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ORIGINAL ARTICLE

Relationship between Circulating Tumor Cells, Blood Coagulation, and Urokinase-Plasminogen-Activator System in Early Breast Cancer Patients

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■ **Abstract:** Cancer is a risk factor for venous thromboembolism (VTE) and plasma d-dimer (DD) and tissue factor (TF) are established VTE associated markers. Circulating tumor cells (CTCs) are associated with the risk of VTE in metastatic breast cancer. This study aimed to correlate CTCs, blood coagulation and the urokinase plasminogen activator (uPA) system in primary breast cancer (PBC) patients. This prospective study included 116 PBC patients treated by primary surgery. CTCs were detected by quantitative RT-PCR assay for expression of epithelial (CK19) or epithelial-mesenchymal transition (EMT) genes (TWIST1, SNAIL1, SLUG, ZEB1, FOXC2). Plasma DD, TF, uPA system proteins were detected by enzyme-linked immunosorbent assays, while expressions of uPA system in surgical specimens were evaluated by immunohistochemistry. CTCs were detected in 27.6% patients. Patients with CTCs had a significantly higher mean plasma DD (ng/mL) than those of patients without CTCs (632.4 versus 365.4, p = 0.000004). There was no association between plasma TF and CTCs. Epithelial CTCs exhibit higher expression of uPA system genes compared to EMT_CTCs. Patients with CTCs had higher plasma uPA proteins than those of patients without CTCs; there was no correlation between tissue expression of uPA system, CTCs, DD or TF levels. In multivariate analysis CTCs and patients age were independent factors associated with plasma DD. We found association between plasma DD and CTCs indicating a potential role for activation of the coagulation cascade in the early metastatic process. CTCs could be directly involved in coagulation activation or increased CTCs could be marker of aggressive disease and increased VTE risk. ■

Key Words: breast cancer, circulating tumor cells, venous tromboembolism

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Cancer is a well-recognized risk factor for venous thromboembolism (VTE). It has been shown that 5–10% of all cancer patients will develop VTE during the course of the disease (1). Evidence suggests that the absolute risk of VTE depends on the tumor type, the stage or extent of the disease and treatment with

antineoplastic agents (1). The diagnosis of VTE was associated with a higher risk of death (2).

Circulating tumor cells (CTCs) are thought to have a crucial role in the metastatic cascade, tumor dissemination and progression. Prognostic value of CTCs was demonstrated by a number of trials for metastatic and primary breast cancer (PBC) (3–5). Increased CTC count and VTE are poor prognostic factors in breast cancer and are linked to inferior survival. Previously, we showed that presence of any CTCs in peripheral blood (PB) of metastatic breast cancer (MBC) patients is associated with increased risk of VTE (6). Fibrinogen, vascular endothelial growth factor (VEGF), d-dimer (DD; hemostasis marker that reflects ongoing fibrin formation and degradation) and tissue factor (TF) are another factors increased in breast patients who subsequently develop VTE (7).

The urokinase plasminogen activator (uPA) system has been found to play a key role in promoting metastasis in breast cancer primarily due to degradation of the basement membrane and extracellular matrix that leads to metastasis and is involved in fibrin degradation as well (8). Overexpression of the uPA system is a negative predictor of patients' outcome in several types of tumors including breast cancer (8).

In this study, we hypothesized that there is a correlation between plasma DD and TF with the presence of detectable CTCs. Moreover, we hypothesized, that activation of the uPA system could be involved in the release of CTCs into PB as well as activation of coagulation. Therefore, we examined whether the expression of these factors in breast tumor tissue, plasma and CTCs correlated with plasma DD and TF levels.

PATIENTS AND METHODS

Study patients

This translational study (Protocol TRU-SK 002; Chair: M. Mego) was approved by the Institutional Review Board (IRB) of the National Cancer Institute of Slovakia and was conducted between March and December 2012. The study included 116 patients with stages I–III PBC who were undergoing definitive surgery. From each patient we obtained PB for CTCs detection and plasma analysis, and whenever possible, corresponding paraffin-embedded tumor tissue. Each patient received a complete diagnostic evaluation to exclude the presence of distant metastasis. Patients on therapeutic anticoagulation therapy including warfarin 1 mg/day or equivalent for port-a-catheter thromboprophylaxis were excluded from the study. Age-matched healthy women donors ($N = 60$) without breast cancer were recruited and consented according to the IRB-approved protocol.

Plasma isolation

Venous PB samples were collected in EDTA-treated tubes in the morning on the day of surgery and centrifuged at 1,000 *g* for 10 minutes at room temperature within 2 hours of venipuncture and processed as described previously (9).

Detection of CTCs in PB

Circulating tumor cells were detected in PB depleted of $CD45^+$ leukocytes by quantitative real-time polymerase chain reaction (qRT-PCR) based assay, as described previously (10,11). Briefly, PB samples were depleted of $CD45^+$ leukocytes using the RosetteSep™ kit (StemCell Technologies, Vancouver, BC, Canada) and isolated RNA from CD45-depleted PB samples were reversed transcribed to cDNA and subsequently subjected to qRT-PCR for expression of epithelial (CK19) or epithelial-mesenchymal transition (EMT) genes (TWIST1, SNAIL1, SLUG, ZEB1, FOXC2). Patient samples with higher CK19 gene transcripts than those of healthy donors were scored as epithelial CTCs positive (CTC_EP), while patient samples with higher EMT gene transcripts than those of healthy donors were scored as CTC_EMT positive (10,11).

Measurement of DD, TF, uPA, and PAI-1 in plasma

Plasma TF, DD, uPA, and PAI-1 were analyzed by enzyme-linked immunosorbent assays (ELISA). Plasma TF was analyzed by ELISA assay using the Quantikine Human Coagulation Factor III/Tissue Factor Immunoassay (R&D Systems, Minneapolis, MN). D-dimer in plasma samples was determined using the IMUCLONE® D-Dimer ELISA (American Diagnostica, Greenwich, CT) system. Plasma uPA was measured using the Human u-Plasminogen Activator/Urokinase Quantikine ELISA kit (R&D Systems). Plasma plasminogen activator inhibitor 1 (PAI-1) was measured

using the Human Serpin E1/PAI-1 Quantikine ELISA Kit (R&D Systems).

Immunohistochemistry

Pathology review was conducted at the Department of Pathology, Faculty of Medicine, Comenius University, by two pathologists (ZC and PJ) associated with the study. Tumor samples were processed and tissue microarray was constructed as described previously (11). Primary goat polyclonal antibodies against uPA receptor (uPAR) (Santa Cruz Biotechnology, Dallas, TX (N-19):sc-9793), uPA (Santa Cruz Biotechnology (C-20):sc-6830) and PAI-1 (Santa Cruz Biotechnology (C-20):sc-6642) were used for tumor staining according to the manufacturer's instructionsAs positive control, normal breast tissue included on the tissue microarrays was used. As negative control, breast tissue was used, omitting the primary antibody from the staining protocol. The result of the immunohistochemical analyses was expressed by an ImmunoReactive Score (IRS), so-called German IRS, evaluating both the percentage of positive cells and the staining intensity of the nuclei or cytoplasm (12).

Statistical analysis

Patient characteristics were tabulated. The patients' characteristics were summarized using the median (range) for continuous variables and frequency (percentage) for categorical variables. Normality of distribution was tested by the Kolmogorov–Smirnoff test. If normally distributed, sample means were tested by Student's *t*-test or ANOVA with Bonferroni's or Tamhane's corrections depending on homogeneity of variance. The nonparametric Mann–Whitney *U* or Kruskal–Wallis *H*-test were was used for non-normally distributed data. Pearson's or Spearman's correlations tests were used according to the normality of data. Categorical data were tested by Fisher's exact test or Chi square test. Median follow-up period was calculated as a median observation time among all patients and among those still alive at the time of their last follow-up.

A multivariate hazards model was used to assess association with DD on the basis of CTCs, hormone receptor status, HER-2 status (normal or amplified), age, tumor grade, tumor histology, tumor stage, and nodal stage. Step-wise regression techniques were used to build multivariate models using a significance level of 0.10 to remain in the model. All p-values presented are two-sided, and associations were considered significant if the p-value was less or equal to 0.05. Statistical analyses were performed using NCSS 2007 software (Hintze J, Kaysville, UT, 2007).

RESULTS

The study population consisted of 116 PBC patients with median age of 60 years (range: 35–83 years). Patients' characteristics are shown in Table 1.

CTC detection

Totally, CTCs were detected in 32 (27.6%) of patients. CTCs with only epithelial markers (CTC_EP) were present in PB of 13 (11.2%) patients; CTC with EMT phenotype (CTC_EMT) were present in 15 (12.9%) of patients, while in four (3.4%) of patients CTCs exhibited both epithelial and mesenchymal markers (Table 1). In one patient sample, there was overlap in overexpression of EMT-inducing TF gene transcripts (SLUG and TWIST).

CTC and plasma DD and TF

Patients with any CTCs in PB had significantly higher plasma DD levels than those of patients without CTCs (Table 1). High tumor grade and advanced tumor stage were associated with higher plasma DD levels as well (Table 1). In multivariate analysis, CTCs and patients' age were independent factors associated with plasma DD levels, while CTCs was the strongest predictor of high plasma DD level (Table 2). There was no association between plasma TF levels and CTCs and other patients'/tumor characteristics (Table 1).

CTC and plasma uPA, PAI-1

Based on observed association between CTCs and DD, we hypothesized, that activation of the uPA system could be involved in the release of CTCs into the PB as well as in coagulation activation. Therefore, we determined plasma uPA and PAI-1 proteins levels. Patients with any CTCs had higher mean plasma uPA (ng/mL) (374.5 versus 309.4, $p = 0.04$) and PAI-1 (pg/mL) levels (5.4 versus 4.4, $p = 0.23$), than those of patients without CTCs. However, we do not observe

Table 1. Association between D-Dimer, Tissue Factor and Patients/Tumor Characteristics

		D-dimer (ng/mL)		Tissue factor (pg/mL)	
Variable	*N* (%)	Mean ± SEM	p-value	Mean ± SEM	p-value
All	116 (100)	411.1 ± 36.1	NA	64.7 ± 1.55	NA
T-stage					
1	72 (62.1)	371.2 ± 45.6	0.07	61.6 ± 1.92	0.07
>1	44 (37.9)	476.4 ± 58.3		69.6 ± 2.46	
N-stage					
0	71 (61.2)	392.9 ± 46.2	0.31	63.6 ± 1.98	0.55
≥1	45 (38.8)	439.8 ± 58.0		66.4 ± 2.49	
Grade					
1 and 2	66 (56.9)	326.4 ± 46.7	0.02	64.6 ± 2.06	0.28
3	48 (41.4)	532.9 ± 54.7		64.7 ± 2.42	
Unknown	2 (1.7)				
Histology					
Invasive ductal carcinoma	96 (82.8)	413.6 ± 39.8	0.97	64.8 ± 1.71	0.51
Other	20 (17,2)	399.3 ± 87.2		63.8 ± 3.74	
Hormone receptor status					
Positive for either	99 (85.3)	393.2 ± 39.0	0.93	64.6 ± 1.68	0.89
Negative for both	17 (14.7)	515.3 ± 94.0		65.1 ± 4.06	
HER2 status					
Amplified	19 (16.4)	398.1 ± 89.5	0.61	61.4 ± 3.83	0.15
Normal	97 (83.6)	413.7 ± 39.6		65.3 ± 1.69	
Ki 67 (cutoff 14%)					
Low	61 (52.6)	371.4 ± 49.7	0.54	64.9 ± 2.14	0.63
High	55 (47.4)	455.1 ± 52.3		64.4 ± 2.26	
CTC_EP					
Present	17 (14.7)	677.5 ± 90.7	0.009	60.6 ± 4.04	0.55
Absent	99 (85.3)	365.4 ± 37.6		65.4 ± 1.67	
CTC EMT					
Present	19 (16.4)	561.3 ± 39.0	0.0003	64.4 ± 3.84	0.40
Absent	97 (83.6)	381.7 ± 88.2		64.7 ± 1.70	
CTC any					
Present	32 (27.6)	642.6 ± 64.1	0.000004	62.9 ± 2.95	0.86
Absent	84 (72.4)	322.9 ± 39.5		65.4 ± 1.82	

Table 2. Multivariate Analysis of Factors Associated with Plasma D-Dimer

Independent variable	Regression coefficient b(i)	SE	Lower 95% CI	Upper 95% CI	T-value to test H0:B(i)=0	p-value
Age (continuous)	12.6	2.8	7.0	18.2	4.46	<0.00001
CTC (present versus absent)	326.0	70.7	185.8	466.1	4.61	<0.00001
Grading (3 versus 1 and 2)	121.6	64.2	−5.5	248.8	1.896	0.06

any correlation between DD, TF and plasma uPA and PAI-1 protein levels respectively.

CTC and tumor tissue expression of uPA, uPAR, PAI-1

Next, we analyzed tissue expression of uPA system that could be involved in CTCs release and coagulation activation. We analyzed tissue expression of uPA, uPAR, and PAI-1 by immunohistochemistry. Paraffin embedded tumor tissue was available for 102 (87.9%) patients. There was neither association between any subtype of CTCs and expression of analyzed proteins nor association between plasma DD and these proteins.

Expression of uPA, uPAR, and PAI-1 on CTCs

To test the hypothesis that expression of the uPA system on CTCs could be responsible for coagulation activation, we analyzed expression of uPA, uPAR, and PAI-1 genes in PB enriched for CTCs (CD45-depleted fraction). We analyzed 30 samples (15 CTCs positive and 15 CTCs negative). CTC_EP samples had a significantly higher expression of uPA, uPAR, and PAI-1, compared with those of CTC_EMT samples or CTC negative samples (Fig. 1). However, there was no correlation between uPA, uPAR, and PAI-1 genes expression on CTCs and plasma DD or TF (data not shown).

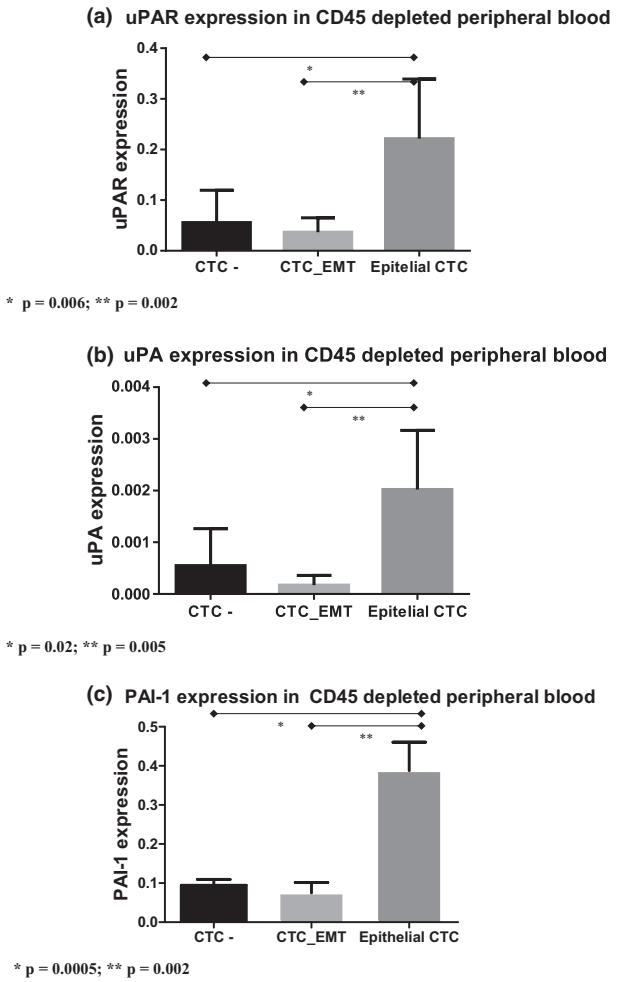

Figure 1. (a) uPAR expression in CD45 depleted PB. (b) uPA expression in CD45 depleted PB. (c) PAI-1 expression in CD45 depleted PB.

CTC and VTE

In median follow-up of 13.7 months (range: 8.7–18.0 months), one patient (0.9%) experienced thrombosis of the left popliteal vein 6 months after surgery for PBC, when she was receiving adjuvant hormonal therapy with tamoxifen. This patient was positive for CTC_EMT at the time of surgery.

DISCUSSION

In this prospective translational study, we showed that the presence of CTCs in PB of PBC patients is associated with a higher level of plasma DD, but not with plasma TF. Moreover, this observation is consistent for both CTC_EP and EMT_CTCs. Whereas DD is a hemostasis marker that reflects ongoing fibrin formation and degradation, uPA system is involved in fibrin degradation. Therefore, we tested the hypothesis that uPA system could be responsible for CTCs generation and DD production. We observed an association between plasma uPA and CTCs, while there was no correlation between tissue expression of uPA, uPAR, PAI-1 and presence of CTCs. We also evaluated expression of uPA genes on CTCs and our data suggest that uPA is overexpressed on CTC_EP, but not on CTC_EMT. We did not find any correlation between plasma DD and uPA system on CTCs as well as there was no correlation between plasma DD and uPA system in plasma or tumor tissue.

Previously, we showed that CTCs are associated with increased risk of VTE in patients with MBC. The risk is increased in MBC patients with any CTC before starting new line of therapy (6). In that retrospective study, CTCs were detected by the CellSearch System (3) that identifies CTC_EP and EMT_CTCs (6), as was done in the current study. Nevertheless, our current data are consistent with our previous results despite the different methods employed for CTCs detection and invariably link CTCs and coagulation activation in breast cancer patients. In this study, we observed only one patient with VTE; nonetheless, that occurred in a patient that was positive for CTCs. More events are needed to establish clinical relevance of our observation in PBC patients.

The expression of several components of the uPA system in tumor tissues has been shown to have prognostic significance in many human cancers, including those of the breast, prostate, lung, brain, ovary, stomach, colon, rectum, oral cavity, kidney, and bone (13). Expression of uPAR was showed in disseminated tumor cells in bone marrow of patients with several types of cancers including breast cancer and this expression correlated with aggressive biological feature and poor prognosis (14,15). In our study, we showed for the first time, that the uPA system is overexpressed on CTC_EP, but not on EMT_CTCs. Moreover, we showed that cancer tissue expression of uPA system is not associated with presence of CTCs in PB. We suppose that CTCs could be directly involved in coagulation activation in breast cancer patients, or increased CTCs count could be a marker of more aggressive disease and increased risk of VTE. Based on our data, we suggest, that other signaling pathways than uPA system could be related both to increased CTCs in PB and coagulation activation as well.

In conclusion, in this prospective study we showed for the first time a positive association between plasma DD levels and CTCs indicating a potential role for activation of the coagulation cascade in the early metastatic process. Together with our previous observation of correlation between CTCs and VTE in MBC, these data invariably link CTCs and coagulation activation. Moreover, we for the first time demonstrated a differential expression of the uPA system in CTC_EP (epithelial) and CTC_EMT (mesenchymal) CTCs respectively. These data further stress the potential contributions of the CTC subpopulations on the heterogeneity of microscopic disease. Moreover, our data suggest that activity of uPA system on CTCs, plasma or in tumor tissue is not responsible for observed higher DD in patients with detected CTCs. Future studies will need to address the prognostic implications of these observations with the potential for therapeutic interventions of anticoagulation therapy for the metastatic process.

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CONFLICTS OF INTEREST

None.

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