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Endothelial cell-derived extracellular vesicles are an important biomarker in cancer-related thrombosis patients

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ABSTRACT

Thrombosis is a common complication in the clinical course of cancer. Vascular endothelial cells and/or the hemostatic-coagulatory system are thought to play important roles in cancer-related thrombosis (CRT). Additionally, several new cancer drugs increase risk of therapy-related thrombosis. Extracellular vesicles (EVs) are small membrane vesicles that are released from many different cell types via exocytic budding of the plasma membrane in response to cellular activation or apoptosis. The merits of detecting tissue factor-expressing EVs in cancer patients make EVs an important feature in current clinical applications. We assessed 240 cancer patients for endothelial cell-derived EVs (EDEVs) and thrombosis-related biomarkers. Among the 240 patients, 23 had CRT within 6 months after their first examination. Plasma concentrations of EDEVs, and soluble endothelial protein C receptor (sEPCR), high mobility group box protein 1 (HMGB1) and plasminogen activator inhibitor-1 (PAI-1) were higher in cancer patients than healthy controls. Additionally, the elevated EDEVs in CRT patients were significantly higher than those in non-CRT patients. Finally, the levels of EDEVs, and soluble EPCR and HMGB1 were negatively correlated with survival times; in particular, EDEV levels were significantly lower in patients who lived for more than 901 days after their first examination compared with previous data. These results suggested that EDEVs are associated with the hypercoagulable state of cancer patients, and that the elevated risk of thrombosis conferred by hypercoagulability could be predicted by measuring serum EDEVs.

KEYWORDS: EDEV, PAI-1, sEPCR, HMGB1, cancer-related thrombosis.

INTRODUCTION

Many cancer patients are also in a hypercoagulable state, and the elevated risk of thrombosis conferred by hypercoagulation increases patient morbidity and mortality [1-3]. Cancer patients frequently develop venous thromboembolism (VTE), and various potential predictive biomarkers have been evaluated for associations with VTE during cancer progression [4-7]. For example, analyses of blood cells can effectively predict the risk of VTE development [5]. Additionally, measuring D-dimer, prothrombin fragment 1 + 2, and soluble P-selectin levels can accurately predict VTE risk [7]. Furthermore, analysis of high mobility group box protein 1 (HMGB1), plasminogen activator inhibitor-1 (PAI-1) and soluble endothelial protein C receptor (sEPCR) can effectively predict the risk of VTE development [8-10].

Extracellular vesicle (EV) levels are also an accurate marker of VTE risk [11-13]. EVs are small membrane vesicles that are released from many cell types via the exocytic budding of the plasma membrane in response to cellular activation or apoptosis [14-17]. EVs disseminate various

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bioactive effectors from their parental cells. Thus, EVs can alter vascular functions and may induce biological responses involved in vascular homeostasis [18]. Although most EVs in human blood originate from platelets, they are also released from leukocytes, erythrocytes, endothelial cells, smooth muscle cells, and cancer cells [19-24]. EVs have been documented in almost all thrombotic diseases occurring in venous or arterial beds [25-28]. Tissue factor (TF)-EVs are related to cancer and are increased in patients with certain malignancies, such as pancreatic and breast cancer [24].

Herein, we evaluated the utility of endothelial cell-derived EVs (EDEVs) in cancer patients. We identified EDEVs, HMGB1, PAI-1, and sEPCR as useful prognostic indicators for cancer-related thrombosis (CRT).

MATERIALS AND METHODS

Subjects

Cancer patients and healthy volunteers were recruited from Kansai Medical Hirakata Hospital and Kansai Medical University (Osaka, Japan) between September 2012 and July 2016. In total, 240 cancer patients were analyzed, and 23 (9.58%) had thrombotic complications within 6 months after their first examination (Table 1). The types of cancer studied were acute myeloblastic leukemia (AML; n = 18), chronic myeloblastic leukemia (CML; n = 32), malignant lymphoma (ML; n = 41), multiple myeloma (MM; n = 39), and lung cancer (LC; n = 110) (Table 1). This study was conducted in accordance with the Declaration of Helsinki and was performed with approval from the Institutional Review Board of Kansai Medical University. Written informed consent was obtained from all participants.

Measuring HMGB1, sEPCR and PAI-1

Patient blood samples were collected in plain or sodium citrate-containing tubes and left at room temperature for a minimum of 1 h. Serum and citrated plasma were isolated by centrifugation for 20 min at 1000 ×g at 4 °C. Serum was divided into aliquots and frozen at -30 °C until use. Recombinant products and standard solutions provided with commercial kits served as positive controls. Plasma concentrations of PAI-1 were measured using monoclonal antibody-based ELISA kits (Invitrogen, Carlsbad, CA, USA). HMGB1 was measured using the HMGB1 ELISA Kit II (Shino-test Corp., Kanagawa, Japan). Plasma sEPCR levels were measured using ELISA (R&D Systems Inc., Minneapolis, MN, USA). All kits were used according to the manufacturers' instructions. Normal ranges were as follows: HMGB1: 1.2-4.8 ng/mL, sEPCR: 30-150 ng/mL, and PAI-1: 1.1-10.5 ng/mL.

Measuring EDEVs

Blood samples were collected using a 21-gauge needle from a peripheral vein into vacutainers

Table 1. Patient characteristics.

	N	Age (mean)	M/F	Thrombosis (n)*
AML	18	39	12/6	1
CML	32	44	24/8	3
ML	41	46	18/23	2
MM	39	58	22/17	6
LC	110	54	90/20	11

N: patient number; AML: acute myeloblastic leukemia; CML: chronic myeloblastic leukemia; ML: malignant lymphoma; MM: multiple myeloma; LC: lung cancer.
*: thrombotic complication within 6 months after first examination.

containing ethylenediaminetetraacetic acid (NIPRO Co. Ltd., Osaka, Japan) to minimize platelet activation. The samples were handled as described in the manufacturer's protocol. Briefly, the samples were gently mixed by inverting the tube once or twice, stored at room temperature for 2-3 h, and centrifuged at 8000 ×g for 5 min at room temperature. Storing samples at room temperature for 2-3 h did not affect EDEV levels. EVs were analyzed using an FACS Cant II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All flow cytometry data of forward light scatter (FSC), side scatter (SSC) and fluorescence intensity (FL) were analyzed in log space. EDEVs were identified and quantified based on their FSC/SSC characteristics according to their size and reactivity to the endothelial cell-specific monoclonal antibody (CD144). The lower detection limit was placed at a threshold above the electronic background noise of the flow cytometer, and the upper threshold for FSC (1μm) was set with the use of standard beads (Megamix, BioCytex, Tokyo, Japan) (Figure 1A). To identify positive-stained events, thresholds were set based on FITC-CD144 (G1 gate) (Figure 1B). Finally, events in the G1 gate were expanded to FSC/SSC (G2 gate) (Figure 1C). The density of EDEV in the G2 gate was set to less than 10 events/μl by using blood samples from healthy volunteers.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and were analyzed using multiple regression (stepwise method), as appropriate. Between-group comparisons were made using the Newman-Keuls test and Scheffe's test. All statistical analyses were performed using StatFlex v6 software, with P-values < 0.05 being considered statistically significant.

RESULTS

Levels of biomarkers in cancer patients

The levels of EDEVs, sEPCR, HMGB1 and PAI-1 were higher in cancer patients compared with

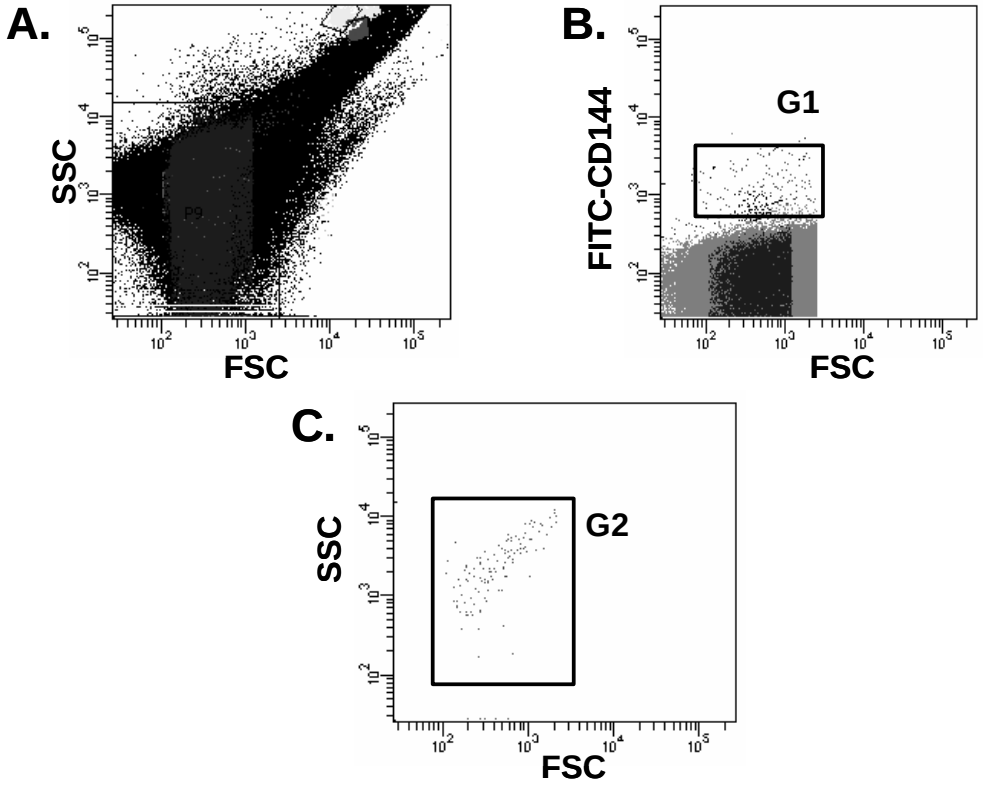

Figure 1. Gating and staining strategy for the detection of EDEVs in FACS analysis. SSC: side scatter; FSC: forward scatter.

controls (Table 2). In particular, EDEVs and sEPCR were very significantly elevated in CML patients compared with controls ($p < 0.001$; Table 2).

Analysis of four VTE biomarkers in relation to thrombotic complications

Next, we compared the concentrations of these four biomarkers (EDEVs, sEPCR, HMGB1 and PAI-1) in cancer patients divided according to whether they had CRT or not (CRT-positive and negative; Figure 2). EDEV and sEPCR levels were higher in CRT-positive patients compared with CRT-negative patients (Figure 2). In particular, EDEV levels in CRT-positive patients were very significantly elevated compared with CRT-negative patients ($p < 0.01$; Figure 2).

Table 2. Various biomarker levels in cancer patients.

	EDEV (ev/μl)	sEPCR (ng/ml)	HMGB1 (ng/ml)	PAI-1 (ng/ml)
Control	3.1 ± 2.5	58 ± 12	3.3 ± 0.9	9.2 ± 2.9
AML	9.2 ± 3.9*	120 ± 41*	9.3 ± 2.2*	13.1 ± 4.2$^{N.S.}$
CML	62.2 ± 10.8***	274 ± 64***	24.9 ± 7.2**	19.1 ± 7.2*
ML	10.3 ± 3.2*	107 ± 32*	6.1 ± 1.9$^{N.S.}$	11.7 ± 4.9$^{N.S.}$
MM	52.9 ± 12.3**	205 ± 47**	12.3 ± 4.1*	22.5 ± 8.9*
LC	39.3 ± 9.5**	236 ± 36**	21.5 ± 8.9**	24.7 ± 7.8*

Data represent the means ± S.D. EDEV: endothelial cell-derived extracellular vesicle; sEPCR: soluble endothelial protein C receptor; HMGB1: high mobility group box 1; PAI-1: plasminogen activator inhibitor. The p values are for control vs. patients. N.S.: not significant. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

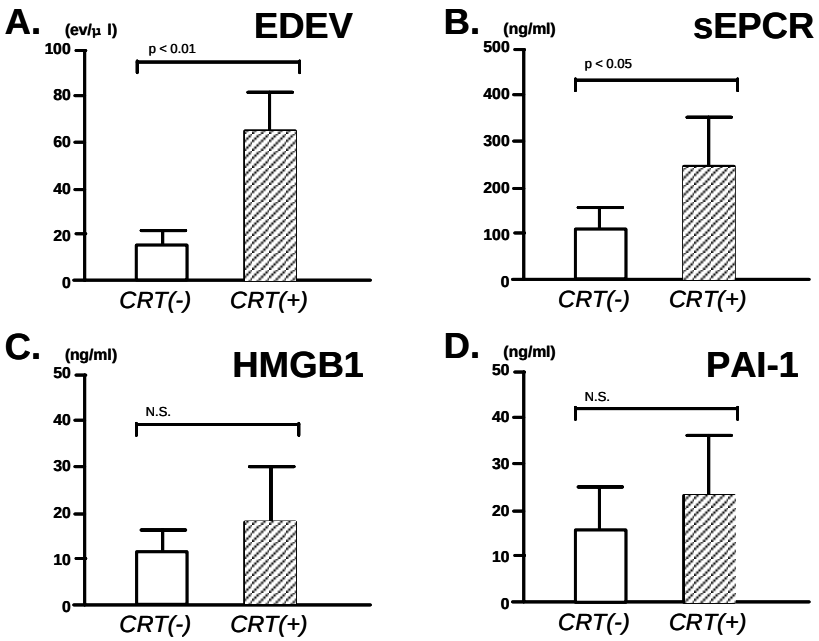

Figure 2. Four biomarkers in CRT-negative and -positive cancer patients. Data represent the means ± S.D. CRT: cancer-related thrombosis; EDEV: endothelial cell-derived extracellular vesicle; sEPCR: soluble endothelial protein C receptor; HMGB1: high mobility group box 1; PAI-1: plasminogen activator inhibitor. The p values are for CRT(-) vs. CRT(+) patients. N.S.: not significant.

Table 3. Survival analysis using biomarkers.

Day	EDEV (ev/μl)	sEPCR (ng/ml)	HMGB1 (ng/ml)	PAI-1 (ng/ml)
0 ~300	54.5 ± 9.8	204 ± 45	22.3 ± 7.2	19.1 ± 5.9
301 ~600	42.2 ± 11.9 N.S.	177 ± 52 N.S.	19.3 ± 6.9 N.S.	18.2 ± 6.2 N.S.
601 ~900	33.4 ± 7.7*	161 ± 39*	16.7 ± 9.3 N.S.	16.2 ± 7.1 N.S.
901 ~	23.2 ± 5.6**	144 ± 55*	15.3 ± 7.6*	15.7 ± 7.4 N.S.

Data represent the means ± S.D. EDEV: endothelial cell-derived extracellular vesicle; sEPCR: soluble endothelial protein C receptor; HMGB1: high mobility group box 1; PAI-1: plasminogen activator inhibitor. The p values are for control vs. patients. N.S.: not significant. *: $p < 0.05$, **: $p < 0.01$.

Survival analysis in relation to the four VTE biomarkers

The concentrations of all four VTE biomarkers were higher in patients who died within 300 days of their first examination (Table 3). EDEV, sEPCR and HMGB1 levels showed a negative correlation with survival time; in particular, EDEV levels were significantly lower in patients who lived for more than 901 days after their first examination than previous data ($p < 0.01$; Table 3).

DISCUSSION

This study assessed the plasma concentrations of several biomarkers of hemostasis, coagulation and endothelial dysfunction in cancer patients. We found that the concentrations of EDEV, sEPCR, HMGB1 and PAI-1 were higher in cancer patients than in healthy controls. These results suggest that cancer patients likely have coagulation- and/or endothelial cell activation-related risk factors for coagulation abnormalities. The clinical significance of HMGB1 and PAI-1 in cancer patients has been previously reported; these markers have been shown to be potential prognostic factors for non-small cell lung cancer (NSCLC) [8, 9, 29, 30]. Although Naumnik *et al*. [8] identified increased HMGB1 levels in advanced NSCLC patients undergoing chemotherapy, they concluded that HMGB1 concentration did not influence survival times following NSCLC treatment because there was no significant difference in HMGB1 levels before and after chemotherapy. In contrast, Wang *et al*. [29] reported that HMGB1 was highly expressed in NSCLC and may be a valuable prognostic predictive marker for this disease. In this study, HMGB1 levels were not different in cancer patients with and without CRT. Conversely, Su *et al*. [9] reported that high PAI-1 expression in NSCLC correlated with poor prognoses. However, they also observed that this effect of PAI-1 was dependent on PAI-2. Therefore, the individual relevance of PAI-1 for NSCLC prognosis remains unclear. In this study, although PAI-1 was at high concentrations in cancer patients, it did not show a relationship with either CRT or survival time.

We found that the combination of CRT and high sEPCR levels in cancer patients was associated with poor prognosis. Activated protein C, combined with its cofactor, protein S, acts as an anticoagulant, inactivating factor Va and factor VIIIa [31]. EPCR, which is a transmembrane glycoprotein found on endothelial cells, enables protein C activation [32]. EPCR is also found in a soluble form, sEPCR, which binds activated protein C, in competition with cell-surface EPCR [33]. Therefore, sEPCR is a biomarker of cancer-related hypercoagulability in human malignancies [10, 34]. In this study, 23 of 240 cancer patients showed CRT, and those with CRT exhibited significantly increased sEPCR levels. Additionally, sEPCR levels were

associated with survival times. However, in this study, the strongest correlation between biomarker levels and survival times was EDEV.

EDEV levels have also been identified as a prognostic biomarker for NSCLC [35-37]. Fleitas *et al*. [35] reported that levels of circulating EDEV and circulating endothelial cells were correlated with prognosis and could be useful prognostic markers for advanced NSCLC patients. Congruently, Wang *et al*. [36] suggested that circulating EDEVs may be a predictive biomarker for 1-year mortality in end-stage NSCLC patients. Furthermore, Tseng *et al*. [37] reported that of all microparticles investigated, only increased EDEV levels were significantly associated with lung cancer. Unfortunately, we could not fully elucidate the relationship between EDEVs and sEPCR in this study. Therefore, it remains unknown whether the high EDEV levels in cancer patients are directly linked with sEPCR levels.

CONCLUSION

Our findings have two potential implications. First, we showed that the combined increase in EDEV and sEPCR levels is related to CRT in cancer patients. Second, we described how endothelial dysfunction may result from increased levels of these biomarkers to contribute to poor cancer patient prognosis. Nevertheless, our study had some limitations. We were unable to determine whether any relationship exists between EDEV and sEPCR. Additionally, we did not investigate how different therapeutic strategies affect the utility of the prognostic markers identified. Further confirmation of our observations in prospective studies is necessary.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests regarding the publication of this study.

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