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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 rombocytopenia Syndrome

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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 ine ective clearance of

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. P l h l h l ll l l h h l l . *A*, P l h l HC h h h m m (₣) . HC, = 40; ₣ ; = 112; 208 m l h - l . *B*, ; m l l DNA, MPO DNA m l (OD), LF DNA m l . HC, = 40; ₣ ; = 112; 224 m l h l . D m h l . *P* l h M - h . A : DNA, !!- DNA; HC , h l h l ; LF DNA, l DNA; MPO DNA, m l DNA; OD, l .

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 signi cantly higher levels of cfDNA in patients who su ered encephalopathy than in patients without this symptom (Figure 2A). Compared with patients who survived, patients who died from SFTS had signi cantly 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.

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

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

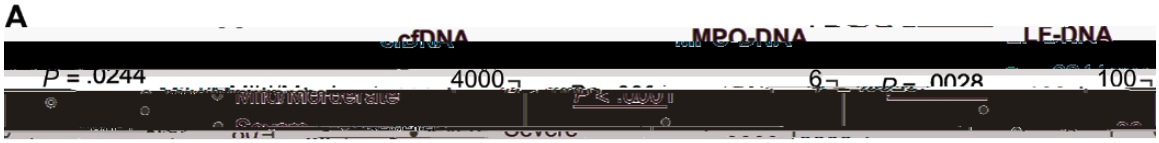

Figure 3. A h h ! DNA h h m m ( ). *A*, m ! DNA, MPO DNA m ! (OD), LF DNA m ! h m ! /m (= 72) (= 40) *B*, ROC A C h ! h h . h ! h 30 h m ! /m 25 h h h ! h 40 h m ! /m 17 h *C*, C m h ! h DNA ! ! >711.7 /mL (= 42) h ! DNA ! ! (= 70). D h m ! h . D m h ! . *P* ! h M - h . A : A C, h h ; DNA, !- DNA; LF DNA, ! DNA; MPO DNA, m ! DNA; OD, ! ; ROC, h .

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.

e previously identi ed 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 speci c organs. However, SFTS is characterized by dysfunction in a variety of tissues and organs. us, 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 identi ed cfDNA as re ecting multiple pathological lesions, including coagulation disorder, hypocalcemia, and dysfunctions of the heart, liver, or brain. is nding not only indicated that cfDNA is an e ective 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. us, 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 quanti cation 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. ese 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.

is nding is highly signi cant in clinical practice because the cuto 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. is nding was consistent with those of previous studies [11, 13], which revealed that LDH and decreased level of consciousness were signi cant predictors of severe illness and a fatal outcome. us, 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 in uenza patients [24]. In contrast, in this study, we found that high levels of NETs were accompanied by decreased numbers of neutrophils. is di erent phenomenon may be due to differences in the pathological mechanisms underlying in uenza and SFTS. ere are several possible reasons for neutropenia in patients with SFTS. First, platelets could be activated by the SFTSV, subsequently coagulating with neutrophils [19]. ese 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. ird, a substantiutrexpansion 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 polymor-

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