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Circulating levels of DNA-histone complex and dsDNA are independent prognostic factors of disseminated intravascular coagulation

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ABSTRACT

Introduction: Neutrophils can be induced to release DNA combined with histones. The resulting neutrophil extracellular trap (NET) provides a scaffold for growing hemostatic plug. Therefore, the NET formation may be inevitable in clinical conditions that are characterized by formation of vascular thrombi. Thus far, there have been no reports on the clinical significance of NET in disseminated intravascular coagulation (DIC). Therefore, we investigated circulating levels of NET in DIC and analyzed their potential values to assess coagulation severity and predict clinical outcome.

Methods: The plasma levels of DNA-histone complexes and double-stranded DNA (dsDNA), considered to be in vivo markers of NET, were measured in 199 patients suspected of having DIC and 20 healthy controls.

Result: The circulating levels of DNA-histone complexes and dsDNA were significantly elevated in overt-DIC. The increased levels of these two markers correlated with the severity of coagulopathy including DIC score and D-dimer. Multivariable Cox regression analysis, adjusted for the conventional DIC markers, revealed that elevated DNA-histone complexes and dsDNA are poor independent prognostic markers.

Conclusion: The circulating levels of NET release reflect the coagulation activation and adverse clinical outcomes in patients with DIC, thereby providing potential clinical relevance for mortality prediction in DIC.

Introduction

In recent studies, neutrophils have been reported to play an essential role in thrombosis [1]. When pathogens such as bacteria invade the circulation, neutrophils defend against the pathogens, through the release of neutrophil extracellular trap (NET). The NET not only binds to the pathogens, it also entraps the platelets and red blood cells that are the main components of vascular thrombi. Meshwork of DNA strands around histones from the main skeleton of NET [1]. Histones can activate platelets, induce endothelial cell damage, and inhibit anticoagulant protein C activation [2,3]. Therefore, it is likely that they promote thrombin formation.

NET is triggered not only by infectious pathogens, but also by reactive oxygen species, antibodies, and activated platelets [4]. As a result, an increase of NET formation has been reported in various clinical conditions including sepsis, trauma, autoimmune diseases, deep vein thrombosis, atherosclerosis, and thrombotic microangiopathy [5–10].

Disseminated intravascular coagulation (DIC) is characterized by systemic activation of coagulation and anticoagulation pathways leading to florid fibrin deposition and formation of microthrombi [11,12]. The underlying conditions of DIC such as sepsis and trauma are known to promote NET formation [2,10]. Since NET contributes to thrombus formation, we presumed that DIC process is closely related with increased NET formation and the resulting NET may in turn promote hypercoagulability. Hence, in the present study we investigated circulating levels of NET in patients suspected of having DIC and analyzed their potential values to assess coagulation severity and to predict clinical outcome.

Materials and Methods

Study Population

A total of 199 patients who were clinically suspected of having DIC and who underwent DIC screening battery tests were enrolled in the

Abbreviations: NET, neutrophil extracellular trap; DIC, disseminated intravascular coagulation; dsDNA, double-stranded DNA; ISTH, International Society of Thrombosis and Haemostasis; PT, prothrombin time; aPTT, activated partial thromboplastin time; ROC, receiver operating characteristic; DNase, deoxyribonuclease

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Table 1
The characteristics of the study population.

	According to DIC diagnosis[a]		According to prognosis[b]	
	No overt-DIC (n = 146)	Overt-DIC (n = 53)	Survivors (n = 166)	Non survivors (n = 33)
Age, yrs,	57.1 ± 18.4	53.0 ± 17.8	55.2 ± 18.4	59.8 ± 17.6
Male/Female (%)	88 (60.3)/ 58 (39.7)	34 (64.2)/19 (35.8)	104 (62.7)/62 (37.3)	18 (54.5)/15 (45.5)
Clinical diagnosis, n (%)				
Sepsis/severe infection	23 (15.8)	8 (15.1)	23 (13.9)	8 (24.2)
Solid malignancies	63 (43.2)	20 (37.7)	72 (43.4)	11 (33.3)
Hematologic malignancies	25 (17.1)	13 (24.5)	25 (15.1)	13 (39.4)
Organ destruction	6 (4.1)	2 (3.8)	8 (4.8)	0 (0.0)
Trauma	4 (2.7)	0 (0.0)	4 (2.4)	0 (0.0)
Severe hepatic failure	4 (2.7)	8 (15.1)	11 (6.6)	1 (3.0)
Severe toxic or immunologic reaction	9 (6.2)	0 (0.0)	9 (5.4)	0 (0.0)
Vascular abnormalities	6 (4.1)	2 (3.8)	8 (4.8)	0 (0.0)
Obstetrical calamities	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other[†]	6 (4.1)	0 (0.0)	6 (3.6)	0 (0.0)
Neutrophil ($x10^9$/L)	7.20 ± 8.76	6.22 ± 6.42	6.95 ± 8.49	6.90 ± 6.57
Platelets ($x10^9$/L)	160 ± 116**	60.3 ± 67.9	142 ± 115*	88.4 ± 97.0
PT (sec)	14.1 ± 9.37*	18.4 ± 5.08	15.1 ± 9.18	16.0 ± 5.25
aPTT (sec)	33.8 ± 8.72**	39.1 ± 10.9	34.7 ± 9.40	37.8 ± 10.4
D-dimer (μg/mL)	5.11 ± 11.2*	10.7 ± 16.4	6.16 ± 13.0	8.87 ± 13.1
Fibrinogen (mg/dL)	360 ± 119**	269 ± 164	343 ± 135	302 ± 152
Protein C (%)	74.9 ± 30.7**	43.0 ± 28.9	69.2 ± 33.2*	52.3 ± 30.5
Antithrombin (%)	75.7 ± 21.9**	52.6 ± 23.5	72.1 ± 24.2*	56.7 ± 22.2
DNA-histone complex (AU)	146 ± 246*	232 ± 286	131 ± 198**	356 ± 412
dsDNA (ng/ml)	2.99 ± 2.51**	4.62 ± 4.13	2.94 ± 2.29**	5.89 ± 4.98

Values are presented as the mean ± standard deviation. [†]Other clinical diagnoses included organ destruction (n = 1), diabetes mellitus (n = 3), and chronic kidney disease (n = 2).
[a] Total population was classified into two groups in terms of overt-DIC criteria. [b] Total population was divided into survivor and non-survivor according to 150-day mortality.
* $P < 0.05$, ** $P < 0.001$.
Abbreviations: DIC, disseminated intravascular coagulation; PT, prothrombin time; aPTT, activated partial thromboplastin time, dsDNA; double stranded DNA.

study. Written consent from patient was exempted, since all data were acquired anonymously and retrospectively from laboratory information system without any additional blood sampling. Exclusion criteria were thrombotic or bleeding disorders, use of warfarin or heparin medications within 3 days of blood collection, or pediatric patients. In addition, 20 healthy adults were enrolled under written informed consent in order to determine the reference range of circulating NET levels. The Institutional Review Board of Seoul National University Hospital approved this study.

The patients' characteristics are described in Table 1. Patients were diagnosed as having overt-DIC, which was defined as a cumulative score of ≥5 by the International Society of Thrombosis and Haemostasis (ISTH) Sub-Committee scoring system [12]. We arbitrarily classified the patients who did not meet the criteria of overt-DIC as no overt-DIC.

Blood Samples and Assays

Peripheral blood was collected in sodium citrate tubes (Becton Dickinson, San Jose, CA, USA). The whole blood samples were centrifuged for 15 minutes at 1550 x g within 2 hours after sample withdrawal. The prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen were assayed on an ACL 3000 automated coagulation analyzer (Instrumentation Laboratory, Milan, Italy) using HemosIL RecombiPlasTin and SynthASil reagents (Instrumentation Laboratory). D-dimer was tested by an immunoturbidimetric assay (Instrumentation Laboratory). Antithrombin and protein C activity were measured by a chromogenic assay (HemosIL Antithrombin and Protein C; Instrumentation Laboratory).

The circulating levels of NET in plasma were quantified using two commercial kits; The DNA-histone complex was quantified using an

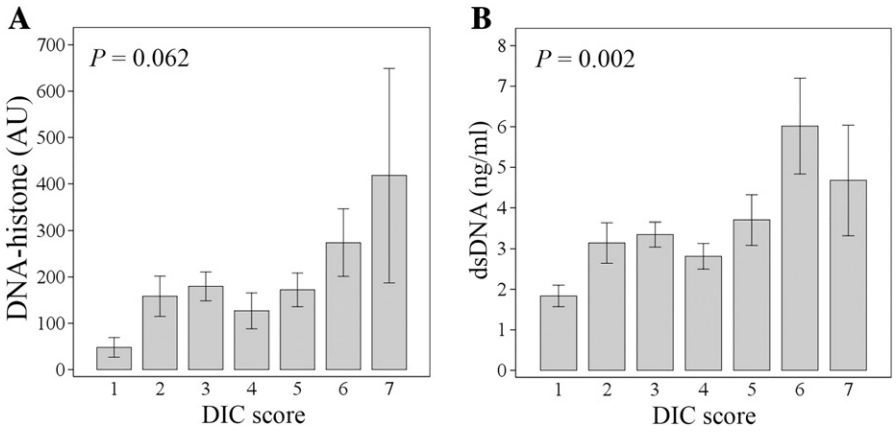

Fig. 1. Trend of circulating levels of DNA-histone complex (A) and dsDNA (B) based on disseminated intravascular coagulation (DIC) score in all patients.

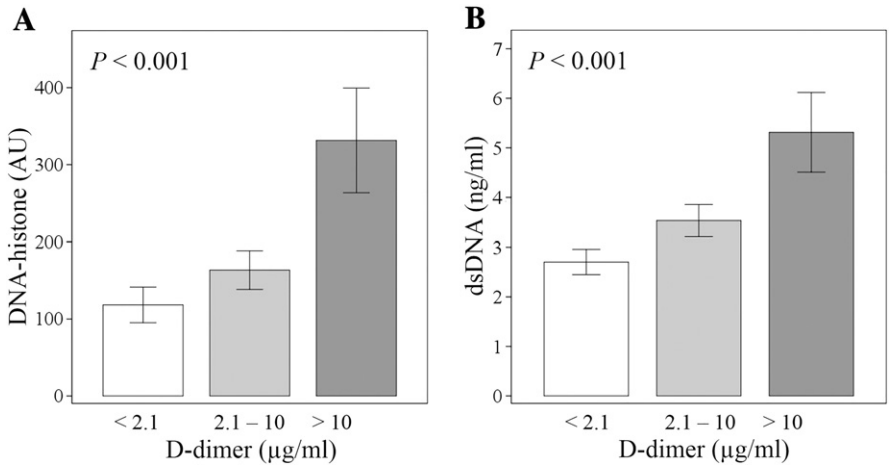

Fig. 2. Association of circulating levels of DNA-histone complex (A) and dsDNA (B) with the D-dimer levels in all patients.

ELISA kit (Cell Death Detection ELISA, Roche Diagnostics, IN, USA); the double-stranded DNA (dsDNA) was measured using Quant-iT PicoGreen dsDNA reagent (Molecular Probes, Eugene, OR, USA) and a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's guidelines.

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Data comparisons were carried out using the t-test or chi-square test. Receiver operating characteristic (ROC) curve analysis was carried out for mortality prediction of each parameter. ROC curve analysis using MedCalc (Mariakerke, Belgium) was used to determine the optimal cutoff value. Kaplan–Meier survival analysis was used to analyze the cumulative survival curves. Univariable and multivariable Cox regression analyses were carried out to identify significant prognostic markers. Two-sided *P*-values of <0.05 were considered to be statistically significant.

Results

Circulating Levels of DNA-histone Complex and dsDNA in Relation to Coagulation Severity

According to the DIC scoring system criteria, 53 out of the total 199 patients were found to be with overt-DIC (Table 1). There were no differences seen with respect to age and sex, between overt-DIC group and no overt-DIC group. The well-known DIC parameters including platelets, PT, aPTT, D-dimer, and fibrinogen showed significant changes in overt-DIC. The decrease in protein C and antithrombin levels was found to be more in overt-DIC than in no overt-DIC. Overt-DIC patients showed significantly high levels of DNA-histone complexes and dsDNA as compared to the levels in no overt-DIC. The reference range of DNA-histone complex and dsDNA established by testing 20 normal healthy controls (mean age 46; 12 males and 8 females) were 0-56 AU and 1.71-1.99 ng/mL, respectively. Even no overt-DIC patients were found to have markedly higher levels of DNA-histone complexes (146 ± 246 AU) and dsDNA (2.99 ± 2.51 ng/mL) than those in healthy controls. Statistically, the DNA-histone complex levels strongly correlated with dsDNA levels ($r = 0.544$, $P < 0.001$).

The circulating levels of DNA-histone complexes and dsDNA were found to increase gradually in relation to the DIC score (Fig. 1). When divided into 3 tertile groups according to the D-dimer levels, the mean values of DNA-histone complex and dsDNA correlated with D-dimer levels (Fig. 2).

Prognostic Values of Circulating DNA-histone Complex and dsDNA Levels

Non-survivors were arbitrarily defined as patients who were dead within 28-hospital days. Out of the total 199 patients, 33 were categorized as non-survivors (Table 1). There were no significant differences in age and sex between survivors and non-survivors. Platelet counts were decreased in non-survivors whereas PT, D-dimer, and fibrinogen levels were not significantly different between survivors and non-survivors. Plasma levels of protein C and antithrombin were significantly decreased in non-survivors as compared to that in survivors. The increase in the circulating levels of DNA-histone complexes and dsDNA was significantly higher in non-survivors than in survivors.

The prognostic values of DNA-histone complexes and dsDNA were evaluated using ROC curves. The AUC of DNA-histone complexes and dsDNA were slightly higher than that of platelet and D-dimer (Fig. 3).

For Kaplan-Meier analysis, the total population was divided into two groups according to the cutoff values that provided the best prognostic power in the ROC analysis. Among all patients, the group with high

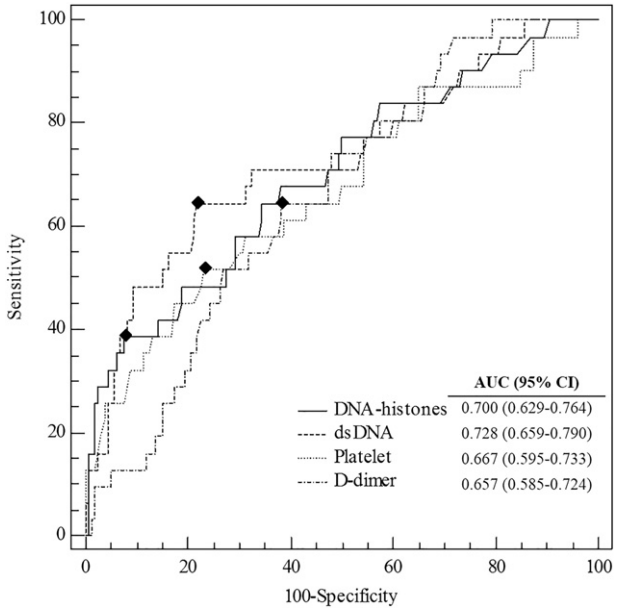

Fig. 3. Receiver operating characteristic (ROC) curves and the area under the ROC curves (AUC) for DNA-histone complexes, dsDNA, platelets and D-dimer for mortality prediction in disseminated intravascular coagulation. Diamond points (◆) represent the optimal cutoff point corresponding to Youden index.

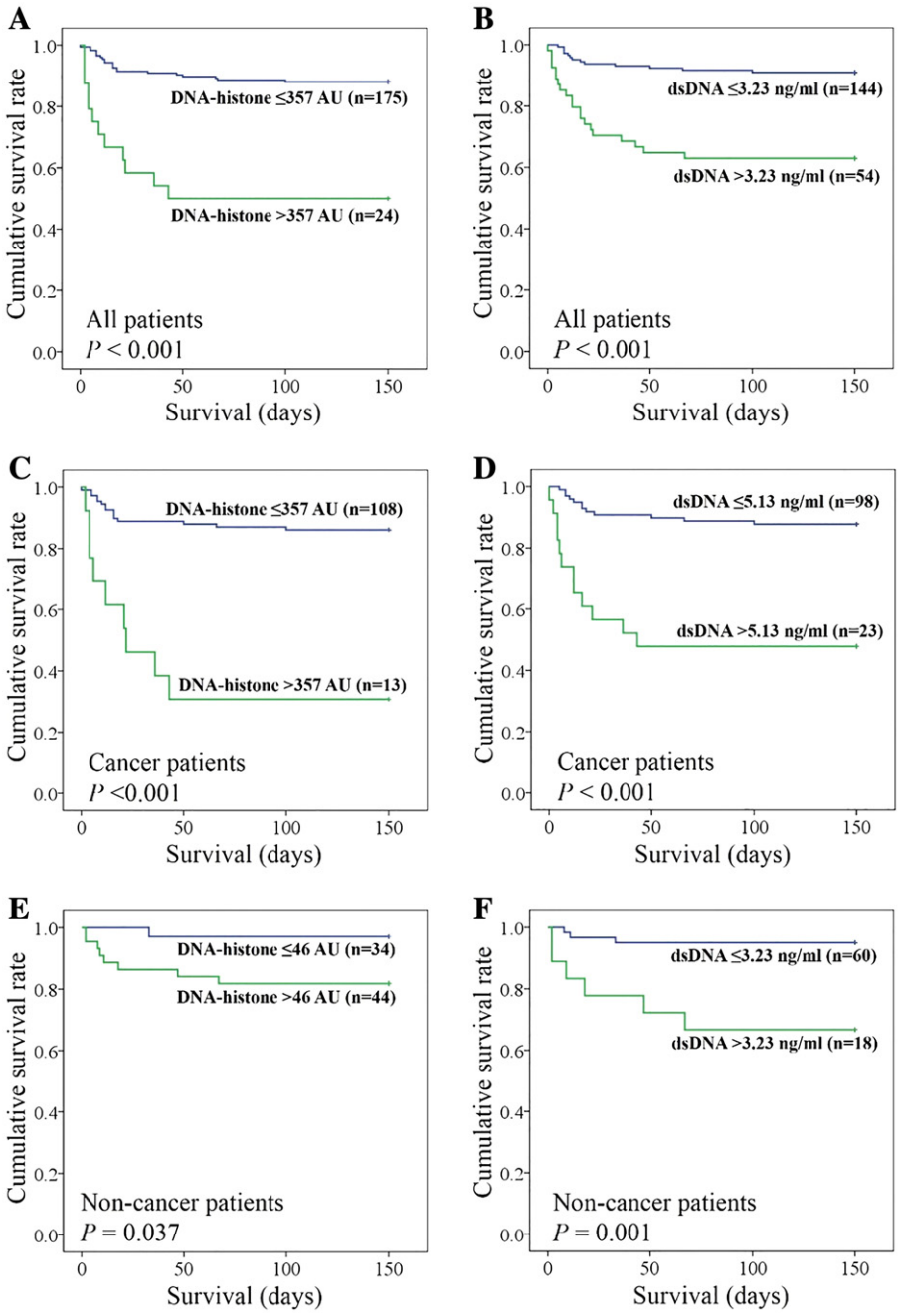

Fig. 4. Kaplan-Meier survival analysis stratified for DNA-histones complex and dsDNA levels in all patients (A, B), cancer patients (C, D), and non-cancer patients (E, F). Values with the highest prognostic power to predict the 28-day mortality were set as the cut-off values.

Table 2
Univariable and multivariable Cox regression analysis for mortality prediction in all patients.

	Univariable			Multivariable		
Variables	HR	95% CI	*P* value	HR	95% CI	*P* value
Platelet (≤55 x 10^9 vs. >55 x 10^9/L)	3.15	1.57–6.32	0.001	2.24	1.10–4.59	0.027
PT (>13.2 vs. ≤13.2 sec)	2.96	1.40–6.22	0.004	1.96	0.84–4.58	0.119
D-dimer (>3.69 vs. ≤3.69 mg/mL)	2.41	1.20–4.85	0.014	1.31	0.60–2.85	0.493
Fibrinogen (≤274 vs. >274 mg/dL)	0.47	0.24–0.93	0.031	0.75	0.36–1.56	0.439
Antithrombin (≤62 vs. >62 μg/L)	2.53	1.18–5.44	0.018	1.08	0.45–2.62	0.862
Protein C (≤72 vs. >72 pg/mL)	3.99	1.54–10.35	0.004	2.51	0.78–8.14	0.124
DNA-histone (>357 vs. ≤357 AU)	5.66	2.78–11.52	0.000	3.89	1.65–9.15	0.002
dsDNA (>3.23 vs. ≤3.23 ng/ml)	4.93	2.45–9.91	0.000	2.41	1.04–5.55	0.039

The cut-off values were determined as the values which produced the best prognostic value.
Abbreviation: HR, hazard ratio; CI, confidence interval; PT, prothrombin time; dsDNA, double stranded DNA.

DNA-histone (>357 AU) or dsDNA (>3.23 ng/mL) exhibited poor survival rates compared to the group with low DNA-histone or dsDNA (Fig. 4A, B). Similarly, among cancer patients, the group with high levels of DNA-histone (>357 AU) or dsDNA (>5.13 ng/mL) showed poorer survival than the group with low levels of DNA-histone or dsDNA (Fig. 4C, D). Likewise, in non-cancer patients, the group with high levels of DNA-histone or dsDNA showed poor prognosis, although the statistical significance of non-cancer patients was slightly less than that of cancer patients (Fig. 4E, F).

Univariable Cox regression analysis showed significant hazard ratios for platelets, PT, D-dimer, fibrinogen, antithrombin, and protein C (Table 2). Notably, the hazard ratios of DNA-histone (5.66) and dsDNA (4.93) were higher than that of D-dimer (2.41) (Table 2). In multivariable Cox regression analysis, the conventional DIC markers except platelet count lost their prognostic significance of mortality. Interestingly, DNA-histone and dsDNA were revealed as independent prognostic factors.

Discussion

Neutrophils can be induced by pathogens or other stimuli to release DNA combined with histones and cytoplasmic components such as neutrophil elastase, myeloperoxidase, and cathepsin G [13]. The resulting NET provides a scaffold to grow a hemostatic plug. Therefore, the NET formation may be inevitable in clinical conditions that are characterized by formation of vascular thrombi. Even though NET is known to be an important scaffold for vascular thrombi, there has been no data on the clinical significance of NET in DIC. Our study demonstrated that the levels of circulating DNA-histone complexes and dsDNA gradually increased in accordance with DIC severity. In addition, the elevated levels of circulating DNA-histone complexes and dsDNA showed independent prognostic significance.

Our data provide an interesting insight for the association of NET with coagulation severity. There have been several reports that explain how the NET contributes to coagulation activation [1,3,7]. Nucleic acids can bind coagulation factor XII and XI, thereby activating the intrinsic pathway [14]. The histones can increase thrombin formation by impairment of thrombomodulin-dependent protein C activation [15]. Histones can also activate platelets and endothelial cells [2,3]. In animal models, histone infusion has been shown to increase platelet-rich microthrombi [16]. Considering these effects of NET on coagulation activation in the previous reports, it is plausible that the increased levels of DNA-histone complex and dsDNA play a role in the amplification of coagulation activation in DIC processes.

One of the intriguing findings of our study was that the increased levels of DNA-histone complexes and dsDNA showed powerful prognostic values that were independent of the already known prognostic DIC markers. This suggests that the continuous formation of NET in patients with DIC could not only amplify the coagulation activation and but also affect other detrimental damage to the host [17]. As activated protein C is known to cleave histones [2], the decreased level of activated protein C in patients with advanced DIC may aggravate histone removal and result in high mortality. In other words, NET formation is thought to play a role in a vicious cycle of hypercoagulability.

Several reports have indicated the presence of extracellular nuclear proteins that are released from dying cells during sepsis [17,18]. Elevated levels of circulating nucleosome, high mobility group box-1, and plasma DNA were observed in critically ill patients [17,19,20]. Furthermore, high nucleosome levels have also been reported in advanced cancer patients [21]. This increase may originate from enhanced cell death or from impaired degradation of nucleosomes in serum by deoxyribonuclease (DNase) [9,22]. In view of various underlying clinical conditions in our study population, it is not easy to clearly identify the kind of factor(s) that increased the NET formation. In the present study, the main underlying conditions of DIC were sepsis, severe infections, and malignancies; therefore, the main contributor of NET formation is likely to be cell death induced by various stimuli.

The neutrophil elastase has been found to digest cross-linked fibrin in DIC, finally increasing plasma level of cross-linked fibrin degradation product by elastase (e-XDP) [23]. Although our study didn't measure the e-XDP level, it is likely to correlate the NET markers with e-XDP, because NET formation induces a large amount of neutrophil elastase which increases the e-XDP level.

Circulating levels of DNA-histone complexes and dsDNA may be dependent on neutrophil counts partly, because they are released from neutrophil. However in our study the DNA-histone complexes and dsDNA levels were not different among subtype of hematologic malignancies (data not shown). Future study may be required to investigate the effect of peripheral neutrophil count on DNA-histone complexes and dsDNA levels in resting status.

Cancer is considered to be a significant contributor to the formation of circulating NET [21]. Therefore, we divided the test population into cancer and non-cancer groups to exclude the effect of cancer on circulating NET formation. Interestingly, circulating NET levels showed significant prognostic values in both cancer patients as well as non-cancer patients (Fig 3). In addition, the association of circulating NET levels with coagulation severity remained unchanged, when analyzed in each cancer and non-cancer patient (data not shown). These findings suggest that the circulating NET levels generated in DIC processes provide prognostic significance regardless of the presence of cancer.

Specimen type is important when measuring circulating DNA levels because *in vitro* clot formation in serum results in an increase of artificially generated DNA increment in specimen tube [24]. Hence, using plasma instead of serum can assess more accurate values reflecting *in vivo* circulating levels of DNA. Our study measured the circulating levels of DNA-histone complexes and dsDNA in plasma, thus representing true *in vivo* condition.

The current study has a few limitations. First, although we measured the circulating DNA-histone complexes and dsDNA but we could not visualize the real NET formation due to methodological limitations. Second, we could not explain the detailed changes associated with circulating DNA-histone complexes and dsDNA because this was designed to be a cross-sectional study representing one specific point in time. Third, we did not evaluate the NET levels with respect to cancer stage. The cancer patients in this study were of various types including stomach, lung, pancreas, breast and prostate cancers, which made it unfeasible to analyze the relationship for each cancer type. However, the significant correlation of NET levels with coagulation severity was observed, suggesting it to be a good representative of DIC severity regardless of cancer stage. Fourth, the assay kits for DNA-histone complexes and dsDNA testing could not be used for clinical use so far. Future studies are required to evaluate the test performance and to validate the usefulness.

Conclusions

We demonstrated that circulating DNA-histone complexes and dsDNA correlated well with DIC severity. In addition, the high levels of circulating DNA-histone complexes and dsDNA showed significantly independent prognostic values in DIC. These findings provide a new insight into the potential clinical relevance of circulating DNA-histone complex and dsDNA, which serve as powerful markers for mortality prediction of DIC in future.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Acknowledgement

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