Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

Histopathological Features of Cancer-Associated Venous Thromboembolism: Presence of Intrathrombus Cancer Cells and Prothrombotic Factors

Toshihiro Gi (魏 峻洸), Aya Kuwahara (桑原 彩), Atsushi Yamashita (山下 篤), Shuntaro Matsuda (松田 俊太郎), Kazunari Maekawa (前川 和也), Sayaka Moriguchi-Goto (盛口 清香), Yuichiro Sato (佐藤 勇一郎), Yujiro Asada (浅田 祐士郎)

BACKGROUND: Cancer-associated venous thromboembolism (VTE) is a critical complication in patients with cancer. However, the pathological findings of VTE are limited. Here, we investigated the histopathological features of cancer-associated VTE in human autopsy cases.

METHODS: We clinically examined the autopsy cases of VTE with (n=114) and without cancer (n=66) and immunohistochemically analyzed the expression of prothrombotic factors in intrathrombus cancer cells, the thrombus contents of erythrocytes, fibrin, platelets, citrullinated histone H3, and degree of organization.

RESULTS: Vascular wall invasion or small cell clusters of cancer cells was observed in thrombi in 27.5% of deep vein thrombosis and 25.9% of pulmonary embolism cases. The majority of the cancer cells in deep vein thrombi appeared to be invading the vessel wall, whereas the majority of pulmonary thrombi had cancer cell clusters, consistent with embolization via blood flow. These cancer cells were immunohistochemically positive for TF (tissue factors) or podoplanin in up to 88% of VTE cases. The frequency of TF-positive monocyte/macrophages in thrombi was higher in cancer-associated VTE than that in VTE without cancer. Citrullinated histone H3 was predominantly observed in the early stages of organizing thrombi. There was no significant difference in thrombus components between VTE with cancer and without cancer groups.

CONCLUSIONS: Vascular wall invasion or cancer cell clusters in thrombi might influence thrombogenesis of cancer-associated VTE. TF and podoplanin in cancer cells and in monocyte/macrophages may induce coagulation reactions and platelet aggregation. Neutrophil extracellular traps may play a role in the early stages of VTE, regardless of cancer status.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: carcinoma ■ extracellular traps ■ fibrin ■ pathology ■ platelet aggregation ■ tissue factor ■ venous thromboembolism

Cancer-associated venous thromboembolism (VTE) is a critical complication affecting mortality in patients with cancer.[1,2] Patients with cancer-related venous thrombosis have a 30-fold increased risk of death during follow-up compared with disease-free subjects.[2] Among patients with pancreatic cancer, the median overall survival of patients estimated after VTE was 5.5 months, compared with 13.4 months in patients without VTE.[3] Primary organs and histological types of cancer likely affect the underlying mechanisms of cancer-associated VTE.[4–6] Cancer cells are associated with the development of VTE by local

See accompanying editorial on page 160

Correspondence to: Atsushi Yamashita, MD, PhD, Department of Pathology, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Email atsushi_yamashita@med.miyazaki-u.ac.jp

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.122.318463.

For Sources of Funding and Disclosures, see page 157.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

Cit-H3	citrullinated histone H3
CK	cytokeratin
DAMPs	damage-associated molecular patterns
DVT	deep vein thrombosis
GPIIb/IIIa	glycoprotein IIb/IIIa
HE	hematoxylin and eosin
NETs	neutrophil extracellular traps
PAI-1	plasminogen activator inhibitor-1
PE	pulmonary embolism
TF	tissue factor
tPA	tissue-type plasminogen activator
VTE	venous thromboembolism

Highlights

- Autopsy cases of cancer-associated venous thromboembolism showed the presence of cancer cells in thrombi with a direct invasive or small cluster pattern.
- Cancer cells in thrombi frequently showed immunohistochemical expression of tissue factor or podoplanin and necrosis with platelet aggregation and fibrin formation.
- The frequency of tissue factor–positive monocyte/macrophages in thrombi was higher in cancer-associated venous thromboembolism than in venous thromboembolism without cancer.
- Neutrophil extracellular traps in the thrombi were related to the early phase of the thrombus-organizing process, regardless of whether patients were in a cancer-bearing state.

venous stasis and the release of various prothrombotic factors, such as mucin, TF (tissue factor), podoplanin, plasminogen activator inhibitor-1 (PAI-1), cytokines, and other procoagulant substances.[3,5–9] In addition, previous studies have reported that cancer cells promote venous thrombus formation and stability via cancer cell-derived microvesicles, cell-free DNA or DAMPs (damage-associated molecular patterns) from cancer cells, and NETs (neutrophil extracellular traps).[7,10,11] These molecules contribute to the activation of coagulation via TF, factor X, and factor XII, in addition to platelet aggregation via histone and C-type lectin receptor 2.[7,10–13] Several previous studies have suggested the histopathological expression of procoagulant factors in human cancer tissue and its association with VTE.[14–17] Several autopsy studies of cancer-associated VTE[4,18,19] have been previously reported. However, there is no pathological evidence that cancer cells directly contribute to cancer-associated VTE. Thus, whether cancer cells are present in VTE, express thrombogenic factors, and their effect on thrombus components remains unknown. This study aimed to investigate the histopathological features of cancer-associated VTE in human autopsy cases.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Selection of Cancer-Associated VTE Cases

This retrospective study was approved by the Ethics Committee of the University of Miyazaki approved (protocol number: O-0681). The study design is summarized in Figure S1. First, we selected VTE cases (n=180) and detected deep vein thrombosis (DVT) or pulmonary embolism (PE) in post-mortem observations based on the autopsy records of consecutive cases at the University of Miyazaki Hospital between 1977 and 2019 (n=2564). We separated these VTE cases into VTE without cancer (n=66) and cancer-associated VTE (n=114), analyzed the clinicopathological backgrounds of each group, and examined the primary organs and histological types of cancers in the latter group. Second, we investigated the histopathological findings in available hematoxylin and eosin (HE)–stained VTE specimens from each group (65 and 109 specimens, respectively). Finally, we performed immunohistochemistry with paraffin-embedded VTE tissue blocks (55 and 79 specimens, respectively). We selected the largest DVT or PE as representative specimens when several thrombotic specimens were available for the same case.

Histological Analysis With HE-Stained Sections

All the VTE specimens were fixed in 20–30% formaldehyde, embedded in paraffin, and cut into 2.5-μm thick sections. Paraffin sections of thrombi were stained with HE. The sizes of the thrombi (DVT and PE) were measured in sections under a microscope using NIS-Element-D 3.2 image analysis software (Nikon, Tokyo, Japan). When >1 DVT or PE thrombus sections were obtained from the same case, we selected the largest thrombus section to minimize histological heterogeneity. All specimens of DVT and PE contained adjacent veins and pulmonary arteries, respectively. The sites of PE thrombi were classified into central or peripheral levels according to the accompanying bronchi or bronchioles, respectively. We classified the degree of neutrophil infiltration into semiquantitative categories: grade (G) 0 (none), G1 (<5% per total thrombus area), G2 (5%–30%), and G3 (<30%; Figure S2A). We further examined the presence of lytic changes in neutrophils and the organization of thrombi.[20–22] Lytic changes in neutrophils were defined as loss of cellular morphology, karyolysis, or nuclear fragmentation (Figure S2B).[21,23] The organization of thrombi was defined based on the following findings: proliferation of the endothelium, proliferation of fibroblasts, collagen deposition, and hemosiderin deposition[22] (Figure S2C through S2F). The area of these organizing processes per total thrombus area was classified into semiquantitative categories: G0 (none), G1 (<33% per total thrombus area), G2 (33%–66%), and G3 (>66%). These histological examinations were performed using a blinded experimental design. VTE sections from the cancer-associated group were examined for the presence of cancer cells and necrotic changes.

Immunohistochemistry and Immunofluorescence of VTE Tissue

In this immunohistochemical study, we focused on epithelial malignancies (carcinomas). We excluded lymphoma from further analysis because lymphomas (nonepithelial malignancies) have different growth patterns and biological behaviors compared with carcinomas. The paraffin sections of thrombi were stained for glycophorin A (erythrocyte marker, mouse monoclonal, clone JC159; DAKO, Glostrup, Denmark), platelet GP (glycoprotein) IIb/IIIa (platelet marker, sheep polyclonal; Affinity Biologicals, Inc, Hamilton, CA), fibrin (mouse monoclonal, clone 59D8; EMD Millipore Corp., Burlington, MA), Cit-H3 (citrullinated histone H3)[24] (NET marker, rabbit polyclonal; Abcam, Cambridge, MA), and pan-CK (cytokeratin; epithelial marker, mouse monoclonal, Abcam), TF (mouse monoclonal, clone H-9; Santa Cruz Biotechnology, Inc., Dallas, TX), and podoplanin (mouse monoclonal, clone D2-40; DAKO). Additionally, VTE specimens with intrathrombus cancer cells were stained for tPA (tissue-type plasminogen activator; rabbit polyclonal; Gene Tex, Irvine, CA) and PAI-1 (rabbit polyclonal; Novus Biologicals, Centennial, CO). Consecutive VTE sections with cellular positivity for TF or podoplanin were also stained for CD163 (monocyte/macrophage marker, rabbit monoclonal; clone EPR19518, Abcam). For the VTE specimens with intrathrombus cancer cells, we classified the predominant thrombus content surrounding intrathrombus cancer cells as follows: erythrocyte-rich, fibrin/platelet-rich, and organizing content. The detailed primary antibodies used are described in Table S1. The sections were stained with EnVision anti-mouse, rabbit immunoglobulin (DAKO), or anti-sheep secondary antibody (Jackson ImmunoResearch). Horseradish peroxidase activity was visualized using 3,3'-diaminobenzidine solution containing hydrogen peroxide, and the sections were counterstained with Mayer hematoxylin. Immunostaining controls were stained with the secondary non-immune mouse, rabbit, or sheep IgG, without primary antibodies.

The specificity of the immunohistochemistry antibodies against TF, podoplanin, tPA, and PAI-1 was examined using non-neoplastic tissue samples (Figure S3). We confirmed immunoreactivity for TF in pancreatic ducts, bronchial epithelium, arterial adventitial cells, and glomerular mesangial cells (Figure S3A and B), Additionally, immunoreactivity was evaluated for podoplanin in lymphatic endothelium and basal cells in squamous epithelium (Figure S3C). Immunoreactivity was examined for tPA in the endothelium and urothelium (Figure S3D) and PAI-1 in vascular smooth muscle cells and syncytiotrophoblasts of the placental villi (Figure S3E). We excluded the specimens with inadequate immunopositivity for intrinsic control cells in the same specimens of VTE tissue (procedure no. 3 of Figure S1). Representative intrinsic positive control tissue samples were as follows: epithelial cells and adventitia of vessels for TF; lymphatic vessels for podoplanin; endothelial cells for tPA; and smooth muscle cells of the media of vessels for PAI-1 (Figure S3B through S3E).[25–28]

Areas that were immuno-positive for fibrin, glycophorin A, GPIIb/IIIa, and CK were semi-quantified using the Win Roof color image analysis software (Mitani, Fukui, Japan).[21] Immuno-positive areas were extracted as green areas using specific protocols based on the color parameters of hue, lightness, and saturation (Figure S4A through S4D). These areas are expressed as the ratio of positively stained areas per total thrombus area. Areas that were immuno-positive for Cit-H3 were expressed as a mean value of the three highest density fields under a 20× objective lens. The data showed the ratios per the corresponding thrombus area since the immuno-positive area for Cit-H3 was too small to measure in a low-magnification image. The method used to calculate immuno-positive areas was also described in Figure S4. We also examined presence or absence of leukocyte lysis in the highest density area of Cit-H3 with the corresponding HE section. We defined cancer cells as those expressing TF, podoplanin, tPA, or PAI-1, in which more than 10% of cancer cells showed immunopositivity for these proteins.

We performed immunofluorescence to examined TF and podoplanin expression in cancer cells and macrophages, and Cit-H3 expression in neutrophils in representative VTE specimens. VTE sections were stained with anti-CD66b antibody (neutrophil marker, mouse monoclonal; clone 6/40c, BioLegend, San Diego, CA), anti-Cit H3 antibody (rabbit polyclonal, Abcam), anti-TF antibody (mouse monoclonal; clone H-9, Santa Cruz Biotechnology), anti-CK antibody (pan-cytokeratin, rabbit polyclonal; Abcam) and CD163 (monocyte/macrophage marker, rabbit monoclonal; clone EPR19518, Abcam). CF568 conjugated-donkey anti-mouse IgG (Biotium, Hayward, CA), and CF488 conjugated-donkey anti-rabbit IgG (Biotium) were used as secondary antibodies. The VTE sections were mounted using a 4, 6'-diamidino-2-phenylindole-containing reagent. The fluorescent images were captured using a microscope digital camera (DP74, Olympus, Tokyo, Japan) and merged using an imaging software (cell Sens Standard 2.3, Olympus).

Statistical Analysis

All data are presented as the medians and ranges, in bar graphs and dot plots. Differences between individual groups were tested as follows: Fisher exact test or the χ^2 test for categorical data, Mann-Whitney *U* test or Kruskal-Wallis test with Dunn multiple comparison test for continuous variables. The Mann-Whitney *U* and Kruskal-Wallis tests were used for comparison between 2 groups and among >3 groups, respectively. Statistical analyses were performed using GraphPad Prism 8.43 (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $P<0.05$.

RESULTS

Clinicopathological Characteristics of Cancer-Associated VTE From the Autopsy Records

Table 1 summarizes the clinicopathological findings of VTE cases from the autopsy records (procedure no. 1 of Figure S1). There were no significant differences in age or sex between the VTE without cancer and cancer-associated VTE groups. Patients in the postoperative state reached about 25% of cases in both groups. A history of chemotherapy or radiation therapy was frequently found in the cancer-associated VTE group (55.3% and 24.6%, respectively). There was one case of chemotherapy history in the VTE without cancer group, which was treated with methotrexate for rheumatoid arthritis.

Table 1. Clinicopathological Characteristics in Autopsy Cases With VTE

	Total VTE (n=180)	Non-CAT (n=66)	CAT (n=114)	*P* value
Clinical information				
Age, y, median (range)	66 (0–96)	66 (0–89)	66 (9–96)	0.51
Male, n (%)	103 (57.2)	42 (63.6)	61 (53.5)	0.21
Postoperative state, n (%)*	45 (25.0)	17 (25.8)	28 (24.6)	0.86
Chemotherapy, n (%)	64 (35.6)	1 (1.5)	63 (55.3)	<0.001
Radiation therapy, n (%)	28 (15.6)	0 (0)	28 (24.6)	<0.001
Clinical massive PE, n (%)	11 (6.1)	5 (7.6)	6 (5.3)	0.54
Autopsy findings (from autopsy records)				
DVT only, n (%)	59 (32.8)	25 (37.9)	34 (29.8)	0.32
PE only, n (%)	52 (28.9)	18 (27.3)	34 (29.8)	0.74
DVT/PE both, n (%)	69 (38.3)	23 (34.8)	46 (40.4)	0.53
Pulmonary infarction, n (%)	24 (13.3)	8 (12.1)	16 (14.0)	0.82
Severe infection, n (%)†	79 (43.9)	37 (56.1)	42 (36.8)	0.013
DAD, n (%)	29 (16.1)	14 (21.2)	15 (13.2)	0.21
DIC, n (%)	13 (7.2)	4 (6.1)	9 (7.9)	0.77
Arterial thrombosis, n (%)	38 (21.1)	19 (28.8)	19 (16.7)	0.061
NBTE, n (%)	30 (16.7)	8 (12.1)	22 (19.3)	0.30
Affected death by VTE, n (%)	26 (14.4)	10 (15.2)	16 (14.0)	0.83

Analysis with Mann-Whitney *U* test for age, and Fisher exact test for the others. This population was based on procedure no. 1 of Figure S1. Non-CAT denotes VTE cases without cancer. CAT indicates cancer-associated VTE; DAD, diffuse alveolar damage; DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; NBTE, nonbacterial thrombotic endocarditis; PE, pulmonary embolism; and VTE, venous thromboembolism.

*Postoperative state was defined as recent major surgery for cancer or traumatic disease.

†Severe infection was defined as clinicopathologically diagnosed infectious disease, related to cause of death.

Clinically diagnosed acute PE accounted for 7.6% of the VTE without cancer group and 5.3% of the cancer-associated VTE group. Pathologically diagnosed PE accounted for 62.1% in the VTE without cancer group and 70.2% in the cancer-associated VTE groups. The frequency of severe infection was significantly higher in the VTE without cancer group than that in the cancer-associated VTE group (56.1% versus 36.8%, *P*=0.013). There were no significant differences in other thrombotic diseases (disseminated intravascular coagulation, arterial thrombosis, and nonbacterial thrombotic endocarditis). These differences were comparable in VTE cases assessed with HE staining and immunohistochemistry, except for frequency of arterial thrombosis. Arterial thrombosis was higher in the VTE without cancer group than that in the cancer-associated VTE groups in cases with immunohistochemical assessment (procedure no. 2 and no. 3 in Figure S1; Table S2 and S3). In VTE cases without cancer, approximately half of the patients had severe systemic infections (Table S4). In addition, ≈15% of VTE cases without cancer occurred in patients with autoimmune diseases, such as systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosus, anti-neutrophil cytoplasmic antibody-related vasculitis, and unclassified vasculitis (Table S4). The DVT were most commonly located in the iliac and femoral veins (51.6%), inferior vena cava (34.4%), cervical vein (18.8%), and subclavian vein (17.2%; Table S5).

Table 2 shows the primary organs and histological types of cancer-associated VTE. The primary organs of the cancers were lung (n=22, 19%), stomach (n=13, 11%), hematopoietic and lymphoid system (n=12, 11%), pancreas (n=11, 10%), colorectal system (n=7, 6%), esophagus (n=6, 5%), biliary tract (n=6, 5%) and others. Four cases (4%) had multiple primary cancers. The

Table 2. Primary Organs and Histological Types of Cancer-Associated VTE, n (%)

Primary organ (n=114)		Histological type (n=114)	
Lung	22 (19.3)	Adenocarcinoma	53 (46.5)
Stomach	13 (11.4)	Lymphoma	13 (11.4)
Hematopoietic and lymphoid	12 (10.5)	Neuroendocrine carcinoma	12 (10.5)
Pancreas	11 (9.6)	Squamous cell carcinoma	11 (9.6)
Colon and rectum	7 (6.1)	Malignant melanoma	4 (3.5)
Esophagus	6 (5.3)	Carcinosarcoma	3 (2.6)
Biliary tract	6 (5.3)	Undifferentiated carcinoma	3 (2.6)
Central nerve system	4 (3.5)	Others	11 (9.6)
Skin	4 (3.5)	Multiple	4 (3.5)
Others	25 (21.9)		
Multiple	4 (3.5)		

This population was based on the procedure no. 1 of Figure S1. VTE indicates venous thromboembolism.

histological cancer types were adenocarcinoma (n=53, 47%), lymphoma (n=13, 11%), neuroendocrine carcinoma (n=12, 11%), squamous cell carcinoma (n=11, 10%), and others.

Histopathological Characteristics of Cancer-Associated VTE Tissue

Histological sections of cancer-associated VTE revealed the presence of cancer cells in thrombi (26.6%, 29/109, based on HE-stained sections; procedure no. 2 of Figure S1). Cancer cells in the thrombi were observed in 27.5% (14/51) of DVT and 25.9% (15/58) of PE. There were 2 patterns of the presence of cancer cells in the thrombi or emboli. The first involved direct vascular wall invasion of cancer cells with thrombus formation (Figure 1A). The cancer cells directly invaded the venous or pulmonary arterial walls and penetrated the vascular lumen at thrombus sites. Another pattern involved the presence of small or large clusters of cancer cells in thrombi without direct vessel invasion (Figure 1B). Direct invasion pattern accounted for 51.7% of total VTE, 71.4% of DVT, and 33.3% of PE with cancer cells (Figure 1C), whereas the cancer cell cluster pattern in thrombi accounted for 48.3% of total VTE, 28.6% of DVT, and 66.7% of PE with cancer cells (Figure 1C). There was a significant difference in the frequency between DVT and PE (P=0.040, χ^2 test). Some PE cases with cancer cells show small tumor emboli with thrombus formation and organization.

Among VTE specimens with CK-immuno-positive cancer cells (n=25), the median value of CK-immuno-positive area was 13.1% per total thrombus area (procedure no. 3 of Figure S1). The CK-immunopositive areas did not differ between DVT and PE (P=0.30, Mann-Whitney U test; Figure 1D). Three of the 5 PE cases with direct invasion patterns were not accompanied by DVT.

DAMPs can initiate thrombus formation via activation of platelets and coagulation factor XII.[7,13] We examined cancer cell necrosis in VTE and found it in approximately 40% of DVT and PE (Figure 1E and 1F). There was no significant difference in the frequency of cancer cell necrosis between DVT and PE (P=0.88, χ^2 test). The cancer and necrotic cells were surrounded by fibrin and GPIIb/IIIa-positive platelets. Fibrin formation was also observed in the necrotic area (Figure 1E). Hematoxylin-positive nuclei and immunohistochemistry for CK highlighted the exposure of nuclear fragments and cytokeratin within the thrombi.

Expression of Prothrombotic and Fibrinolytic Factors in Cancer Cells in VTE Tissue

Figure 2A through 2D, Figure S5, and Table 3 show the expression of prothrombotic and fibrinolytic factors in cancer cells in the thrombi and thrombus components. Cancer cells in thrombi immunohistochemically expressed TF, podoplanin, tPA, or PAI-1 (76.0%, 44.0%, 36.0%, and 20.0%, respectively; Figure 2A and 2C, Figure S5, and Table 3). Immunofluorescence of representative specimens showed coexpression of TF and CK (Figure 2B) and of podoplanin and CK (Figure 2D) in the intrathrombus cancer cells. These data indicate that cancer cells express TF and/ or podoplanin in the thrombus.

Cancer cells were accompanied by fibrin/platelet-rich (21/25, 84%; Figure 2A and 2C), organizing (3/25, 12%), or erythrocyte-rich content (1/25, 4%). The cancer cells in VTE frequently expressed TF or podoplanin in any primary organ; however, primary gastric cancer showed the lowest frequency of expression of podoplanin (16.7%, 1/6). All histological types expressed TF, with adenocarcinoma (75.0%, 9/12) and squamous cell carcinoma (75.0%, 3/4) most frequently expressing TF. However, podoplanin expression tended to depend on the histological type, and squamous cell carcinoma showed consistent expression of podoplanin. Cancer cells in VTE expressed tPA in one-third of the cases. Among the histological types, adenocarcinoma frequently expressed tPA (41.7%, 5/12). Cancer cells in VTE infrequently expressed PAI-1 among all cases (20%, 5/25; Table 3 and Figure S5).

Thrombus Contents of Cancer-Associated VTE and of VTE Without Cancer

Table S6 shows the histopathological characteristics of VTE without cancer and cancer-associated VTE, based on HE-stained sections. Cancer-associated DVT areas (n=51) were significantly larger than those of DVT without cancer (n=38; median 14.5 mm^2 vs 5.6 mm^2, P=0.0010). There was no significant difference in PE size between the groups; however, PE cases without cancer were significantly localized to the central sites of the pulmonary artery. The degree of neutrophil infiltration (G0–G3) was significantly different between the DVT with and without cancer (P=0.0070, χ^2 test; Table S6). The predominant degree on neutrophilic infiltrate in DVT without cancer was G3 (41.2%). Conversely, for cancer-associated DVT the degree was G1 (42.1%). Thus, DVT without cancer was associated with a high degree of neutrophilic infiltration. However, there was no difference in the frequency of leukocyte lytic changes between groups. VTE tissue showed various organizing processes in both groups. In particular, endothelial proliferation, fibroblast proliferation, and hemosiderin deposition were more frequent in cancer-associated DVT than in DVT without cancer. In contrast, there was no significant difference in the organization process of PE between the groups.

We immunohistochemically examined the VTE content of erythrocytes, platelets, and fibrin. Both VTEs with and without cancer were immuno-positive for glycophorin A, GPIIb/IIIa, and fibrin (Figure S4 and Figure S6). Although the glycophorin A-immuno-positive area in

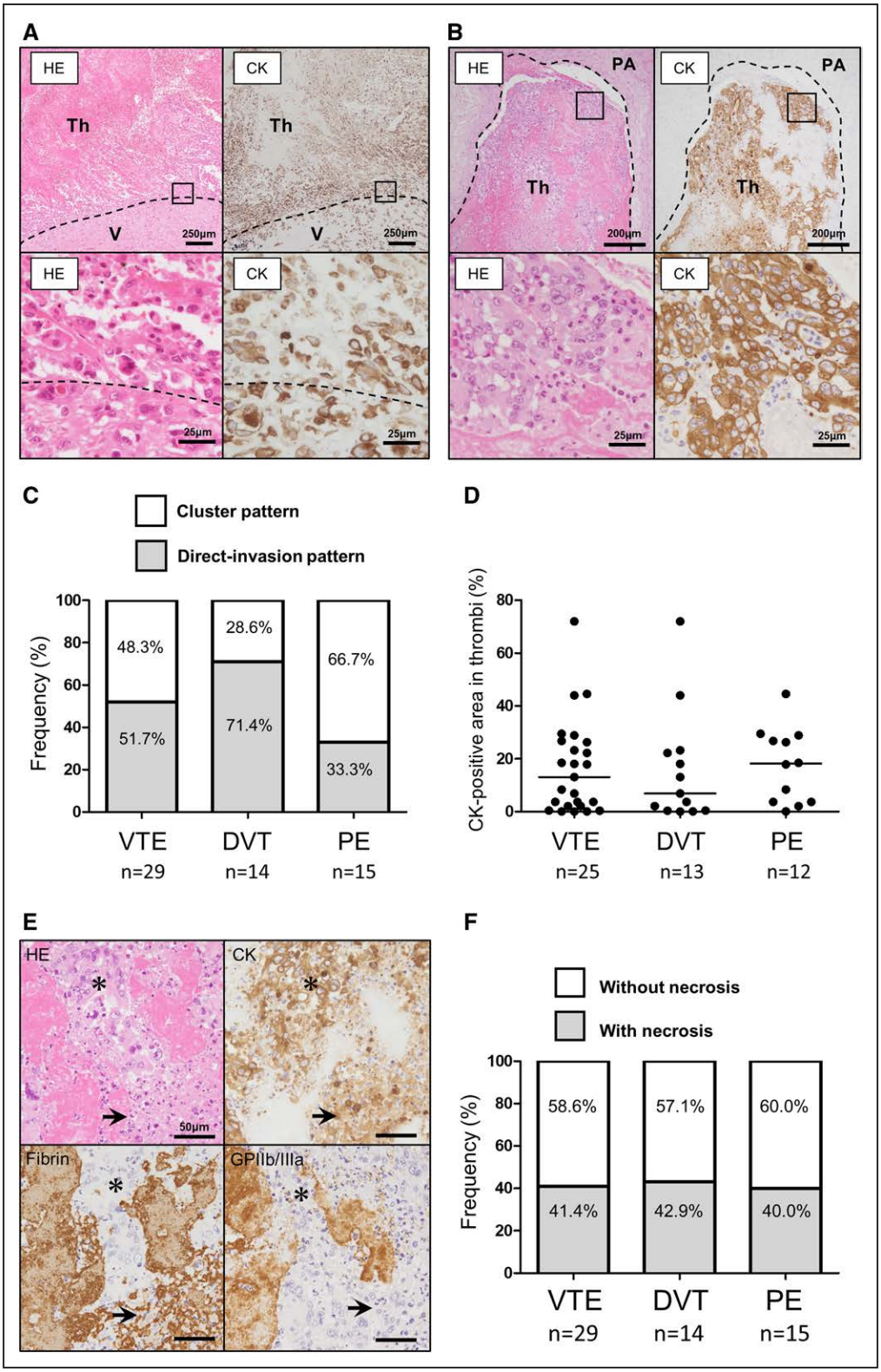

Figure 1. Histopathology of cancer cells in venous thromboembolism (VTE) tissue of autopsy cases.
A, Venous thrombus of inferior vena cava with pancreatic adenocarcinoma. Dashed line indicates the interface between the thrombus (Th) and the venous wall (V). The thrombus occupies the venous lumen. Cancer cells, CK (cytokeratin)-positive cells, show direct invasion into the thrombus from the venous wall, associated with thrombus formation. **B**, Pulmonary thrombi with gastric adenocarcinoma. Cancer clusters are observed in a variety of sizes. There is no direct cancer invasion from the pulmonary artery (PA). Squares of the upper figures are corresponding to the lower figures in high-power fields (**A** and **B**). **C**, Frequency of cancer pattern in cancer-associated VTE tissue with cancer cells (specimen n=29). There was a significant difference in the frequency between deep vein thrombosis (DVT) (*Continued*)

cancer-associated DVT tended to be larger than that in DVT without cancer, there was no significant difference in the immuno-positive areas between groups (Figure S6A). Platelet and fibrin contents were higher than erythrocyte content in any DVT or PE (Figure S6B). There was no significant difference in the glycophorin A-, GPIIb/IIIa-, fibrin-, and Cit-H3-immunopositive area in VTE among the histological types of cancer (adenocarcinoma n=50, squamous cell carcinoma n=11, neuroendocrine carcinoma n=8, and others n=10; P=0.071 for glycophorin A, P=0.78 for GPIIb/IIIa, P=0.25 for fibrin, and P=0.23 for Cit-H3, Kruskal-Wallis test).

Expression of Prothrombotic Factors in Monocyte/Macrophages in VTE Tissue

We also examined the expression of TF and podoplanin in non-cancer cells in cancer-associated VTE and VTE without cancer. We analyzed immunohistochemical localization with consecutive sections: CD163- and TF-positive cells; and CD163- and podoplanin-positive cells. Certain CD163-positive cells expressed TF (Figure S7A) or podoplanin (Figure S8A) mainly in the organizing area of the thrombi. We also performed immunofluorescence in representative specimens to show coexpression of CD163 and TF (Figure S7B) and of CD163 and podoplanin (Figure S8B). The frequency of TF expression in CD163-positive cells was higher in the cancer-associated VTE than that in VTE without cancer (46.8%, 37/79 versus 16.4%, 9/55, P<0.001, Fisher exact test). The frequency of podoplanin expression in CD163-positive cells was 1.8% (1/55) and 2.5% (2/79) in the cancer-associated VTE and VTE without cancer groups, respectively. This difference was not statistically significant (P=1.0, Fisher exact test).

Relationships Between Leukocyte Lysis, Cit-H3–Positive Cells, and Organization of Thrombi

Figure 3A shows that Cit-H3 immunoreaction in the leukocyte lytic area was rich in platelets and fibrin and less abundant in erythrocytes. Immunofluorescence images of CD66b (a neutrophil marker), Cit-H3, and 4',6-diamidino-2-phenylindole (a DNA marker) showed the presence of Cit-H3 positive DNA with CD66b in the leukocyte lytic area (Figure 3B) but not in the nonlytic fresh area (Figure 3C). We also examined the presence or absence of leukocyte lysis in the highest density area of Cit-H3 with the corresponding HE section. Cit-H3 immunopositivity was observed in 59.7% (80/134) of the VTE tissue. Ninety-five percent (76/80) of the thrombi with Cit-H3–positive cells corresponded to lytic leukocytes. Only 5% (4/80) of the thrombi with Cit-H3–positive cells were localized in areas with nonlytic leukocytes.

We also examined the relationship between the Cit H3 immuno-positive area and the grade of the total thrombus organizing area (G1–G3). The Cit-H3 immuno-positive area was measured in the highest density field under high magnification and expressed as a mean value of three fields in each VTE. There was no significant difference in Cit-H3 positive areas between the VTE without cancer and cancer-associated VTE, DVT, or PE groups (Figure 3D). In addition, the Cit-H3 immunopositive areas were larger in the VTE with none (G0) or small organizing areas (G1) compared with large organized areas (G3; Figure 3E). For VTE without cancer, the Cit-H3 immuno-positive areas were larger in VTE without organizing areas (G0) than in those with large organized areas (G3; Figure 3E). For cancer-associated VTE, the Cit-H3 immuno-positive areas were larger in VTE with small organizing areas (G1) than in those with large organized areas (G3; Figure 3E). Among the low-grade organizing VTE (G0 and G1), there was no significant difference in the Cit-H3 immuno-positive area between VTE without cancer (n=36; median, 0.39; range, 0 to 14.8) and that of cancer-associated VTE (n=37; median, 1.5; range, 0 to 12.3; P=0.36, Mann-Whitney U test).

DISCUSSION

We showed that cancer cells were present in thrombi as cancer cell clusters or as direct vascular wall invasion in one-quarter of cancer-associated VTE. Additionally, these cancer cells expressed TF or podoplanin in three-fourths or half of the cases, respectively. Cancer-associated DVT showed a more advanced organization process than DVT without cancer. TF-positive monocyte/macrophages in VTE tissue were more frequently observed in cancer-associated VTE groups than in VTE without cancer. NET formation was predominantly found in less-organized VTE tissue and was not affected by the cancer-bearing state.

The primary organs of cancer were found to be associated with the risk of cancer-associated VTE. In particular, tumors of the brain, pancreas, kidney, lung, ovary, and hematopoietic and lymphoid systems are known to be at high risk of VTE.[5,29] Similarly, histological types of cancer could affect the risk of cancer-associated VTE. For example, the results of some cohort studies have indicated

Figure 1 Continued. and pulmonary embolism (PE; P=0.040, χ^2 test). **D**, CK-positive area per total thrombus area with cancer cells (specimen n=25). There was no significant difference between DVT and PE (Mann-Whitney U test). **E**, Necrosis of cancer cells in pulmonary thrombus with gastric adenocarcinoma. Cytoplasmic lysis and nuclear fragmentation indicating necrosis (arrow) in the CK immunopositive area adjacent to cancer cells (*). The cancer and necrotic cells are surrounded by fibrin and platelets (GPIIb/IIIa [glycoprotein IIb/IIIa]). Fibrin formation is also observed in the necrotic area (arrow). **F**, Frequency of necrotic changes in VTE tissue with cancer cells (specimen n=29). There was no significant difference in the frequency between DVT and PE (P=0.88, χ^2 test). HE indicates hematoxylin and eosin.

that lung adenocarcinoma had a higher risk of VTE than squamous cell carcinoma of the lung.[4,30,31] We have previously reported that patients with clear cell carcinoma of the ovary had a higher frequency of VTE complications than other ovarian carcinomas, in a manner associated with TF expression in the surgical cancer tissue.[17] In the present study, the primary organs of cancer-associated VTE were high in the lung, stomach, hematopoietic and lymphoid system, and pancreas. Among the histological types, approximately half of the cancer-associated VTE cases were adenocarcinomas. These results are comparable to those of previous reports.

Little is known about the histopathological findings of VTE in patients with cancer. Sakuma et al[19] analyzed characteristics of cancer-associated PE (n=65 181) using a Japanese autopsy record and reported that thrombotic PE was recognized in 2.3% of the autopsy cases, which tended to be most commonly observed in cases of ovarian cancer, hematopoietic malignancy, and pancreaticobiliary cancers, and in the histological types of large cell carcinoma, leukemia, and adenocarcinoma. The authors further reported that pulmonary tumor emboli were found in 0.2% of cases and was associated with cancer invasion into large veins. A Swedish population-based autopsy study of patients with cancer (n=23 796) reported a PE prevalence of 23% and adenocarcinoma was independently associated with PE risk.[4] However, these 2 studies showed a lack of

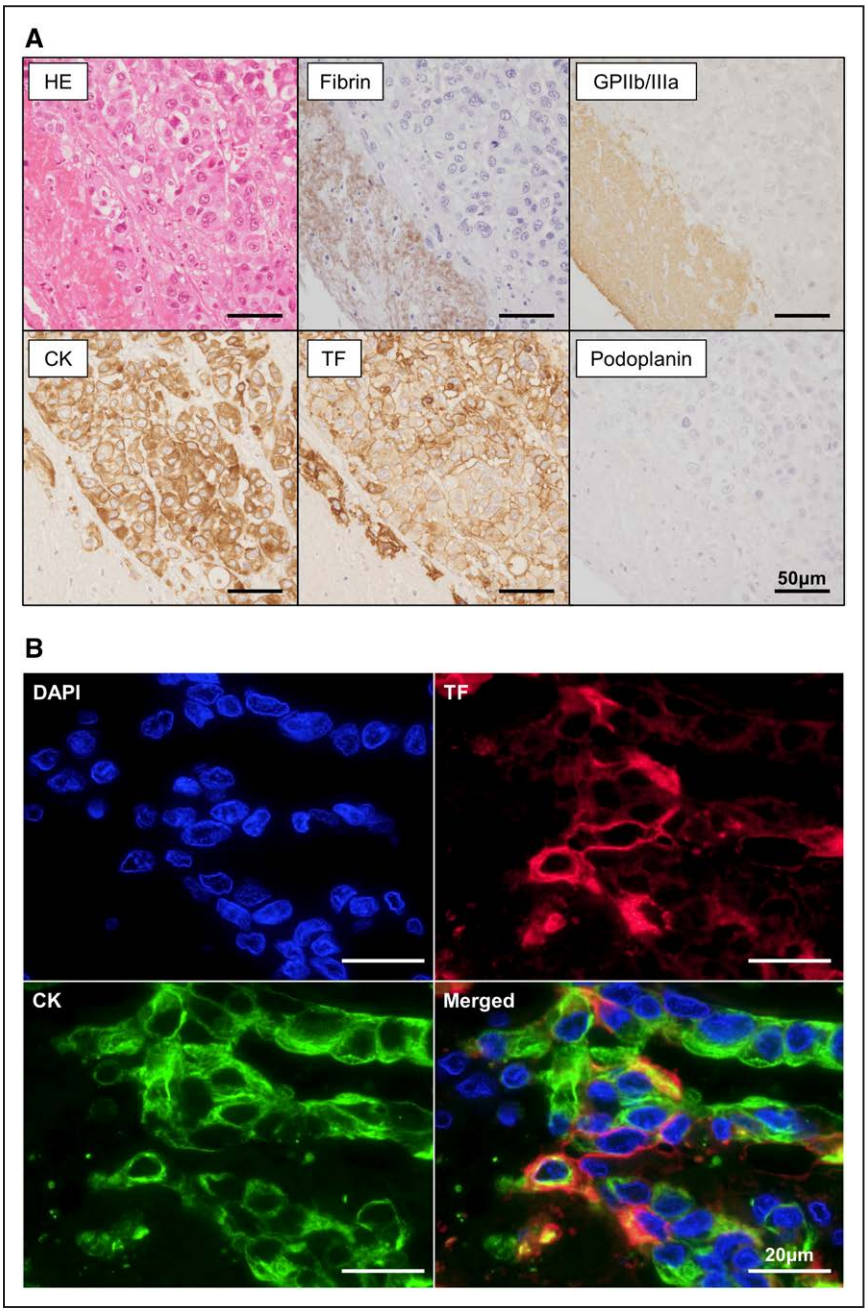

Figure 2. Expression of prothrombotic factors of cancer cells in venous thromboembolism tissue. Representative microphotographs of cancer cells in a venous thrombus, accompanied by TF (tissue factor) expression in a case of pancreatic adenocarcinoma (**A**) and in a case of gastric adenocarcinoma (**B**). Immunohistochemically, cancer cells in thrombi correspond to CK (cytokeratin)-positive cells (**A**). Additionally, the cancer cells express TF (**A**). No podoplanin is expressed in the cancer cells. Immunofluorescence shows heterogeneous TF expression in CK-positive cells (**B**). (*Continued*)

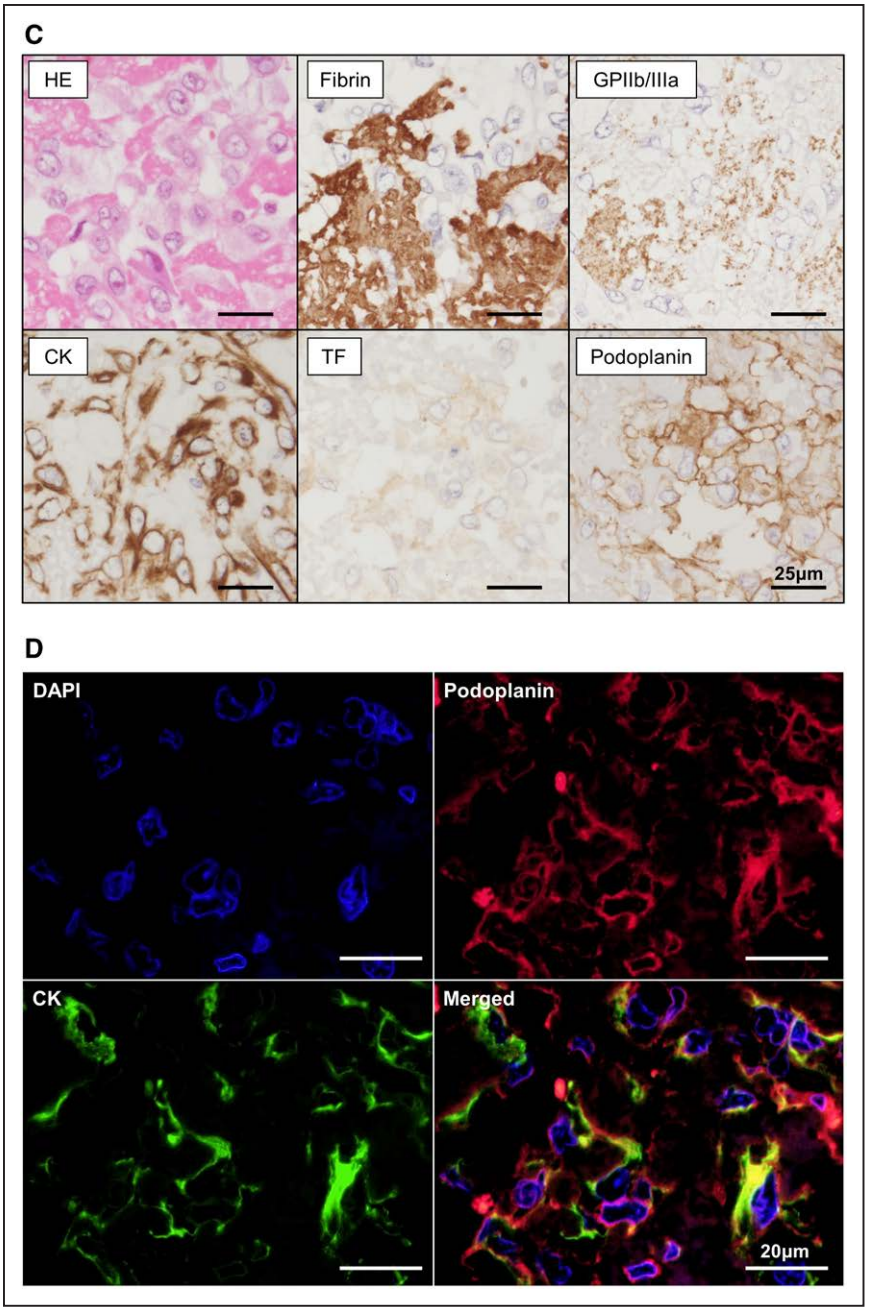

Figure 2 Continued. Representative microphotographs of cancer cells in a pulmonary thrombus, accompanied by podoplanin expression (**C** and **D**) in a case of cutaneous squamous cell carcinoma. The cancer cells are highlighted with CK, and diffusely expressed podoplanin (**C**). In contrast, the cancer cells weakly and focally expressed TF (**C**). Immunofluorescence shows that CK-positive cells are also positive for podoplanin (**D**). In these cases, fibrin formation and platelet (glycoprotein [GP] IIb/IIIa) aggregation are observed in the surrounding cancer cells (**A** and **C**). DAPI indicates 4',6-diamidino-2-phenylindole; and HE indicates hematoxylin and eosin.

histopathological analysis of VTE. A Dutch autopsy study of patients with cancer with PE (n=1191) was classified as thrombotic embolism, tumor embolism, septic embolism, fat tissue embolism, and bone marrow embolism.[18] However, the study did not evaluate other thrombus contents and these associations. Venous invasion and compression by cancer are considered factors that influence VTE.[32] However, there is no pathological evidence that cancer cells are present in cancer-associated VTE within human VTE tissue. In the present study, direct vascular wall invasion suggests that vascular wall damage, endothelial denudation, and exposure of thrombogenic vascular surface and cancer cells all contribute to venous thrombus initiation. Additionally, 60% of cancer-associated PE with cancer cell invasion were not accompanied by DVT. These findings imply the presence of cancer-associated pulmonary thrombosis, in addition to PE. Further, circulating tumor cells in the blood can play an important role in thrombosis.[33] Feinauer et al[34] reported that circulating cancer cells injected into the left cardiac ventricle and arrested in brain vessels initiated local thrombus formation and promoted metastasis in a mouse model. Previous clinical studies have also indicated that circulating tumor cells might indicate an increased risk of VTE in patients with breast cancer.[35,36] The intrathrombus cancer cell clusters may be derived from circulating cancer cells and play a role in the initiation or propagation of

Table 3. Expression of Prothrombotic Factors of Cancer Cells in VTE Tissue

		Tissue factor	Podoplanin	Tissue factor+podoplanin		tPA	PAI-1
				Copositive	Conegative		
VTE tissue with cancer cell	(n=25)	19 (76.0)	11 (44.0)	8 (32.0)	3 (12.0)	9 (36.0)	5 (20.0)
Primary organs							
Stomach	(n=6)	5 (83.3)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)
Lung	(n=4)	3 (75.0)	2 (50.0)	2 (50.0)	1 (25.0)	1 (25.0)	1 (25.0)
Pancreas	(n=4)	3 (75.0)	2 (50.0)	1 (25.0)	0 (0)	3 (75.0)	0 (0)
Thyroid	(n=2)	2 (100)	2 (100)	2 (100)	0 (0)	2 (100)	2 (100)
Skin	(n=2)	2 (100)	2 (100)	2 (100)	0 (0)	1 (50.0)	0 (0)
Others	(n=7)	4 (57.1)	3 (42.9)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)
Histological types							
Adenocarcinoma	(n=12)	9 (75.0)	5 (41.7)	3 (25.0)	1 (8.3)	5 (41.7)	2 (16.7)
Squamous cell carcinoma	(n=4)	3 (75.0)	4 (100)	3 (75.0)	0 (0)	1 (25.0)	1 (25.0)
Neuroendocrine carcinoma	(n=2)	1 (50.0)	0 (0)	0 (0)	1 (50.0)	0 (0)	0 (0)
Others	(n=7)	6 (85.7)	2 (28.6)	2 (28.6)	1 (14.3)	3 (42.9)	2 (28.6)

PAI-1 indicates plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; and VTE, venous thromboembolism.

venous thrombi, in addition to the propagation of a pulmonary embolus.

TF and podoplanin are associated with the cancer microenvironment, invasion, metastasis, and prognosis.[8,16,37,38] In general, adenocarcinoma and squamous cell carcinoma cells tend to express TF and podoplanin, respectively.[5,6,39] The present study showed that most cases of adenocarcinoma and squamous cell carcinoma express TF or podoplanin in VTE. In addition, 25% of adenocarcinomas and 75% of squamous cell carcinomas revealed coexpression of TF and podoplanin. These cancer nests in VTE are surrounded by fibrin and aggregated platelets. In addition, nuclear debris and cytoplasmic fragments due to cancer cell necrosis have been detected in patients with VTE. DAMPs, such as extracellular chromatin, proteins, and metabolites, can activate coagulation via factor XII and platelet aggregation.[12,13] The present study provides pathological evidence that cancer cells directly contribute to thrombogenesis via thrombogenic factor expression and exposure to DAMPs in cancer-associated VTE.

The plasminogen activator system, including tPA, urokinase-plasminogen activator, urokinase-plasminogen activator receptor, and PAI-1, plays an important role in fibrinolysis, fibrosis, and cancer cell biology.[40,41] Among patients with pancreatic cancer, patients with high levels of serum PAI-1 had poor prognosis, compared with those with lower levels.[3] In addition, human pancreatic cancer-bearing mice had higher plasma levels of PAI-1 than control mice, and this was associated with venous thrombus weight.[9] However, expression of these factors in human cancer-associated VTE tissue remains unknown. In the present study, we showed that cancer cells in VTE tissue expressed tPA and PAI-1 in various histological types of cancer. Pancreatic cancer and adenocarcinoma frequently expressed tPA However, PAI-1 expression was less frequent. These results suggest that intrathrombus cancer cells affect thrombus formation and organization via PAI-1 and tPA.

Monocytes are known as TF-expressing cells under cancer-associated states and systemic infective diseases, associated with procoagulant activity.[42–44] We showed that TF-positive monocyte/macrophages were highly frequent in cancer-associated VTE tissue compared with VTE tissue without cancer. Among patients with lung cancer, TF expression in circulating monocytes of patients with VTE was higher than that in patients without VTE.[45] Our findings suggest that monocyte/macrophage TF expression in thrombi is enhanced in patients with cancer. The higher frequency of TF-positive monocytes/macrophages may be affected by enhanced organization in cancer-associated VTE (Table S6) since the number of macrophages increased in a time-dependent manner in DVT.[20,21]

Hisada et al[46] reported that the venous thrombi of human pancreatic cancer-bearing mice weighed significantly more than that of control. Although we did not measure the VTE volume in this study, we nevertheless showed that the areas of cancer-associated DVT were significantly larger than that of patients without cancer. These results suggest that cancer-associated states promote venous thrombi growth. However, there was no difference in the PE size between groups. Moreover, PE in patients with cancer tended to be located in the peripheral pulmonary arteries. We observed that some cancer-associated PE cases showed multiple small thrombotic emboli with or without cancer cells. These findings might explain the tendency of PE to be located in the peripheral arteries.

Despite the presence of thrombogenic factor expression and necrosis in cancer-associated VTE, there was no significant difference in the area ratios of the erythrocyte, platelet, and fibrin components between patients with and without cancer. In this study, patients without cancer had various thrombogenic conditions,

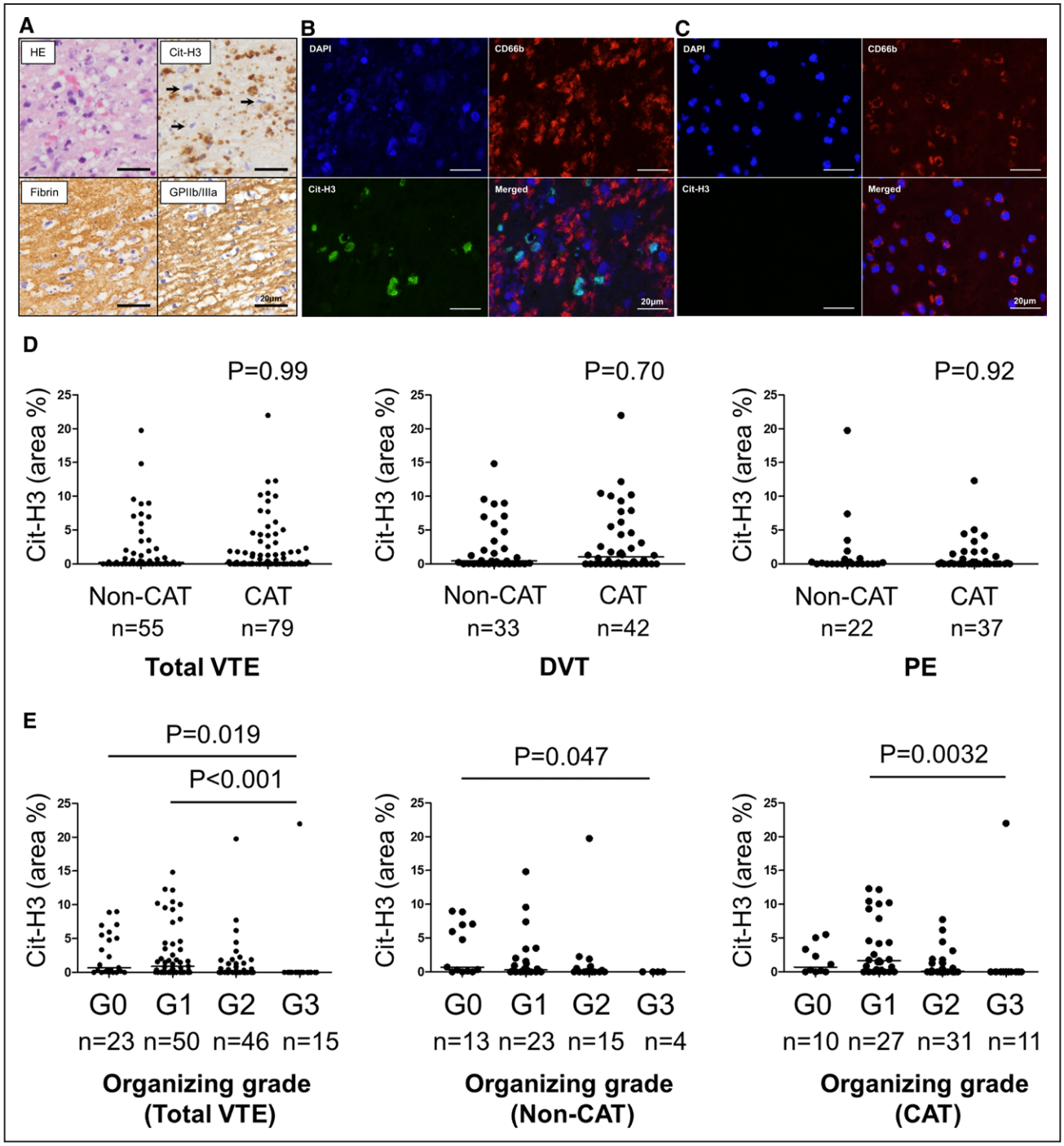

Figure 3. Analysis of NETs (neutrophil extracellular traps) in venous thromboembolism (VTE) tissue.
A, Representative micrographs of Cit-H3 (citrullinated histone H3; as a NET marker) in a venous thrombus. Hematoxylin and eosin (HE) staining shows lysis and fragmentation of neutrophil chromatin, corresponding to Cit-H3 expression. The surrounding thrombus is composed of fibrin and platelets. Nonlytic neutrophils are immuno-negative for Cit-H3 (arrows). **B**, Representative immunofluorescence images of NETs in VTE tissue. Lytic cells show delobulation and fragmentation of nuclei (4',6-diamidino-2-phenylindole [DAPI], blue) and dispersed CD66b (neutrophil marker, red) immunoreaction. Triple immunofluorescence images reveal the presence of Cit-H3 positive (green) DNA (cyan) with CD66b in the lytic area. **C**, Representative immunofluorescent images of neutrophils without NET formation in VTE tissue. This image is obtained from different fields within the same thrombus from **B**. Nonlytic segmented cells express CD66b (neutrophil marker, red). Triple immunofluorescence images reveal the absence of Cit-H3–positive DNA (blue) in the nonlytic area. **D**, Immuno-positive area ratio of Cit-H3 between VTE without cancer (non-CAT) and cancer-associated VTE (CAT). The Mann-Whitney *U* test was used for statistical analysis. **E**, Immuno-positive area ratio of Cit-H3 between organizing grades (G0–G3) of total VTE, non-CAT, and CAT. The Kruskal-Wallis test with Dunn multiple comparison test was used for statistical analysis. DVT indicates deep vein thrombosis; and PE, pulmonary embolism.

 Arterioscler Thromb Vasc Biol. 2023;43:146–159. DOI: 10.1161/ATVBAHA.122.318463

including severe infection, postoperative states, and systemic autoimmune diseases. Their cancers were heterogeneous in terms of the primary organs, histological types, thrombogenic factor expression, and history of cancer-related therapy. These conditions and situations may affect thrombus formation and result in a wide range of immuno-positive areas for erythrocytes, fibrin, and platelets in both the groups. The lack of difference among the components in both groups suggests that the simultaneous activation of blood coagulation and platelet aggregation is a consistent process in VTE formation. A previous study reported that cancer-derived TF leads to increased fibrin density in venous thrombi in human pancreatic cancer-bearing mice using scanning electron microscopy.[47] We showed that both non-cancer and cancer-associated VTE were composed of various phases of cellular and organizing reactions. This finding is comparable with previous histopathological studies of human VTE.[21,48] The evidence suggests that human DVT has repeated thrombus formation phases and that the mechanisms of thrombus formation differ from those in animal models, in which thrombus formation is induced with a single artificial initiation.

It has been proposed that cancer-bearing states promote NET formation, which contributes to cancer-related thrombogenicity.[11] Mauracher et al[49] reported that the plasma Cit-H3 level could be used as a predictive marker of VTE occurrence in patients with cancer. Mangold et al[50] reported the presence of NETs in DVT using immunohistochemistry for DNA histones. In addition, neutrophils and NETs were found to contribute to venous thrombus formation in human pancreatic cancer-bearing mice but not in control mice.[47] In contrast, the present study showed a higher degree of neutrophil infiltration in VTE without cancer than in cancer-associated VTE, although there was no significant difference in NET formation between the groups. These results suggest that neutrophil numbers do not always reflect NET formation in human patients with VTE and that neutrophils in patients with cancer may be more prone to NET formation. Half of the patients without cancer in this study also had severe infectious diseases until shortly before death. Therefore, inflammatory conditions in these patients might be related to a higher infiltration of neutrophils without NET formation. NETs were predominantly observed in thrombi during the organizing stages rather than in fresh thrombi in eleven cases of human VTE.[51] In comparison with this study, NET formation was found to be greater in VTE with no or a small organizing area compared with cases of organized VTE. The present results support the notion that local NETs in thrombi predominantly play a role after, rather than during, thrombus formation, in VTE with and without cancer.

This study had several limitations. First, the study cohort consisted of autopsy cases; thus, almost all cancer cases were in the advanced stage. Second, we could not obtain detailed blood sample data, such as D-dimer, neutrophil counts, and serum NET markers, and could not analyze the relationships between these blood sample data and histopathological findings. Third, since this was a retrospective cohort study, we could not define the precise length of fixation which may have affected the immunoreactivity of each antigen. To standardize immunoreactivity, we excluded the VTE tissue with inadequate immunopositivity in intrinsic control cells of the same sections. Fourth, although thrombus components are heterogenous, we did not evaluate the entire thrombus. To minimize heterogeneity, we selected the largest section of each thrombus. Finally, we could not statistically examine the expression of prothrombotic factors in intrathrombus cancer cells by histopathological type due to the small number of cases.

In conclusion, this study revealed the presence of cancer cells as direct vascular wall invasion or small clusters in thrombi in 27% of cancer-associated VTE specimens. Intrathrombus cancer cells might play a role in thrombogenesis of cancer-associated VTE. Moreover, expression of TF and podoplanin in cancer cells and necrosis-related DAMP exposure may promote coagulation reactions and platelet aggregation.

ARTICLE INFORMATION

Received July 15, 2022; accepted November 2, 2022.

Affiliations

Department of Pathology (T.G., A.Y., K.M., Y.A.), Department of Laboratory Center (A.K.), Department of Diagnostic Pathology, University of Miyazaki Hospital (S.M.-G., Y.S.), Department of Medicine and Community Health (S.M.), Faculty of Medicine, University of Miyazaki, Japan.

Acknowledgments

The authors are grateful to Nahoko Udatsu, Kyoko Ohashi, Kensuke Kawamoto, Takuya Kinoshita, and Mariko Fujikawa for their technical assistance. The authors thank Professor Hisanori Horiuchi for the valuable discussions and Editage (www.editage.com) for the English language editing.

Sources of Funding

This study was partly supported by Grants-in-Aid for Scientists from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Numbers JP18K15083, JP19K07437, JP20K08085, JP21K15403), Setsuro Fujii Memorial The Osaka Foundation for Promotion of Fundamental Medical Research, and the Cooperative Research Project Program of the Joint Usage/Research Center at the Institute of Development, Aging and Cancer, Tohoku University.

Disclosures

None.

Supplemental Material

Tables S1–S6
Figures S1–S8
Major Resources Table

REFERENCES

1. Zamorano JL, Lancellotti P, Rodriguez Munoz D, Aboyans V, Asteggiano R, Galderisi M, Habib G, Lenihan DJ, Lip GYH, Lyon AR, et al; ESC Scientific Document Group. 2016 ESC Position Paper on cancer treatments and cardiovascular toxicity developed under the auspices of the ESC Committee for Practice Guidelines: The Task Force for cancer treatments and

cardiovascular toxicity of the European Society of Cardiology (ESC). *Eur Heart J.* 2016;37:2768–2801. doi: 10.1093/eurheartj/ehw211
2. Timp JF, Braekkan SK, Versteeg HH, Cannegieter SC. Epidemiology of cancer-associated venous thrombosis. *Blood.* 2013;122:1712–1723. doi: 10.1182/blood-2013-04-460121
3. Moik F, Prager G, Thaler J, Posch F, Wiedemann S, Schramm T, Englisch C, Mackman N, Pabinger I, Ay C. Hemostatic biomarkers and venous thromboembolism are associated with mortality and response to chemotherapy in patients with pancreatic cancer. *Arterioscler Thromb Vasc Biol.* 2021;41:2837–2847. doi: 10.1161/ATVBAHA.121.316463
4. Ogren M, Bergqvist D, Wahlander K, Eriksson H, Sternby NH. Trousseau's syndrome - what is the evidence? A population-based autopsy study. *Thromb Haemost.* 2006;95:541–545. doi: 10.1160/TH05-10-0694
5. Hisada Y, Mackman N. Cancer-associated pathways and biomarkers of venous thrombosis. *Blood.* 2017;130:1499–1506. doi: 10.1182/blood-2017-03-743211
6. Date K, Ettelaie C, Maraveyas A. Tissue factor-bearing microparticles and inflammation: a potential mechanism for the development of venous thromboembolism in cancer. *J Thromb Haemost.* 2017;15:2289–2299. doi: 10.1111/jth.13871
7. Abdol Razak NB, Jones G, Bhandari M, Berndt MC, Metharom P. Cancer-associated thrombosis: an overview of mechanisms, risk factors, and treatment. *Cancers (Basel).* 2018;10:380. doi: 10.3390/cancers10100380
8. Suzuki-Inoue K. Platelets and cancer-associated thrombosis: focusing on the platelet activation receptor CLEC-2 and podoplanin. *Blood.* 2019;134:1912–1918. doi: 10.1182/blood.2019001388
9. Hisada Y, Garratt KB, Maqsood A, Grover SP, Kawano T, Cooley BC, Erlich J, Moik F, Flick MJ, Pabinger I, et al. Plasminogen activator inhibitor 1 and venous thrombosis in pancreatic cancer. *Blood Adv.* 2021;5:487–495. doi: 10.1182/bloodadvances.2020003149
10. Stark K, Schubert I, Joshi U, Kilani B, Hoseinpour P, Thakur M, Grunauer P, Pfeiler S, Schmidergall T, Stockhausen S, et al. Distinct pathogenesis of pancreatic cancer microvesicle-associated venous thrombosis identifies new antithrombotic targets in vivo. *Arterioscler Thromb Vasc Biol.* 2018;38:772–786. doi: 10.1161/ATVBAHA.117.310262
11. Thalin C, Hisada Y, Lundstrom S, Mackman N, Wallen H. Neutrophil extracellular traps: villains and targets in arterial, venous, and cancer-associated thrombosis. *Arterioscler Thromb Vasc Biol.* 2019;39:1724–1738. doi: 10.1161/ATVBAHA.119.312463
12. Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. *Blood.* 2011;118:3708–3714. doi: 10.1182/blood-2011-01-332676
13. Long AT, Kenne E, Jung R, Fuchs TA, Renne T. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. *J Thromb Haemost.* 2016;14:427–437. doi: 10.1111/jth.13235
14. Uno K, Homma S, Satoh T, Nakanishi K, Abe D, Matsumoto K, Oki A, Tsunoda H, Yamaguchi I, Nagasawa T, et al. Tissue factor expression as a possible determinant of thromboembolism in ovarian cancer. *Br J Cancer.* 2007;96:290–295. doi: 10.1038/sj.bjc.6603552
15. Khorana AA, Ahrendt SA, Ryan CK, Francis CW, Hruban RH, Hu YC, Hostetter G, Harvey J, Taubman MB. Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. *Clin Cancer Res.* 2007;13:2870–2875. doi: 10.1158/1078-0432.CCR-06-2351
16. Riedl J, Preusser M, Nazari PM, Posch F, Panzer S, Marosi C, Birner P, Thaler J, Brostjan C, Lotsch D, et al. Podoplanin expression in primary brain tumors induces platelet aggregation and increases risk of venous thromboembolism. *Blood.* 2017;129:1831–1839. doi: 10.1182/blood-2016-06-720714
17. Gi T, Yamashita A, Aman M, Kuwahara A, Asada Y, Kawagoe Y, Onishi J, Sameshima H, Sato Y. Tissue factor expression and tumor-infiltrating T lymphocytes in ovarian carcinomas and their association with venous thromboembolism. *Pathol Int.* 2021;71:261–266. doi: 10.1111/pin.13074
18. Gimbel IA, Mulder FI, Bosch FTM, Freund JE, Guman N, van Es N, Kamphuisen PW, Buller HR, Middeldorp S. Pulmonary embolism at autopsy in cancer patients. *J Thromb Haemost.* 2021;19:1228–1235. doi: 10.1111/jth.15250
19. Sakuma M, Fukui S, Nakamura M, Takahashi T, Kitamukai O, Yazu T, Yamada N, Ota M, Kobayashi T, Nakano T, et al. Cancer and pulmonary embolism: thrombotic embolism, tumor embolism, and tumor invasion into a large vein. *Circ J.* 2006;70:744–749. doi: 10.1253/circj.70.744
20. Fineschi V, Turillazzi E, Neri M, Pomara C, Riezzo I. Histological age determination of venous thrombosis: a neglected forensic task in fatal pulmonary thrombo-embolism. *Forensic Sci Int.* 2009;186:22–28. doi: 10.1016/j.forsciint.2009.01.006
21. Furukoji E, Gi T, Yamashita A, Moriguchi-Goto S, Kojima M, Sugita C, Sakae T, Sato Y, Hirai T, Asada Y. CD163 macrophage and erythrocyte contents in aspirated deep vein thrombus are associated with the time after onset: a pilot study. *Thromb J.* 2016;14:46. doi: 10.1186/s12959-016-0122-0
22. Gi T, Kuroiwa Y, Yamashita A, Mizutani Y, Asanuma T, Miyati T, Maekawa K, Aman M, Imamura T, Asada Y. High signal intensity on diffusion-weighted images reflects acute phase of deep vein thrombus. *Thromb Haemost.* 2020;120:1463–1473. doi: 10.1055/s-0040-1714280
23. Rittersma SZ, van der Wal AC, Koch KT, Piek JJ, Henriques JP, Mulder KJ, Ploegmakers JP, Meesterman M, de Winter RJ. Plaque instability frequently occurs days or weeks before occlusive coronary thrombosis: a pathological thrombectomy study in primary percutaneous coronary intervention. *Circulation.* 2005;111:1160–1165. doi: 10.1161/01.CIR.0000157141.00778.AC
24. Cuthbert GL, Daujat S, Snowden AW, Erdjument-Bromage H, Hagiwara T, Yamada M, Schneider R, Gregory PD, Tempst P, Bannister AJ, et al. Histone deimination antagonizes arginine methylation. *Cell.* 2004;118:545–553. doi: 10.1016/j.cell.2004.08.020
25. Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am J Pathol.* 1989;134:1087–1097.
26. Ordonez NG. Podoplanin: a novel diagnostic immunohistochemical marker. *Adv Anat Pathol.* 2006;13:83–88. doi: 10.1097/01.pap.0000213007.48479.94
27. Grondahl-Hansen J, Kirkeby LT, Ralfkiaer E, Kristensen P, Lund LR, Dano K. Urokinase-type plasminogen activator in endothelial cells during acute inflammation of the appendix. *Am J Pathol.* 1989;135:631–636.
28. Bastelica D, Morange P, Berthet B, Borghi H, Lacroix O, Grino M, Juhan-Vague I, Alessi MC. Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits. *Arterioscler Thromb Vasc Biol.* 2002;22:173–178. doi: 10.1161/hq0102.101552
29. Khorana AA, Connolly GC. Assessing risk of venous thromboembolism in the patient with cancer. *J Clin Oncol.* 2009;27:4839–4847. doi: 10.1200/JCO.2009.22.3271
30. Blom JW, Osanto S, Rosendaal FR. The risk of a venous thrombotic event in lung cancer patients: higher risk for adenocarcinoma than squamous cell carcinoma. *J Thromb Haemost.* 2004;2:1760–1765. doi: 10.1111/j.1538-7836.2004.00928.x
31. Chew HK, Davies AM, Wun T, Harvey D, Zhou H, White RH. The incidence of venous thromboembolism among patients with primary lung cancer. *J Thromb Haemost.* 2008;6:601–608. doi: 10.1111/j.1538-7836.2008.02908.x
32. Mukai M, Oka T. Mechanism and management of cancer-associated thrombosis. *J Cardiol.* 2018;72:89–93. doi: 10.1016/j.jjcc.2018.02.011
33. Xue J, Xie VK, Wang P, Cui J, Gao Y, Lu Z. Interrelationships of circulating tumor cells with metastasis and thrombosis: role of microRNAs. *Curr Pharm Des.* 2014;20:5298–5308. doi: 10.2174/1381612820666140128220152
34. Feinauer MJ, Schneider SW, Berghoff AS, Robador JR, Tehranian C, Karreman MA, Venkataramani V, Solecki G, Grosch JK, Gunkel K, et al. Local blood coagulation drives cancer cell arrest and brain metastasis in a mouse model. *Blood.* 2021;137:1219–1232. doi: 10.1182/blood.2020005710
35. Mego M, De Giorgi U, Broglio K, Dawood S, Valero V, Andreopoulou E, Handy B, Reuben JM, Cristofanilli M. Circulating tumour cells are associated with increased risk of venous thromboembolism in metastatic breast cancer patients. *Br J Cancer.* 2009;101:1813–1816. doi: 10.1038/sj.bjc.6605413
36. Beinse G, Berger F, Cottu P, Dujaric ME, Kriegel I, Guilhaume MN, Dieras V, Cabel L, Pierga JY, Bidard FC. Circulating tumor cell count and thrombosis in metastatic breast cancer. *J Thromb Haemost.* 2017;15:1981–1988. doi: 10.1111/jth.13792
37. Hisada Y, Mackman N. Tissue factor and cancer: regulation, tumor growth, and metastasis. *Semin Thromb Hemost.* 2019;45:385–395. doi: 10.1055/s-0039-1687894
38. Mello FW, Kammer PV, Silva CAB, Parkinson EK, Monteiro L, Warnakulasuriya S, Rivero ERC. Prognostic and clinicopathological significance of podoplanin immunoexpression in oral and oropharyngeal squamous cell carcinoma: A systematic review. *J Oral Pathol Med.* 2021;50:1–9. doi: 10.1111/jop.13041
39. Chuang WY, Chang YS, Yeh CJ, Wu YC, Hsueh C. Role of podoplanin expression in squamous cell carcinoma of upper aerodigestive tract. *Histol Histopathol.* 2013;28:293–299. doi: 10.14670/HH-28.293
40. Bharadwaj AG, Holloway RW, Miller VA, Waisman DM. Plasmin and plasminogen system in the tumor microenvironment: implications for cancer diagnosis, prognosis, and therapy. *Cancers (Basel).* 2021;13:1838. doi: 10.3390/cancers13081838

41. McMahon B, Kwaan HC. The plasminogen activator system and cancer. *Pathophysiol Haemost Thromb*. 2008;36:184–194. doi: 10.1159/000175156
42. Gregory SA, Morrissey JH, Edgington TS. Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. *Mol Cell Biol*. 1989;9:2752–2755. doi: 10.1128/mcb.9.6.2752-2755.1989
43. Aleman MM, Gardiner C, Harrison P, Wolberg AS. Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability. *J Thromb Haemost*. 2011;9:2251–2261. doi: 10.1111/j.1538-7836.2011.04488.x
44. Hisada Y, Sachetto ATA, Mackman N. Circulating tissue factor-positive extracellular vesicles and their association with thrombosis in different diseases. *Immunol Rev*. 2022;312:61–75. doi: 10.1111/imr.13106
45. Deng C, Wu S, Zhang L, Yang M, Lin Q, Xie Q, Ding H, Lian N, Gao S, Huang Y, et al. Role of monocyte tissue factor on patients with non-small cell lung cancer. *Clin Respir J*. 2018;12:1125–1133. doi: 10.1111/crj.12640
46. Hisada Y, Ay C, Auriemma AC, Cooley BC, Mackman N. Human pancreatic tumors grown in mice release tissue factor-positive microvesicles that increase venous clot size. *J Thromb Haemost*. 2017;15:2208–2217. doi: 10.1111/jth.13809
47. Hisada Y, Grover SP, Maqsood A, Houston R, Ay C, Noubouossie DF, Cooley BC, Wallen H, Key NS, Thalin C, et al. Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors. *Haematologica*. 2020;105:218–225. doi: 10.3324/haematol.2019.217083
48. Ro A, Kageyama N, Tanifuji T, Fukunaga T. Pulmonary thromboembolism: overview and update from medicolegal aspects. *Leg Med (Tokyo)*. 2008;10:57–71. doi: 10.1016/j.legalmed.2007.09.003
49. Mauracher LM, Posch F, Martinod K, Grilz E, Daullary T, Hell L, Brostjan C, Zielinski C, Ay C, Wagner DD, et al. Citrullinated histone H3, a biomarker of neutrophil extracellular trap formation, predicts the risk of venous thromboembolism in cancer patients. *J Thromb Haemost*. 2018;16:508–518. doi: 10.1111/jth.13951
50. Mangold A, Alias S, Scherz T, Hofbauer M, Jakowitsch J, Panzenbock A, Simon D, Laimer D, Bangert C, Kammerlander A, et al. Coronary neutrophil extracellular trap burden and deoxyribonuclease activity in ST-elevation acute coronary syndrome are predictors of ST-segment resolution and infarct size. *Circ Res*. 2015;116:1182–1192. doi: 10.1161/CIRCRESAHA.116.304944
51. Savchenko AS, Martinod K, Seidman MA, Wong SL, Borissoff JI, Piazza G, Libby P, Goldhaber SZ, Mitchell RN, Wagner DD. Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development. *J Thromb Haemost*. 2014;12:860–870. doi: 10.1111/jth.12571