Original Article

Neutrophil extracellular traps are increased in cancer patients but does not associate with venous thrombosis

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Contributions: (I) Concept and design: R Oklu; (II) Administrative support: All authors; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background: A single center, prospective tissue-based study was conducted to investigate an association between neutrophil extracellular traps (NETs) and venous thromboembolic disease in patients with malignancy.

Methods: Plasma was collected from 65 patients in which 27 were cancer patients and 38 were age-matched non-cancer patients. Plasma NETs, circulating free DNA (cfDNA), DNase-1, endonuclease-G, endonuclease activity and thrombin-antithrombin III (TAT) complex levels was quantified. Laboratory values were also compared. Additionally, NETs detection and quantification was performed with fluorescent immunohistochemistry (IHC) in tissue-banked tumor sections and fresh human venous thrombus derived from cancer patients.

Results: Plasma samples from cancer patients contained higher levels of nucleosomes (P=0.0009) and cfDNA (P=0.0008) compared to the non-cancer group. Western blot analysis revealed significantly lower DNase-1 protein levels (P=0.016) that paralleled lower nuclease activity (P=0.03) in plasma samples from cancer patients compared to non-cancer patients. Thrombus tissue from cancer patients and tumor tissue from liver and lung cancer also showed marked levels of NETs. However, increased levels of NETs in cancer patients did not correlate with TAT complex activation or prevalence of venous thrombosis in cancer patients.

Conclusions: Further studies are warranted to determine the role of NETs as a procoagulant in human thrombosis.

Keywords: Neutrophil extracellular traps; NETs; thrombosis; nuclease

Submitted May 15, 2017. Accepted for publication Jun 28, 2017.
doi: 10.21037/cdt.2017.08.01
View this article at: http://dx.doi.org/10.21037/cdt.2017.08.01

Introduction

The strong association between malignancy and venous thromboembolic disease has been known for decades (1-3). While several mechanisms have been described, including increased levels of blood clotting factors, increased expression of tissue factor, exposure of phosphatidylserine and release of circulating procoagulant microparticles (4,5), the pathophysiology of this phenomenon still remains largely unknown and may vary between cancer types and stage of disease.

Recently, it was shown that neutrophils, in response to activation, release their genomic DNA into the extracellular space, referred to as neutrophil extracellular traps (NETs).

Table 1 Demographics of patient population

Demographics	Malignancy	No malignancy	P value
n (%)	27 (41%)	38 (59%)	–
Age, mean (range), years	63.6 [30–91]	69.5 [35–91]	0.07
Gender (M/F)	18/9	23/15	0.79
History of VTE (n, %)	7 (26%)	4 (10%)	0.18
Anticoagulation (n, %)	12 (44%)	18 (47%)	1.0

There was no significant difference in age, gender, history of venous thromboembolism (VTE), or anticoagulation between patients with and without malignancy.

NETs, which are comprised of extracellular chromatin studded with cytoplasmic and nuclear proteins, are released through a process termed NETosis—a form of cell death distinct from apoptosis and necrosis (6). NETs are thought to be an important part of the innate immune response and function as mesh-like networks to trap pathogens in a highly concentrated antimicrobial microenvironment (6). NETs have also been shown to have pro-thrombotic properties, through the activation of both the intrinsic and the extrinsic clotting pathways (7,8). NETs can bind to red blood cells (RBCs) and platelets, thus acting as a scaffold for thrombosis (9,10). NETs have also been observed in human deep venous thrombosis (DVT) samples as a component of the thrombus matrix, supporting their role in thrombus formation (11,12).

Despite preclinical data associating NETs with malignancy and with thrombosis, the role of NETs in cancer-associated thrombosis in humans remains unexplored. In this study, we show that intravascular NETs in cancer patients are elevated due to decreased degradation by plasma nucleases. However, increased levels of NETs and cfDNA in plasma and in venous thrombus of cancer patients did not correlate with higher levels of thrombin-antithrombin (TAT) complex activation or prevalence of DVT. Thrombosis is a complex process; the exact mechanism of NETs as a procoagulant in humans remains to be elucidated.

Methods

Study design

Institutional review board (IRB) approval was obtained for this study. Plasma samples were collected from sequential patients who presented to the radiology department for a computed tomography (CT) imaging study. Following sample acquisition, demographic and clinical data were retrospectively analyzed. A total of 65 plasma samples were collected, and of these, 27 patients had a cancer diagnosis (*Table 1*). The control population comprised of non-cancer patients that presented for a CT scan for the evaluation of vascular disease (n=25; aneurysm, dissection, peripheral vascular disease), chest pain (n=4) and abdominal pain (n=9).

Circulating nucleosome levels in plasma

The plasma levels of circulating mono- and oligo-nucleosomes were measured using Cell Death Detection ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN, USA), according to manufacturer instructions.

Circulating free DNA (cfDNA) levels in plasma

Levels of plasma cfDNA were quantified using Quant-iT™ PicoGreen® double stranded DNA Assay Kit (Thermo Fisher Scientific Inc., Grand Island, NY, USA), according to manufacturer's instructions. Each sample was diluted ten-fold in 10 mM Tris-HCl, 1 mM EDTA, pH =7.5 DNase-free buffer, and loaded into a 96 well plate. Fluorescent signal of the plasma and the DNA standard samples were acquired at excitation of 485 nm and emission of 538 nm using fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, USA). The plasma cfDNA concentration was extrapolated from the standard DNA values and expressed in nanogram/mL ± standard error of the mean.

DNase-1 and endonuclease-G protein levels in plasma

To test for differences in DNA degradation between cancer and non-cancer patients, DNase-1 and endonuclease-G protein levels were quantitated using Western blotting. Human plasma was mixed with four volumes of RIPA protein lysis buffer containing 10 µL/mL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The samples were mixed with Laemmli sample buffer and boiled at 95 ℃ for 5 min. Soluble plasma protein was loaded into 4–15% SDS-PAGE gradient gel (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were treated with blocking solution (Sigma-Aldrich) for 1 h at room temperature. Membranes were incubated over night with polyclonal rabbit anti-DNase-1 (SC-30058) or anti-Endonuclease G

(SC-32935, Santa Cruz Biotech, Santa Cruz, CA, USA) IgG at 1:500 dilution followed by 1:4000 diluted horseradish-peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. The membranes were reacted using chemiluminescence system (ECL Advance Western Blotting Detection Kit, Amersham Bioscience, GE Healthcare, Pittsburgh, PA, USA) and the specific chemiluminescence signal was visualized using FluorChem HD2 imaging system (ProteinSimple, Santa Clara, CA, USA). The membranes were stained with Ponceau S to confirm equal sample loading and the band density of 60 kDa protein was obtained. The specific band densities of the DNase-1 and Endonuclease-G were calculated using the imager software and normalized to the band densities of the Ponceau S staining. Data was expressed as arbitrary units (AU).

Plasmid incision assay

In addition to testing plasma levels of endonucleases, we tested nuclease activity using a plasmid incision assay in the following manner. An aliquot of each plasma sample was diluted 20-fold in a buffer containing 50 mM Tris-HCl, 0.5% Triton X-100, 0.25 M sucrose and 10 µL of protease inhibitor cocktail (Sigma-Aldrich). Equal aliquots from each sample were added to a reaction buffer containing 1 µg pBR322 plasmid DNA (New England Biolabs, Beverly, MA, USA), 2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) and 0.5 mM dithiothreitol and incubated at 37 ℃ for 30 min. The reaction was stopped with a buffer containing 0.05% w/v Bromophenol blue, 40% sucrose, 0.1 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 and 1% sodium dodecyl sulfate (SDS). Samples were loaded into 1% agarose gel containing 1 µg/mL ethidium bromide and run at 7 V/cm for 2 h. A digital image of the gel was acquired using FluorChem HD2 imaging system (ProteinSimple) and UV light source. The relative amount of nuclease activity was calculated based on the density of the three bands for plasmid DNA present in covalently closed circular DNA (Type I), open circular DNA (Type II), or linear DNA (Type III), as previously described (13,14).

TAT complex in plasma

Quantitative assessment of TAT complex concentration in the plasma was measured using a human ELISA kit (ab108907, Abcam, Cambridge, MA, USA). The absorbance was read on a microplate reader at a wavelength of 450 nm with wavelength correction at 570 nm. The concentration in the plasma was determined by extrapolating from a regression analysis of the log-log curve-fit of the standard values. Results were expressed as nanograms per milliliter ± standard error of the mean.

Analysis of NETs in human thrombus

The presence of NETs in fresh human venous thromboemboli was assessed in the following manner. Aspirated thrombus specimens were acquired from patients with malignancy (n=5) who presented to interventional radiology for management of acute ileocaval thrombosis and who underwent mechanical thrombectomy. Samples were acquired through 9–12 French sheaths. All patients carried a diagnosis of malignancy: lung cancer (n=3), pancreatic adenocarcinoma (n=1), and bladder cancer (n=1). Thrombus samples were fixed in formalin immediately after acquisition. Immunohistochemistry (IHC) for NETs was then performed in the following manner. Sections were incubated with mouse monoclonal anti-histone H2A/H2B/DNA complex antibody at 1 µg/mL dilution (gift of Dr. Marc Monestier, Temple University, PA, USA). Slides were incubated with biotin conjugated anti-mouse IgG (Vector Labs, Burlingame, CA, USA) followed by HRP-conjugated streptavidin. Specific signal was developed using cyanine-3 tyramide signal amplification kit (Perkin Elmer, Boston, MA, USA) according to the manufacturer's instructions. An adjacent section was treated with human recombinant DNase-1 prior to immunostaining to demonstrate antibody specificity and illustrate the potential therapeutic implications. Digital images were acquired using a digital slide scanner (NanoZoomer2.0-RS, Hamamatsu Corporation, Middlesex, NJ, USA) and quantified using ImageJ software.

Immunohistochemical analysis of NETs from normal and malignant tissue

The presence of NETs in normal and malignant tissue was assessed in the following manner. Paraffin embedded tissue sections of both normal organs and malignant tissue were purchased from a commercial tissue bank (IMH-326/327/343, Imgenex, San Diego, CA, USA). Detailed information about each slide is available online. Briefly, the slides contain both normal tissues (skin, breast, spleen, muscle, lung, liver, stomach, colon, kidney, prostate, placenta, and brain) as well as malignant tissues from a range of organs (breast, lung, liver, esophagus, stomach,

Table 2 Plasma markers in cancer and non-cancer patients

Plasma markers	Non-cancer group	Cancer group	P value
WBC	8.22±0.53	7.32±0.74	0.23
Neutrophil	5.53±0.74	5.50±0.74	0.49
ESR	25.1±15.6	32.5±7.5	0.32
PT	14.2±1.0	14.1±0.7	0.46
INR	1.19±0.10	1.11±0.06	0.26
APTT	26.7±0.8	25.1±0.7	0.13
HCT	39.7±0.8	39.0±1.0	0.29
Platelet	222.7±17.5	248.2±20.1	0.19

The values are shown as mean ± standard error of the mean.

small bowel, colon, kidney, bladder, ovary, and skin). Slide processing and IHC staining was performed according to the manufacturer's instructions. Paraffin embedded tissue sections were deparaffinized and rehydrated then incubated with mouse monoclonal anti-histone H2A/H2B/DNA complex antibody as above. The slides were covered with mounting medium (Vectashield, Vector Labs) containing 4',6-diamidino-2-phenylindole (DAPI). For negative control, the NETs antibody was omitted and substituted with 1μg/mL dilution of non-specific anti-mouse IgG.

Statistical analysis

Statistical analysis was performed using Prism-5 program (Graph pad, La Jolla, CA, USA) using parametric and non-parametric *t*-tests. The results are expressed as the mean ± standard error. Demographic and clinical characteristics were compared using Chi-square for parametric continuous variables and Kruskal-Wallis test for non-parametric data.

Results

Patient population

There was no significant difference in mean age (63.6 *vs.* 69.5 years, P>0.05) or gender (67% *vs.* 64% male, P>0.05) for the non-malignancy and malignancy groups (*Table 1*). Similarly, laboratory values between the two groups, including white blood cell count, neutrophil count, sedimentation rate, C-reactive protein, prothrombin time, international normalized ratio, activated partial thromboplastin time, hematocrit, and platelet levels, were not significantly different (P>0.05) (*Table 2*). There was no significant difference in the clinical history of previous venous thromboembolic events (Chi square =1.59, P>0.1), thromboembolic risk factors (smoking, immobilization, oral contraceptive use; Chi square =0.97, P>0.1), or anticoagulation use (heparin, warfarin, aspirin, dipyridamole and clopidogrel; Chi square =1.07, P>0.1) between the malignancy and non-malignancy groups. However, there was a significantly greater number of patients with thromboembolic risk factors in the malignancy group (Chi square =7.78, P<0.01).

Plasma NETs analysis

Circulating nucleosome levels, indicating NETs in plasma, were markedly higher in the malignancy group compared to the non-malignancy group (0.188±0.046 *vs.* 0.062±0.004 AU, P=0.0009) (*Figure 1*). Similarly, cfDNA levels were higher in the malignancy group (950.34±88.40 *vs.* 625.02±56.87 ng/mL, P=0.0008) (*Figure 1*). Consistent with these results, plasma levels of DNase-1 were found to be lower in the malignancy group (5.2±0.36 *vs.* 5.9±0.19 AU, P=0.016) (*Figure 2*). Endonuclease-G plasma levels, however, were not found to be significantly different between the two groups (7.4±1.1 *vs.* 6.4±0.4 AU, P=0.95) (*Figure 2*). These data imply that diminished degradation of nucleic acids by plasma nucleases such as DNAse-1 could result in higher levels of NETs in cancer patients.

Since there are many nucleases present in plasma, overall nuclease activity was evaluated using a semi-quantitative plasmid incision assay. Plasma nuclease activity was found to be significantly lower in cancer patients compared to non-cancer patients (349±14 *vs.* 406±20 AU, P=0.029) (*Figure 3*). These data further substantiate the hypothesis that increased plasma levels of NETs and cfDNA in cancer patients are due, in part, to reduced nuclease levels and/or activity.

To assess for intravascular coagulation activity, TAT complex levels was quantified (15). TAT complex plasma concentration was slightly elevated in cancer patients (34.19±17.62 ng/mL), in comparison to non-cancer group (31.26±15.05 ng/mL) but this increase was not statistically significant (P=0.29) (*Figure 4*).

Analysis of NETs in human thrombus

Next, we immunostained aspirated thrombus tissues from five cancer patients that presented with acute venous thrombosis; a corresponding plasma analysis for NETs in this subset was not performed. IHC was notable for

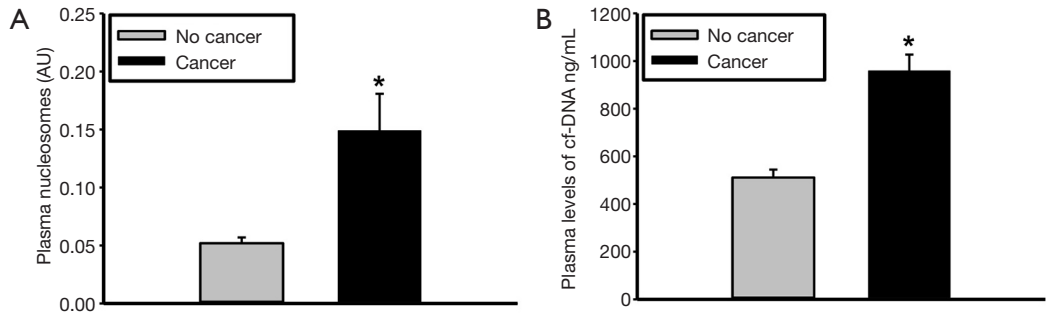

Figure 1 Plasma levels of nucleosomes and circulating free DNA (cf-DNA). Plasma nucleosomes levels (A) were markedly higher in patients with cancer (*, P=0.0009). This was mirrored by higher plasma levels of cf-DNA (B) in cancer patients (*, P=0.0008).

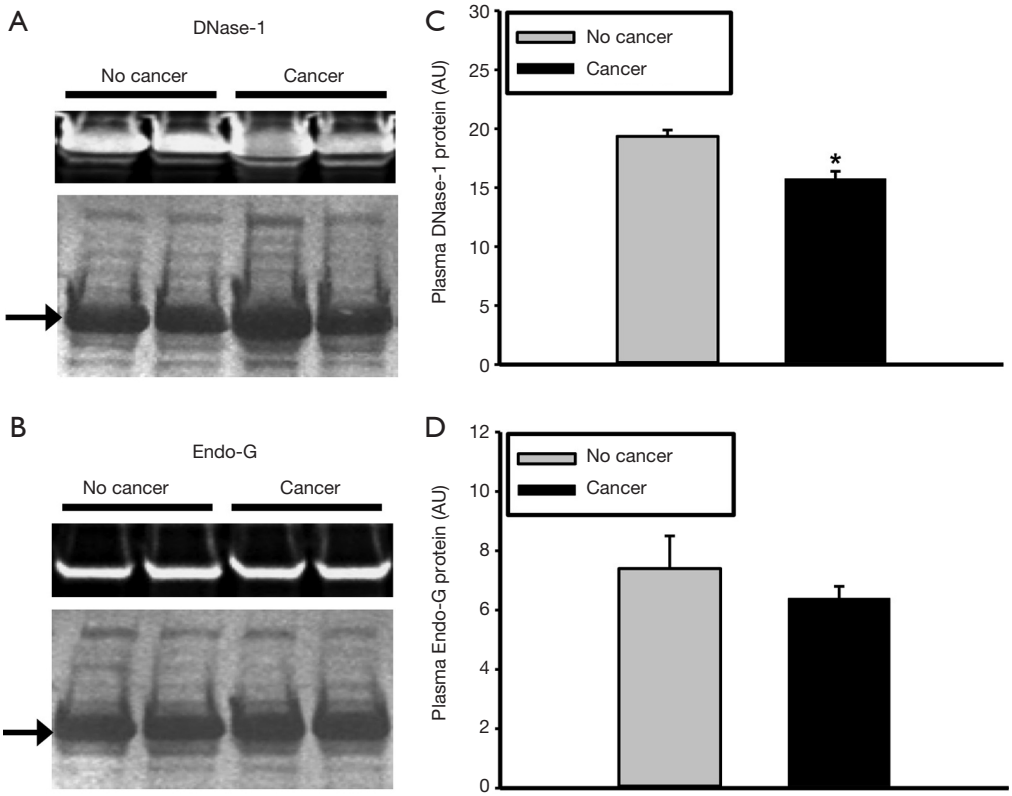

Figure 2 Plasma levels of DNase-1 and Endo-G nucleases. Representative Western blot analysis shows specific protein bands for DNase-1 (A) and Endo-G (B) in plasma samples. Ponceau S staining was used to normalize the densitometric value from each lane (black arrow). Densitometric analysis summary showed significantly lower DNase-1 protein (C) levels in plasma collected from cancer patients (*, P=0.0016). In contrast, Endo-G levels were not significantly different between the two groups (D). Endo-G, endonuclease-G.

extensive extracellular staining, consistent with NETs, throughout the thrombus (*Figure 5*). Moreover, consecutive sections pretreated with DNase prior to immunostaining showed marked reduction in the level of NETs detected in the extracellular matrix demonstrating the specificity of the NETs antibody used in these experiments.

IHC analysis of NETs from normal and malignant tissues

The presence of NETs in an array of both normal and

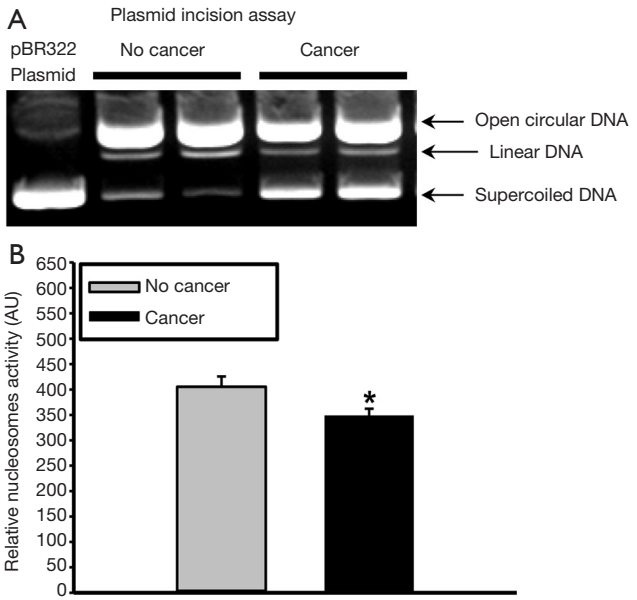

Figure 3 Plasma nuclease activity levels. Nuclease activity in plasma samples obtained from cancer and non-cancer patients was measured with the plasmid incision assay using pBR322 plasmid as the substrate. (A) Two representative reactions from non-cancer and cancer patients stained with ethidium bromide following agarose gel electrophoresis. Nuclease activity is proportional to the conversion rate of the intact supercoiled circular plasmid DNA (lower band), to open circular DNA (upper band) or linear DNA (middle band). The non-cancer samples in (A) show reduced levels of supercoiled DNA indicating that they were cleaved by higher levels of plasma nucleases. The calculated relative nuclease activity (B) demonstrated lower activity in cancer patients compared to the non-cancer group (*, P=0.028).

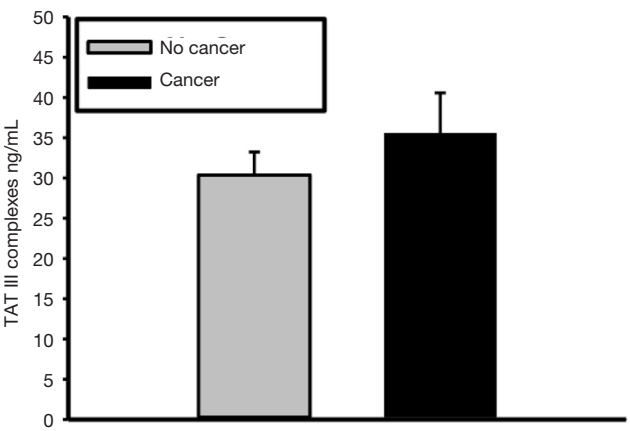

Figure 4 Plasma levels of TAT III complexes. Quantitative analysis of plasma TAT III complexes showed no difference between cancer and non-cancer groups.

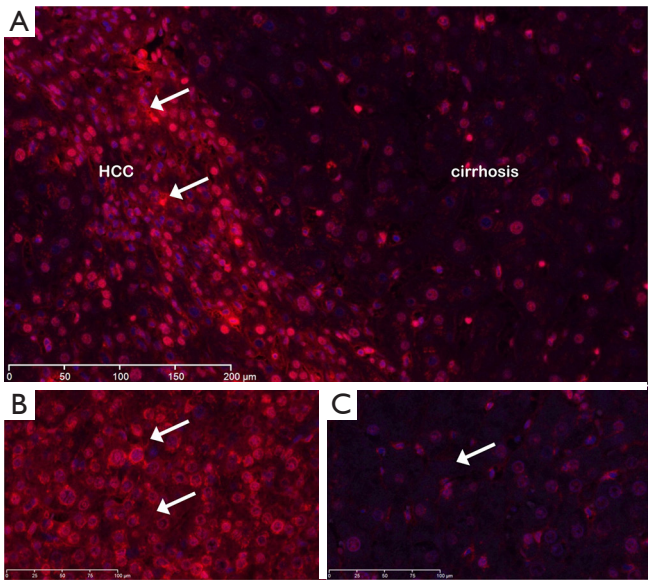

Figure 5 Immunohistochemistry of NETs in cirrhosis and hepatocellular carcinoma (HCC) liver tissue. Red = anti-histone H2A/H2B/DNA complexes (NETs); blue = nuclear counterstaining (DAPI). (A) There are extensive NETs immunostaining in areas corresponding to HCC (arrows) compared to the adjacent non-cancer, cirrhosis segments that show only nuclear immunostaining. (B) Higher magnification of HCC segments demonstrates the extensive extracellular NETs immunostaining (arrows). (C) Higher magnification of a cirrhosis segment demonstrating minimal extracellular NETs signal (arrow); white bar indicates 200 microns in (A) and 100 microns in (B,C). DAPI, 4',6-diamidino-2-phenylindole. NETs, neutrophil extracellular traps.

malignant tissue was evaluated by IHC. Examples are provided in *Figures 6* and 7. Cirrhotic tissue had minimal NETs signal; however, hepatocellular carcinoma (HCC) tissue demonstrated higher cellularity and higher relative levels of NETs signal in the extracellular space (*Figure 6*). In lung cancer, scattered foci within the tumor demonstrated intense NETs signal in the extracellular matrix (*Figure 7*). In other tissue specimens included in the array, NETs signal was predominantly nuclear.

Discussion

NETs are complex extracellular networks of nucleic acids studded with histones and anti-microbial proteins that may promote thrombosis. Increased levels of intravascular NETs have been

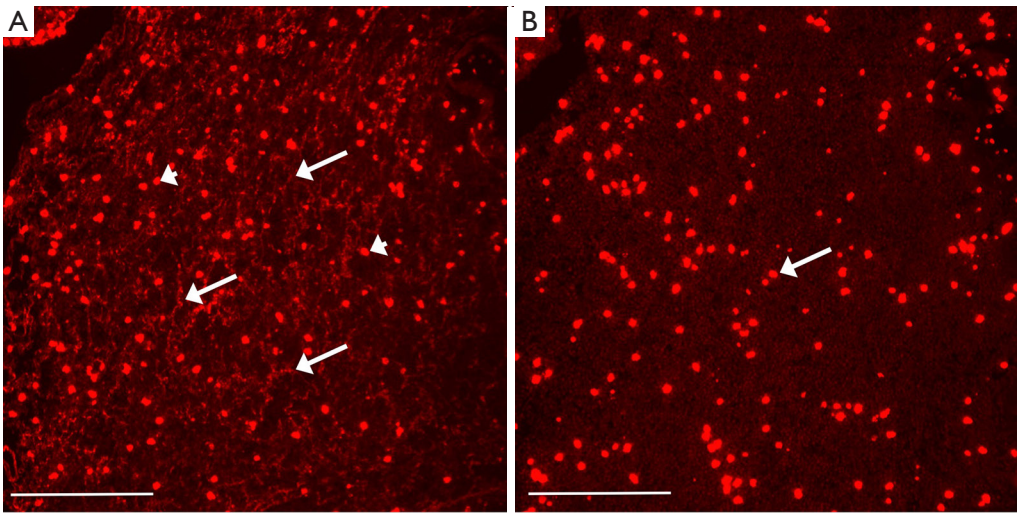

Figure 6 NETs in aspirated thrombus from a patient with malignancy. (A) NETs immunostaining demonstrates both nuclear (arrowheads) and extensive extra-cellular signal (arrows). (B) Following incubation of adjacent tissue sections with DNase-1, immunostaining demonstrated absence of extracellular signal indicating the specificity of the antibody for NETs; only nuclear immunostaining of NETs was detected (arrow), as expected. Red = anti-histone H2A/H2B/DNA complexes (NETs); white bar indicates 100 microns.

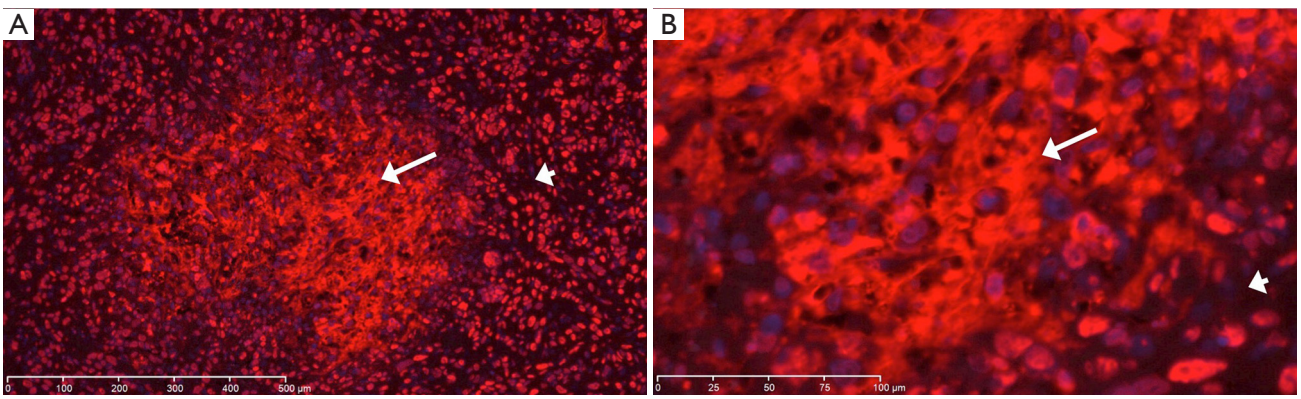

Figure 7 Immunohistochemistry of NETs in small-cell lung cancer. Red = anti-histone H2A/H2B/DNA complexes (NETs); blue = nuclear counterstaining (DAPI). (A) There are intense extracellular NETs signal detected within foci of cancer tissue (arrow) compared to adjacent segments (arrow head). (B) Higher magnification views reveal detection of intense lace-like extracellular network of NETs (arrow) compared to adjacent tissue (arrowhead); white bar indicates 500 microns in (A) and 100 microns in (B).

proposed as a mechanism for the high rate of venous thromboembolic disease in cancer patients (12,16-19). In this study, we tested the hypothesis that intravascular NETs are higher in cancer patients and associate with higher prevalence of venous thrombosis when compared to non-cancer patients. Blood sampled from cancer patients was found to have greater concentrations of NETs and cfDNA, as well as lower concentrations and activity of nucleases, compared to a demographically similar group of non-cancer patients. Extracellular DNA was shown to be a diffuse component of human thrombus samples from cancer patients, and treatment with DNase substantially degraded this scaffolding network to indicate the specificity of the antibody. Finally, NETs were shown to be present extensively within the extracellular matrix of certain malignant tissues.

NETs and cfDNA represent a conserved, common

denominator among cancer, thrombosis, autoimmune diseases and infection, and their role in the pathogenesis of these diseases continues to expand. Patients with sepsis have higher plasma levels of nucleosomes compared to healthy controls (20), and elevated level of cfDNA has been suggested as a prognostic indicator of mortality in intensive care unit patients with severe sepsis. Decreased plasma nucleosome levels have been suggested as predictors for response to cancer chemotherapy and as a prognostic biomarker in various malignancies, including pancreatic, colorectal, lung, breast and liver cancer (21-26); following therapy, patients with progressive disease have been shown to have higher levels of circulating nucleosomes compared to patients with partial or complete response (22,27-29). NETs may also play a role in ischemia-reperfusion injury (7,30), capillary injury (31), and atherosclerosis (32).

Nucleosomes are a cell death by-product, and thus it follows logically that patients with aggressive tumors with high proliferative index would lead to greater nucleosome release especially by circulating tumor cells. Additionally, NETs and cfDNA can also accumulate in plasma due to reduced degradation process by nucleases. Blood nuclease activity is responsible for the degradation of circulating nucleosomes. Its plasma level is known to be decreased in systematic lupus erythematosus patients, causing longer persistence of nucleosomes and sustained autoimmune response stimulation (33). Although reduced *in vitro* activity of endonuclease (DNase) in various tumor cells has been known for more than 50 years, their plasma levels have not been studied in cancer patients until recently (34).

Beyond elucidating a fundamental mechanism for cancer-associated thromboembolic disease, NETs may represent a new therapeutic target for venous thromboembolic disease (12). NETs are a component of thrombus matrix and a scaffold for thrombus formation (11,12). Moreover, in this study DNase was shown to be an effective method of dismantling the NETs scaffold. Thus, NETs may be a potential drug target for cancer-induced thrombosis (35,36). A previous human plasma study suggested that DNase treatment results in increased thrombin generation, possibly due to breakdown of cfDNA scaffold, thus exposing histone protein molecules which possess potent pro-inflammatory and platelet activation properties (20). On the contrary, another study showed that DNase treatment protects mice from flow restriction-induced DVT (9,37). Therefore, any therapeutic potential of recombinant DNase for thrombosis remains to be further investigated.

Lung cancer and HCC tissues demonstrated intense IHC signal for NETs in the extracellular matrix of the tumor tissue. Although the source for these NETs is not known, it is either from cancer cell turn-over or from immune cells such as neutrophils. The significance of these NETs in tumor tissue is also not known; however, high levels of DNA in the extracellular matrix will likely impede chemotherapy distribution due to steric hindrance from the negatively charged DNA and reduce drug efficacy (38).

There are several limitations to this study. While no significant difference in prior venous thromboembolic event was found between the malignancy and non-malignancy cohorts in the plasma NETs experiment, these patients were not followed prospectively to identify subsequent thrombosis events. Additionally, we identified an extensive NETs infrastructure within thrombus aspirated from cancer patients, but we did not compare these results with thrombus obtained from non-cancer patients to assess relative levels of NETs. Furthermore, plasma from these patients with active venous thrombosis was not analyzed because the thrombectomy procedure often leads to cellular injury and this may potentially cause a rise in plasma NETs levels. The source of the NETs detected in the thrombus tissue and in cancer tissue was not investigated; immune cells and/or cancer cells maybe the source.

Conclusions

NETs are elevated in patients with cancer and comprise an extensive scaffolding network within thrombus and certain tumor tissue. These high levels of NETS and cfDNA in cancer patients are associated with reduced DNAse-1 levels and nuclease activity but did not associate with higher prevalence of venous thrombosis. While NETs may potentially become an important biomarker of malignancy, further studies are warranted to determine their role as a procoagulant in human thrombosis.

Acknowledgments

Funding: R Oklu gratefully acknowledges funding from the National Institutes of Health (EB021148, CA172738, EB024403, HL137193) and the Mayo Clinic.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical statement: The study was approved by the Institutional review board (No. 2010P002234). Written consent form showed that all data could be used for publication.

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Cite this article as: Oklu R, Sheth RA, Wong KH, Jahromi AH, Albadawi H. Neutrophil extracellular traps are increased in cancer patients but does not associate with venous thrombosis. Cardiovasc Diagn Ther 2017;7(Suppl 3):S140-S149. doi: 10.21037/cdt.2017.08.01

