characteristic.

and fatal outcomes. More importantly, we identified that a high level of cfDNA (>711.7 ng/mL) predicted severe illness in SFTS patients, and patients with cfDNA levels >711.7 ng/mL were at a higher risk of death. Thus, cfDNA could be used as a biomarker and predictor of disease severity and poor prognosis of SFTS.

The previously identified biomarkers and risk factors for the prognosis of SFTS were almost all clinical or laboratory parameters, and each single biomarker could only indicate damage to 1 or 2 specific organs. However, SFTS is characterized by dysfunction in a variety of tissues and organs. Thus, although a variety of indicators have been demonstrated to be associated with poor prognosis or fatal outcome, such as LDH or encephalopathy, it still lacks one indicator that could link multiple tissue damage together. Here, we identified cfDNA as reflecting multiple pathological lesions, including coagulation disorder, hypocalcemia, and dysfunctions of the heart, liver, or brain. This finding not only indicated that cfDNA is an effective biomarker but also suggested that it may be involved in death-related pathological mechanisms.

In addition to neutrophils, cfDNA could also be released by other damaged cells during apoptosis or necrosis. Thus, the cfDNA detected in the serum of SFTS patients might be derived from both neutrophils (NETs) and the parenchymal cells of solid organs, such as cardiomyocytes, hepatocytes, and endothelial cells. As there was no similar degree of correlation of MPO-DNA or LF-DNA with clinical severity, our results indicated that cfDNA was a more accurate marker of multiple pathological lesions than other markers of NETs. Moreover, the method of cfDNA quantification has been widely used in liquid samples and has several advantages: It requires a small amount of serum (50 μL), the turnover time is short (1 hour), the operation is simple and inexpensive, and the results are stable and reproducible. These methodological advantages make cfDNA an attractive and feasible parameter for the dynamic monitoring of SFTS patients.

Among the 4 independent risk factors for severe illness, cfDNA was the most critical variable with the highest OR.

Table 3. Multivariate Logistic Regression Analysis of Variables Associated With Severe Illness Among Patients With Severe Fever With Thrombocytopenia Syndrome

Variable	OR (95% CI)	*P* Value
cfDNA, ng/mL		
≤711.7	1.00	Reference
>711.7	8.285 (2.049–33.503)	.003
LDH, U/L		
≤2 × ULN	1.00	Reference
>2 × ULN	7.623 (1.971–29.480)	.003
Encephalopathy		
No	1.00	Reference
Yes	5.854 (1.544–22.186)	.009
Leukocyte count, $\times 10^9$/L		
4.0–10.0	1.00	Reference
<4.0	0.236 (.058–.955)	.043

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; LDH, lactate dehydrogenase; OR, odds ratio; ULN, upper limit of normal.

This finding is highly significant in clinical practice because the cutoff value for cfDNA has strong potential to be used in predicting the development and prognosis of SFTS. In addition to cfDNA, LDH levels and encephalopathy also have high predictive values for disease severity. This finding was consistent with those of previous studies [11, 13], which revealed that LDH and decreased level of consciousness were significant predictors of severe illness and a fatal outcome. Thus, a comprehensive evaluation based on cfDNA and LDH levels and encephalopathy may be more accurate for the prediction of prognosis. Early interventions in patients with abnormal manifestations of all 3 indicators may improve their clinical outcome.

We recently found that high levels of NETs were associated with increased numbers of peripheral neutrophils in influenza patients [24]. In contrast, in this study, we found that high levels of NETs were accompanied by decreased numbers of neutrophils. This different phenomenon may be due to differences in the pathological mechanisms underlying influenza and SFTS. There are several possible reasons for neutropenia in patients with SFTS. First, platelets could be activated by the SFTSV, subsequently coagulating with neutrophils [19]. These platelet-neutrophil aggregates may be excluded by the conventional neutrophil counting method. Second, the formation of NETs, also known as NETosis, is one pathway to death taken by neutrophils. Once the hemopoietic system can no longer replenish neutrophils at the rate they are dying (through NETosis, apoptosis, or being engulfed by macrophages), the peripheral neutrophil counts decrease. Third, a substantial expansion of arginase-expressing granulocytic myeloid-derived suppressor cells was observed in patients with SFTS in a recent study [30], which can lead to a decrease in the number of mature polymorphonuclear neutrophils that are released into the periphery.

A large number of studies have shown that components of the complement system are involved in the activation of platelets and neutrophils in the immune response. C3a and C5a are potent inflammatory mediators, and their receptors are expressed on the surface of neutrophils [31–33]. However, we found that the serum levels of C3a and C5a in the patients with SFTS were comparable to those in the healthy controls (Supplementary Figure 2), which suggested that C3a and C5a are not involved in NET production and the progression of SFTS. Further studies are needed to reveal the mechanism underlying NET activation in SFTS, which may be different from the mechanisms observed in previous studies of influenza and other infections.

In conclusion, we found that cfDNA is a novel predictive biomarker for severe illness through a large multicenter cohort of SFTS patients. Further studies exploring the roles and mechanisms by which NETs act during SFTS pathogenesis will determine the therapeutic potential of NET-targeting strategies.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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