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Increased expression of tissue factor and protease-activated receptor-1 does not correlate with thrombosis in human lung adenocarcinoma

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Abstract

A correlation between cancer and hypercoagulability has been described for more than a century. Patients with cancer are at increased risk for thrombotic complications and the clotting initiator protein, tissue factor (TF), is possibly involved in this process. Moreover, TF may promote angiogenesis and tumor growth. In addition to TF, thrombin seems to play a relevant role in tumor biology, mainly through activation of protease-activated receptor-1 (PAR-1). In the present study, we prospectively studied 39 lung adenocarcinoma patients in relation to the tumor expression levels of TF and PAR-1 and their correlation with thrombosis outcome and survival. Immunohistochemical analysis showed TF positivity in 22 patients (56%), most of them in advanced stages (III and IV). Expression of PAR-1 was found in 15 patients (39%), most of them also in advanced stages (III and IV). Remarkably, no correlation was observed between the expression of TF or PAR-1 and risk for thrombosis development. On the other hand, patients who were positive for TF or PAR-1 tended to have decreased long-term survival. We conclude that immunolocalization of either TF or PAR-1 in lung adenocarcinoma may predict a poor prognosis although lacking correlation with thrombosis outcome.

Key words: Lung adenocarcinoma; Thrombosis; Tissue factor; Protease-activated receptor

Introduction

Lung cancer is the most frequent and fatal cancer worldwide, being responsible for up to 2 million deaths each year. In the United States, 215,000 new cases with 161,840 deaths were estimated in 2008, while in Brazil 27,000 new cases were estimated for the same year, with only 10% of these patients expected to survive up to 5 years. The smoking habit is considered to be the main cause of lung cancer, and adenocarcinoma is the most frequent histological type (1-3).

Since the work of Trousseau in the 1860's, there has been increasing evidence of the interference of clotting factors with cancer progression (4,5). In this context, tissue factor (TF) is a 263-amino acid transmembrane glycoprotein that plays a central role in the interface between blood coagulation and tumor biology (6). It is now established that TF expression correlates with the histological grade of malignancy of several types of cancer, being particularly associated with invasive potential and angiogenesis (7-11). There is evidence that TF expression is related to increased blood thrombogenicity in lung cancer (12).

In addition to TF, thrombin seems to play a significant role in cancer progression (13). In fact, the action of thrombin in tumor biology results in part from the proteolytic cleavage of protease-activated receptor-1 (PAR-1) in tumor cells, which elicits several pro-tumoral responses including angiogenesis, cell proliferation and invasion (14). Some studies

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have demonstrated an increased expression of PAR-1 in patients' samples including breast, colon, melanoma, and esophageal cancer (15-18).

In the present study, we attempted to evaluate the expression of TF and PAR-1 in human lung adenocarcinoma samples. Moreover, we followed survival and thrombosis outcome in the same set of patients. Our data showed no correlation between the expression of TF or PAR-1 and risk for thrombosis development. On the other hand, patients who were positive for either TF or PAR-1 tended to have decreased long-term survival.

Material and Methods

We studied paraffin blocks (tumor biopsy or tumor tissue extracted for treatment) obtained from 39 lung adenocarcinoma patients prospectively studied in our service. The criterion of patient selection for immunohistochemistry was paraffin block availability. All patients studied, even non-symptomatic ones, were followed for 2 years or until death, with venous Doppler scan of arms and limbs every 3 months. The study was approved by an internal Instituto Nacional do Câncer Ethics in Research Committee and patients signed an informed free consent term before entering the study.

Immunohistochemistry

All samples were fixed in 10% formalin and embedded in paraffin. Serial tissue sections were cut, mounted on glass slides and dried at 56°C before dewaxing in xylene and rehydration in alcohol according to standard histological procedures. Monoclonal antibodies against TF (#4509; 1:400) were from American Diagnostica, Inc. (USA) and antibodies against PAR-1 were from Santa Cruz Biotechnology (USA) (#sc-13503; 1:10). All sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6.0, for 3 min, followed by inhibition of endogenous peroxidase (peroxidase block, RE7101, Novocastra, UK). Incubation of primary antibodies (anti-TF or anti-PAR-1) was performed overnight at room temperature in TBS containing 2 mM sodium azide and 1 mM BSA. Slides were further submitted to post-primary blockade (RE7111, Novocastra), and then incubated at room temperature with NovoLink™ polymer (RE7112, Novocastra) for 30 min. Slides were further developed with diaminobenzidine chromogen (RE7105, Novocastra) at 1/20 dilution, and finally stained with Mayer hematoxylin, dehydrated and mounted with Canadian balsam.

TF and PAR-1 expression was evaluated semi-quantitatively as percent positive cells after counting 300 cells, being scored as negative (up to 5% of positive cells), or positive (more than 5% cells staining positively). Negative controls were obtained by omitting the primary antibody. Pancreas adenocarcinoma samples were used as positive controls (data not shown).

Table 1. Characteristics of the patients studied.

Characteristics	
Median age in years (range)	58 (45-80)
Gender	
Male	63%
Female	37%
Disease stage	
I and II	23%
III and IV	77%
Treatment	
Chemotherapy	55%
Radiotherarapy	53%
Surgery	12%
Palliative	8%
Previous tobacco use	
Yes	88%
No	12%

Determination of thrombogenic factors in plasma

A blood sample was obtained and analyzed at inclusion in the study and every 6 months up to 2 years of follow-up or death. This evaluation involved the determination of procoagulant factors (fibrinogen and factor VIII) known to induce thrombosis when increased, and of D-dimer for evaluation of fibrinolysis. Fibrinogen levels above 500 ng/dL and 200% factor VIII concentration were considered to be thrombogenic. D-dimer up to 250 ng/mL was considered to be normal. Lupus anticoagulant activity was measured by coagulometry using diluted Russel viper venom. All tests were performed using an automated coagulometer device (Sysmex CA1500, DADE Behring, Newark, USA) according to manufacturer instructions.

Statistical analysis

Data are reported as descriptive statistics (median, minimum and maximum limits and percent). For data analysis, we used the chi-square test with Fisher adjustment when necessary, and $P < 0.05$ was considered to be significant.

Results

Thirty-nine patients diagnosed with lung adenocarcinoma were prospectively evaluated in this study. Patients' characteristics are described in Table 1.

Immunohistochemical analysis of tumor samples showed positive TF staining in 22 patients (56%), as illustrated in Figure 1. Remarkably, TF expression was correlated with disease stage, being more frequent in patients with advanced disease (Table 2). Surprisingly, the frequency

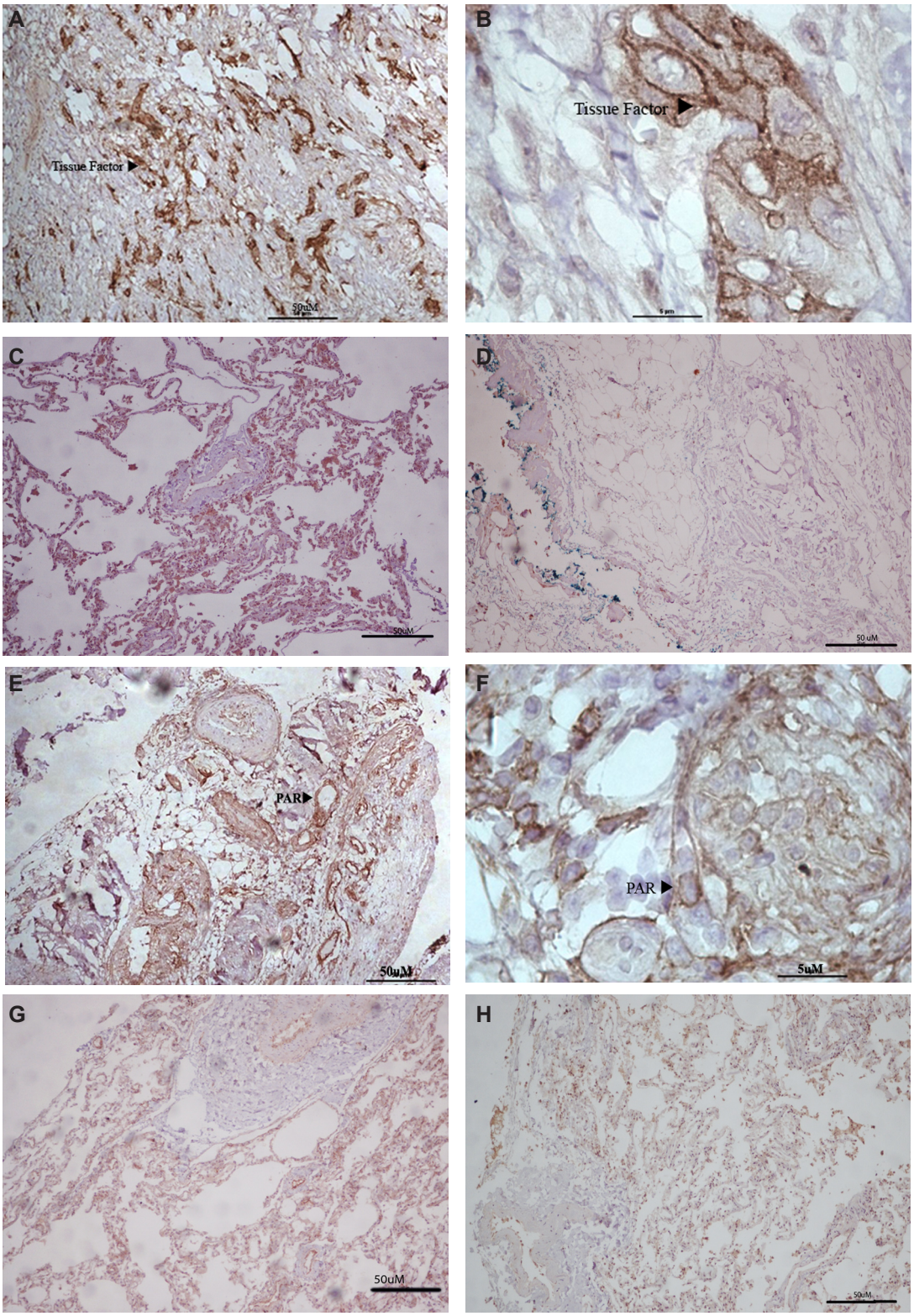

Figure 1. Immunohistochemistry for tissue factor (arrowhead) in lung adenocarcinoma (A, 100X and B, 1000X), normal lung (C), and negative control (D). Immunohistochemistry for protease-activated receptor-1 (PAR-1; arrowhead) stain in lung adenocarcinoma (E, 100X and F, 1000X), normal lung (G), and negative control (H).

of thrombosis showed no correlation with TF expression, as demonstrated in Table 2, with 2 of 5 patients who developed thrombosis demonstrating TF positivity in tumor samples. On the other hand, survival curves showed a trend to decreased survival for TF expression, as demonstrated in Figure 2A.

In addition to TF, immunohistochemistry revealed positivity for PAR-1 expression in a number of patient samples, as illustrated in Figure 1. Table 2 shows that PAR-1 staining was detected in 15 patients (39%), with the expression pattern being also correlated with advanced disease stages. Interestingly, 11 patients were positive for both TF and PAR-1, with this co-expression being more prevalent in the advanced stages of lung adenocarcinoma. Expression of PAR-1 showed no correlation with thrombosis risk (Table 2).

Table 2. Immunohistochemical status of TF and PAR-1 in lung adenocarcinoma.

	TF status	
Disease stage	Negative	Positive
I and II	6 (15.4%)	2 (5.1%)
III and IV	11 (28.2%)	20 (51.3%)
Thrombosis outcome	3/17	2/22[a]
	PAR-1 status	
Disease stage	Negative	Positive
I and II	3 (7.7%)	5 (12.8%)
III and IV	21 (53.8%)	10 (25.6%)
Thrombosis outcome	3/24	2/15[b]

TF = tissue factor; PAR-1 = protease-activated receptor-1. [a]P = 0.52 (chi-square test) and P = 0.63 (Fisher adjusted chi-square test) for TF status compared to disease staging and thrombosis outcome. [b]P = 0.80 (chi-square test) and P = 0.58 (Fisher adjusted chi-square test) for PAR-1 status compared to disease staging and thrombosis outcome.

Furthermore, as seen with TF, positivity for PAR-1 expression was not statistically significant when we attempted to correlate it with overall survival but clearly demonstrated a trend to decreased long-term survival (Figure 2B). In addition, analysis of thrombogenic risk factors showed no correlation with TF or PAR-1 expression (Table 3).

Discussion

A correlation between cancer and blood coagulation activation has been well established, with cancer patients being at increased risk for thrombosis development. In fact, White et al. (19) found that, among the main types of solid cancers, lung cancer showed a high risk for the development of venous thromboembolism. In the present study, we observed a high incidence of thrombosis in a group of lung adenocarcinoma patients. However, it is important to note that the present patients were screened for thrombosis every 3 months even if asymptomatic. This procedure was necessary because it is believed that cancer-related thrombosis is largely undiagnosed.

Several studies have demonstrated that the procoagulant properties of tumor cells mainly derive from the expression of TF, the clotting initiator protein (20,21). Sawada et al. (9) have previously demonstrated increased TF expression in malignant cells from patients with lung cancer. Remarkably, in their study, TF expression levels correlated with the presence of metastatic tumors. Goldin-Lang et al. (12) also demonstrated increased TF expression in non-small cell lung cancer, although no direct correlation between disease stage and TF expression levels was detected. In the present study, we observed an increased TF expression in tumor cells from 22 of 39 lung adenocarcinoma patients, in particular at advanced stages. However, to our surprise, we found no significant correlation between TF expression and the occurrence of thrombosis. There are some possible explanations for this observation. TF expression

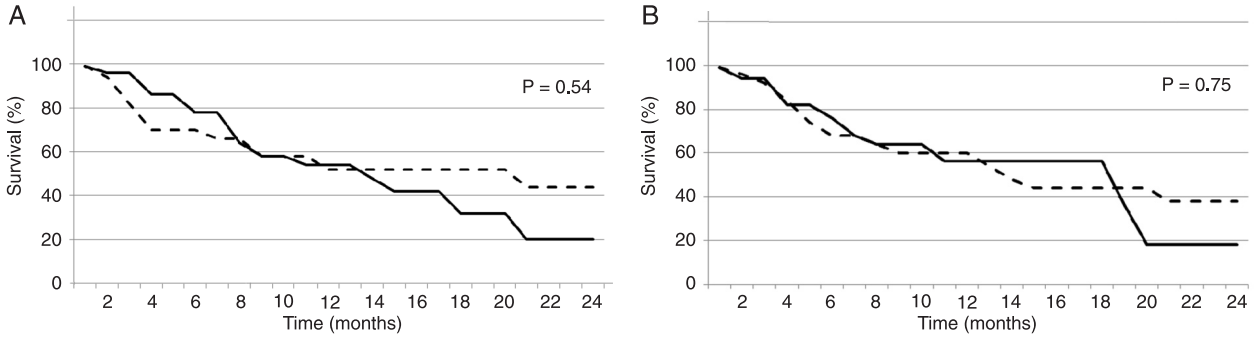

Figure 2. Kaplan-Meier survival curves for lung adenocarcinoma patients regarding TF or PAR-1 expression patterns. *A*, Survival of patients who stained positive (solid lines; N = 22) compared to negative (dashed lines; N = 17) for TF, as analyzed by immunohistochemistry. *B*, Survival of patients who stained positive (solid lines; N = 16) compared to negative (dashed lines; N = 23) for PAR-1, as analyzed by immunohistochemistry. TF = tissue factor; PAR-1 = protease-activated receptor-1.

in lung adenocarcinoma may have no direct correlation with plasma TF levels. Another possibility is that additional thrombogenic factors may have a strong influence on lung adenocarcinoma-related thrombosis. In this regard, we recently observed that positivity for antiphospholipid antibodies is a strong risk factor for thrombosis outcome in lung adenocarcinoma (22). Finally, since post-mortem analyses have not been routinely performed in our Institution, occult pulmonary embolism cannot be excluded in patients with increased TF expression.

Table 3. Thrombogenic risk factors do not correlate with TF or PAR-1 expression.

	TF positivity (relative risk)	PAR-1 positivity (relative risk)
Lupus anticoagulant		
Positive	12	9
Negative	10	7
	RR: 1.36 (0.65-2.82)	RR: 1.31 (0.68-2.52)
	P = 0.39	P = 0.42
Factor VIII		
Increased	8	5
Normal	14	11
	RR: 0.90 (0.39-2.08)	RR: 0.82 (0.33-2.03)
	P = 0.82	P = 0.66
Fibrinogen		
Increased	7	4
Normal	14	11
	RR: 1.55 (0.48-5.01)	RR: 0.88 (0.30-2.60)
	P (Fisher): 0.35	P (Fisher): 0.56
D-dimer		
Increased	13	7
Normal	9	7
	RR: 0.85 (0.52-1.41)	RR: 0.75 (0.41-1.37)
	P (Fisher): 0.41	P = 0.33

TF = tissue factor; PAR-1 = protease-activated receptor-1. The chi-square test and the Fisher chi-square adjusted test were used for statistical evaluation.

In addition to TF, thrombin seems to contribute to tumor progression, mainly through cleavage and activation of PAR-1, a G-protein-coupled receptor (13). Overexpression of PAR-1 has been described in several types of tumor including breast, colon, melanoma, and esophageal cancer (15-18). Moreover, Depasquale and Thompson (23) recently reported PAR-1 expression as a negative prognostic factor in melanomas, which strongly correlated with tumor stage (23). In the present study, we observed increased PAR-1 expression in 15 of 39 lung adenocarcinoma patients. No correlation between PAR-1 expression and thrombosis outcome was detected. However, survival curves showed a trend to decreased long-term survival in PAR-1-expressing patients. It is important to note that 11 patients co-expressed TF and PAR-1, indicating the need for a larger cohort to determine whether these proteins may serve as possible prognostic factors in lung adenocarcinoma.

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