Tissue Factor Expression in Non-small Cell Lung Cancer: Relationship with Vascular Endothelial Growth Factor Expression, Microvascular Density, and K-*ras* Mutation

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Introduction: Tissue factor (TF) is the physiological trigger of blood coagulation, but it could also have an important role in cancer by regulating VEGF expression and angiogenesis.

Methods: TF expression was studied by real-time PCR in lung tumors of 64 patients with non-small-cell lung cancer (NSCLC) and by immunohistochemical analysis. The gene expression of two VEGF isoforms, $VEGF_{165}$ and $VEGF_{189}$, was also evaluated. Microvascular density (MVD) was studied by measuring Von Willebrand Factor (VWF) mRNA levels and by immunohistochemistry using an anti-CD34 antibody.

Results: TF mRNA levels were significantly lower than in corresponding non-affected lung tissues. However, TF expression was higher in T3-T4 tumors and this result was confirmed by immunohistochemistry. $VEGF_{189}$ mRNA levels were ten times higher than those of $VEGF_{165}$ and well correlated with TF mRNA levels. MVD was lower in the inner part of tumors than in the adjacent non-affected lung without being related to TF expression. Finally, codon 12 K-*ras* mutation was found in 8 lung carcinomas, and higher TF and $VEGF_{189}$ mRNA levels were measured in mutated tissues ($p < 0.001$).

Conclusion: These results suggest that high TF expression in lung tumors may result from K-*ras* mutation and contribute to NSCLC progression, probably via mechanisms other than angiogenesis.

Key Words: Tissue factor, VEGF, Non-small cell lung cancer, Microvascular density, K-*ras* mutation.

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Tissue factor (TF), the physiological trigger of blood coagulation, is a 47 KDa glycoprotein whose primary sequence is similar to that of the type II cytokine receptor.[1] Several normal cells such as fibroblasts in the adventitia of blood vessels constitutively express TF, but cytokines, growth factors, and endotoxins can also promote TF gene expression in vascular endothelial cells and circulating monocytes.[2] TF has also been recognized as having a key role in certain cancers, particularly by regulating vascular endothelial growth factor (VEGF) expression and tumor angiogenesis.[3,4] It is now well established that certain tumor cells such as breast,[5] glioma, and pancreatic cancer cells[6] express TF at levels which correlate both with their invasive potential and the stage of the cancer.[7,8]

TF expression by tumor cells can enhance angiogenesis by several mechanisms. On one hand, TF has been shown to directly regulate VEGF expression in fibroblasts,[4] in sarcoma cells,[9] and in melanoma cells,[3] and this effect depends on the intracellular domain of the protein.[3] On the other hand, TF/FVIIa complex triggers local thrombin generation in vivo and thus may also indirectly induce VEGF signaling through the protease-activated receptor (PAR)-1 pathway.[10,11] In addition, the TF/FVIIa complex can also activate PAR-2 and thereby regulate proangiogenic growth factor expression.[12]

VEGF is a major angiogenic factor that stimulates the proliferation and migration of endothelial cells. Four isoforms with 121, 165, 189, and 206 amino acids, respectively, can be synthesized by alternative splicing of VEGF mRNA. The larger isoforms, $VEGF_{189}$ and $VEGF_{206}$, are cell-associated and bind to glycosaminoglycans, whereas the smaller isoforms ($VEGF_{121}$ and $VEGF_{165}$) are secreted in the extracellular matrix.[13] On the other hand, $VEGF_{165}$ can increase TF expression in endothelial cells.[14] Moreover, strong VEGF expression has been reported to be associated with poor prognosis in breast carcinoma,[15] prostate carcinoma,[16] and melanoma.[17,18]

Lung cancer is currently the most frequent cause of cancer-related deaths in the western world, and the major histologic types of lung tumors (that affect about 80% patients) are non-small cell lung cancer (NSCLC). In addition, lung cancer is associated with an increased risk of venous thromboembolism,[19] and by triggering blood coagulation TF could enhance the development of this complication.

The aim of this study was therefore to investigate whether TF expression is increased in NSCLC and whether it correlates with tumor VEGF levels and microvascular density

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(MVD). Finally, we also looked for codon 12 K-*ras* mutation, which has been shown to be a prognostic marker in adenocarcinoma of the lung[20] and was also recently identified as regulating TF gene expression in colorectal cancer cells.[21]

PATIENTS, MATERIALS AND METHODS

Patients and Samples

Tissue samples were analyzed from a prospective series of 64 patients (54 men and 10 women) with NSCLC who had undergone complete surgical resection of lung tumors as initial treatment (i.e., without prior radiotherapy or chemotherapy) between January 2002 and February 2005. Shortly after surgery, tissue samples were selected by a pathologist from the inner part of tumor and from parts of the nonaffected lung in which no cancer cells could be detected. One-third of samples was systematically frozen in nitrogen and used for DNA analysis. Another third was fixed in formalin and paraffin embedded for conventional histopathological analysis. The last third was conserved at +4°C in a "RNA later" solution (Ambion, Austin, TX) inhibiting cellular RNases until further Real time-polymerase chain reaction (RT-PCR) analysis. In addition, three other cores biopsies were sampled from the inner part of tumor for tissue microarray (TMA) analysis. Histologic diagnosis and grade of differentiation were assigned according to the World Health Organization criteria for lung tumors,[22] and pathologic staging was based on the revised tumor, node, metastasis Classification of Malignant tumor.[23]

Cell Lines

One normal epithelial lung cell line (NL20) and 11 human lung cancer cell lines were also studied: two were derived from adenocarcinoma (H23, H522), two from squamous cell carcinoma (Calu 1, H520), one from large cell carcinoma (H460), two from bronchiolo-alveolar carcinoma (A549, H358) and four from small cell lung cancer (nonadherent H69, H209 or adherent DMS53, H69AR). All these cell lines were obtained from American Type Culture Collection (Manassas, USA) and cultured in appropriate medium supplemented with endotoxin-free fetal calf serum. In addition, human umbilical vein endothelial cells (HUVEC) were freshly collected and cultured as previously described.[24]

RNA Extraction and cDNA Synthesis

Total RNA was extracted from all tissue samples using the Perfect RNA Mini Kit (Eppendorf, Hambourg, Germany) according to the manufacturer's instructions and as previously described.[25] For cell lines, total RNA was extracted from 10^6 cells with the TRIzol reagent (Sigma, Saint Quentin Fallavier, France). Total RNA yield and purity were determined by spectrophotometry and only samples with an A_{260}/A_{280} ratio above 1.6 were kept for further experiments. Reverse transcription was performed using 2 μg of total RNA in a final volume of 40 μL comprising 62.5 μM of dNTP, 1.25 μM of random decamers (Ambion), AMV-RT buffer 1X concentrate, 18U of RNase inhibitor and 15U of AMV-RT (Roche Diagnostics, Meylan, France) incubated for 1 hour at 42°C.

Real Time PCR Assays for Tissue Factor, $VEGF_{165}$, $VEGF_{189}$, and Von Willebrand Factor mRNA

RT-PCR assays were developed to evaluate TF, $VEGF_{165}$, and $VEGF_{189}$ mRNA levels in cell lines and tumors. Von Willebrand Factor (VWF) gene expression was also studied to evaluate angiogenesis because VWF is a specific marker for endothelial cells.

The primers used for RT-PCR assays (TF, $VEGF_{165}$, and $VEGF_{189}$) were chosen with the assistance of the primer 3 computer program (Table 1). Previously designed primers were used to study VWF and 18S ribosomal RNA,[26,27] and 18S RNA was therefore used as an internal control to evaluate the amount and quality of cDNA in every sample.

All PCR reactions were performed using an iCycler iQ detection system (Bio-rad, Ivry sur Seine, France) in a total reaction volume of 25 μL containing 2 μL of cDNA obtained from 50 ng of total RNA, 1X Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Cergy Pontoise, France), 0.2X Sybr Green solution (Roche) and 0.375, 0.300, 0.25, 0.15 μM of each primer (Proligo, Paris, France) specific for FT, $VEGF_{165}$, $VEGF_{189}$, and VWF, respectively. The optimal PCR conditions for TF, $VEGF_{165}$, $VEGF_{189}$, and VWF were 65°C for 35 cycles, 63°C for 35 cycles, 60°C for 30 cycles, and 64°C for 30 cycles, respectively. The melting curve was analyzed for each sample to check PCR specificity. The target gene expression was quantified in every sample by measuring the threshold cycle (Ct) value of each reaction and using a calibration curve specific for every gene studied.

To express our results, standard curves for TF, $VEGF_{165}$, $VEGF_{189}$, and 18S RNA were established using purified PCR products specific for each gene studied. Decreasing amounts of these DNA fragments (from 2×10^7–2×10^2 copies) were used as templates and the results obtained were reproducibly linear. Good correlation coefficients between the Ct value and the log number of DNA copies ($r^2 \geq 0.99$) were obtained for the four genes studied, and the efficiency of reactions was between 94 and 99%, with

TABLE 1. Oligonucleotide Primers Used in Real-Time PCR

	PCR Product (bp)	Forward Primer 5′→3′	Reverse Primer 5′→3′
TF	114	CCAAACCCGTCAATCAAGT	TGCTTCACATCCTTCACAATCTCG
$VEGF_{165}$	99	AGGCCAGCACATAGGAGAGA	CAAGGCCCACAGGGATTTT
$VEGF_{189}$	106	TGTGAATGCAGACCAAAGAAAGA	CGTTTTTGCCCCTTTCCC
VWF	84	AGAAACGCTCCTTCTCGATTATTG	TGTCAAAAAATTCCCCAAGATACAC
RNA 18S	120	CGCGGTTCTATTTTGTTGTTTT	TTCGCTCTGGTCCGTCTTG

very low intra and interassay variations (<2% and <1%, respectively). Each sample from both the nonaffected lung and tumor was studied in duplicate. All results were expressed as the number of mRNA copies of each gene for 10^8 copies of 18S RNA.

VWF gene expression was measured to evaluate vascular density in tumors, and standard curves were established using serial dilutions of total RNA extracted from 10^6 HUVEC. Results were expressed as "Endothelial Cell Equivalent" per 10^7 copies of 18S RNA.

Immunoassay for TF

Thirty tumor samples were ground in liquid nitrogen, homogenized in 1 mL of TNC buffer (50 mM Tris-Hcl, 0.15 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN3, 0.05% Brij35), and then centrifuged at 10,000*g* for 15 minutes. The protein concentration of each supernatant was measured by Lowry's method (Total protein Kit, Sigma, Saint Quentin Fallavier, France). Each sample was then diluted to obtain a protein concentration of 1 mg/mL. TF protein level was quantified by Asserachrom TF immunosorbent assay kit (Diagnostica stago, Asnières sur Seine, France), with a range of detection of 0 to 2000 pg/ml.

Immunohistochemical Analysis for TF and VEGF Protein Expression and for Tumor Microvessel Density

Immunohistochemical analysis was performed on formalin-fixed and paraffin-embedded samples from the 64 patients. In 22 cases, both affected and nonaffected lung tissues (4 μm-thick sections) were studied.

In addition, all cancerous samples ($n = 64$) were also analyzed using a TMA technique and results were compared with those obtained by molecular analyses. Core biopsies of 1 mm in diameter were taken from each lung cancer tissue block away from necrotic zones and arrayed in a recipient paraffin block using a tissue microarrayer (Beecher Instrument, Sun Prairie, Wisconsin), as described.[28] Three cores were displayed in the array for each tumor, resulting in histologic spots on corresponding slides. Several tissue samples (from normal lung, normal breast, prostate cancer, and colon cancer) were tested as controls and presented in the array as five cores. Slides were incubated for 1 hour with a murine IgG_1 monoclonal anti-TF antibody (specific for amino acids 1–25 within the extracellular domain; American Diagnostica, Stanford) or with a murine monoclonal anti-VEGF IgG2 antibody specific for the 1 to 140 amino-acid sequence of human VEGF present in both $VEGF_{165}$ and $VEGF_{189}$ isoforms (C-1:sc7269, Santa Cruz Biotechnology, Santa Cruz). Both antibodies were diluted at 1:100 in antibody diluent (Dako, Glostrup, Denmark). Biotinylated goat antimouse IgG were then used as secondary antibodies, and biotin-streptavidin-peroxidase with diaminobenzidine as chromogen (ChemMate Detection kit, Dako) was employed for detection. The same reagents and procedures were used for the conventional immunohistochemical analyses.

Two independent observers (SR and CB) evaluated the topography and intensity of the staining, blinded to clinical and pathologic information.

TF and VEGF staining was graded on a scale from 0 to 3 depending on the intensity of staining and the percentage of positive tumor cells. Grade 0 represented tumors with malignant cells not or weakly stained. When the intensity was significant (moderate or strong), lung tumors were defined as either grade 1 (1–33% of positive cells), or grade 2 (34–66% of positive cells) or grade 3 (>67% of positive cells).[29] Finally, tumors were classified as "Low" (grades 0 and 1) or "High" (grades 2 and 3) for both TF and VEGF.

To evaluate microvessel density, slides were incubated for 30 minutes in 1:25 diluted monoclonal anti-CD34 antibody (IgG_1; C QBEmd 10, Dako) and biotinylated goat antimouse IgG were then used as described above. Individual microvessels were counted independently at high power magnification (×200; area 0.713 mm^2) by two observers. Each score was obtained by adding the vessel counts of 4 × 200 fields, as previously described.[30]

Genotyping of Codon 12 K-*ras* Mutation in NSCLC

Genomic DNA was extracted from 40 tumor biopsies using a DNA purification Kit (Qiagen, Courtaboeuf, France). The K-*ras* codon12 sequence was amplified by an enriched PCR-restriction fragment length analysis method as described by Urban et al.[31] When detected in tumors, the presence of codon12 K-*ras* mutation was also investigated in nonaffected lung tissues collected from the same patients.

Statistical Analysis

The sign-test was used to evaluate the distribution of values in nonaffected lung tissues and tumor samples, and a Mann-Whitney *U* test was used to compare the continuous variables. Spearman's rank correlation was also used to analyze the coexpression of the different genes studied in noncancerous tissues and NSCLC tumors, and a correlation was considered to be significant when p was <0.05. Multivariate analysis was also performed according to principal component analysis using the SPAD software as previously described.[32] Categorical variables were compared using Fisher's exact test, and a p value of 0.05 or less was considered as statistically significant.

RESULTS

Characteristics of Patients

Sixty-four patients were studied (mean age was 64 years, range: 36–83). The majority of patients were men (54 of 64, 84%) and smokers (57 of 64, 89%). The most frequent histologic type was adenocarcinoma (37 of 64; 57.8%), followed by squamous cell carcinoma (19 of 64; 29.5%), and eight tumors (12.7%) were classified as other histologic subtypes, corresponding to large cell carcinoma or mixed type. Tumors were more frequently of small size (T1–T2: 52 of 64; 81%) and without lymph node involvement (39 of 64; 61%) (Table 2).

TF Gene Expression in NSCLC Tumors and in Pulmonary Cell Lines

Specific TF mRNA levels were significantly lower in NSCLC tumors (median: 11,540 per 10^8 copies 18S RNA)

TABLE 2. Relationship Between TF Gene Expression (Number of Copies per 10^8 Copies of 18S RNA) and Immunohistochemical Groups (Low and High Expression) and Clinicopathological Features (Fisher's Exact Test)

Features	*n*	Median TF mRNA Levels (Range)	*p*	High TF Expression *n* (%)	Low TF Expression *n* (%)	*p*
All	64	11540 (60–309,600)		17 (26.5)	47 (73.5)	
Age (yrs)	65 (36–83)	11540 (60–309,600)		65 (44–78)	66 (36–83)	
Sex						
Male	54	10100 (60–309,600)	+0.33	15 (28)	39 (72)	0.98
Female	10	11540 (60–309,600)	−0.33	2 (20)	8 (80)	
Tobacco						
Yes	57	101,000 (60–309,600)	+0.01	14 (83)	43 (91)	0.37
No	7	15,790 (720–85,180)	−0.01	3 (43)	4 (57)	
Histology						
AC	37	9320 (60–309,600)	+0.27	9 (24)	28 (76)	0.78
SCC	19	11,590 (720–154,800)	+0.39	5 (26)	14 (74)	
Other	8	13,130 (1680–256,000)	+0.079	3 (37.5)	5 (62.5)	
Differentiation						
Yes	46	8600 (60–154,800)	−0.08	11 (24)	34 (76)	0.91
No	18	11,280 (100–309,600)	+0.08	5 (28)	13 (72)	
Tumor status						
T1 and T2	52	8530 (100–154,800)	−0.04	10 (19)	42 (81)	0.01
T3 and T4	12	19,150 (60–309,600)	+0.04	7 (58)	5 (42)	
Node status						
N0	39	10,500 (100–309,600)	+0.4	10 (26)	28 (74)	0.95
N1–N2	25	7150 (60–154,800)	−0.4	7 (28)	19 (72)	
Metastasis						
M0	53	11,590 (60–309,600)	+0.06	15 (28)	38 (72)	0.49
M1	11	5520 (110–137,430)	−0.06	2 (18)	9 (82)	
Stage						
I–II	34	9780 (100–272,400)	−0.03	8 (23)	26 (77)	0.58
III–IV	30	17710 (60–309,600)	+0.03	9 (30)	21 (70)	

AC, adenocarcinoma; SCC, squamous cell carcinoma.

than in corresponding nonaffected lung tissues (34,350 copies; $p = 0.0085$) (Figure 1).

TF gene expression was also compared with clinicopathological features using a descriptive multivariate analysis and was significantly higher in T3–T4 tumors than in T1–T2 tumors ($p = 0.04$) and in samples collected from patients with stages III–IV ($p = 0.03$). No other significant differences were found according to sex, histologic type, and differentiation or lymph node status (Table 2).

TF gene expression was also evaluated in 12 pulmonary cell lines, 11 derived from lung tumors (7 NSCLC and 4 small cell lung cancers) and 1 from normal lung tissue (NL20) (Figure 1). TF gene expression was variable from one cell line to another (3–57,250 copies/10^8 copies of 18S RNA) and almost undetectable in the two SCLC cell lines that were nonadherent (H69 and H209).

TF Protein Expression in NSCLC Tumors

TF antigen levels measured in 30 NSCLC tumors with a specific immunoassay (range = 22–164 pg/mg of total protein) were significantly correlated with mRNA levels (Spearman correlation, r = 0.84; $p < 0.0001$) (data not shown).

Immunohistochemical study was performed on nonaffected lung samples and tumors collected from 22 patients to identify the cells expressing TF. Normal bronchial epithelial cells were positive for TF in all nonaffected lung biopsies, particularly in the basal pole. On the other hand, TF was detected in a few alveolar macrophages and in some capillary endothelial cells. The TF staining was variable from one cancerous biopsy to another and TMA was then performed on all 64 tumors. In the majority of tumors ($n = 47$, 73.5%), less than one-third of cells were positive for TF staining (grade ≤ 1) and these samples were classified in the "Low TF" group. Only 17 NSCLC tumors (26.5%) exhibited strong TF protein expression (staining score = grade 2 or 3) and they were therefore categorized in the "High TF" group (Figure 2). As expected, median TF mRNA levels were higher in "High TF" tumors than in "Low TF" samples (66,440 versus 9315 copies/10^8 18S; $p < 0.0001$).

TF protein expression evaluated by immunohistochemistry was also compared with the clinicopathological features. A significant correlation was found between high TF expression and tumor status (T3–T4) ($p = 0.01$; Fisher's exact test) but none with the stage or other features of NSCLC (i.e., sex,

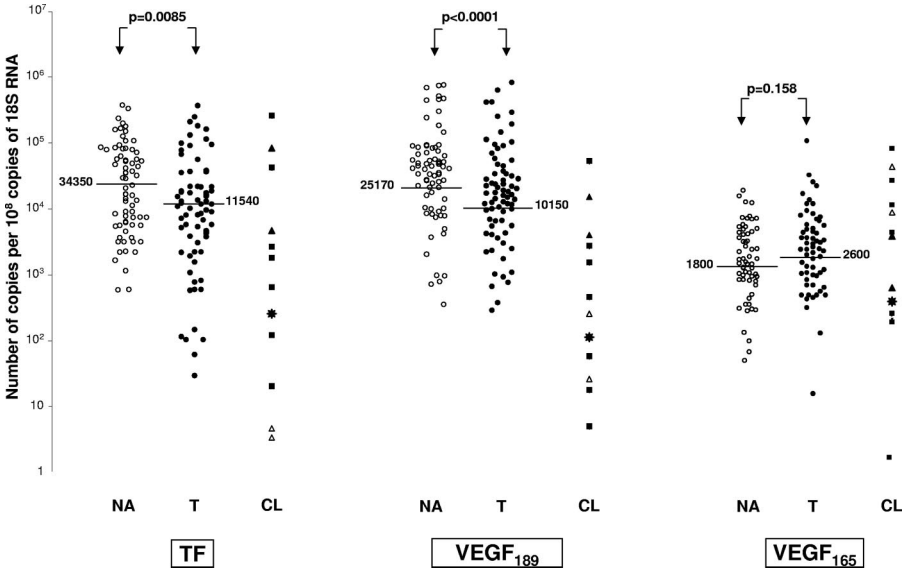

FIGURE 1. TF, $VEGF_{189}$ and $VEGF_{165}$ gene expression in noncancerous lung tissues (NA, empty circles), nonsmall cell lung tumors (T, filled circles) and pulmonary cell lines (CL) from a normal lung (NL20, ↑), NSCLC (■, all adherent) and SCLC (adherent ▲ and nonadherent △). Median values are presented as plain bars. Each point is representative of two (tissues) or three (cell lines) separate experiments.

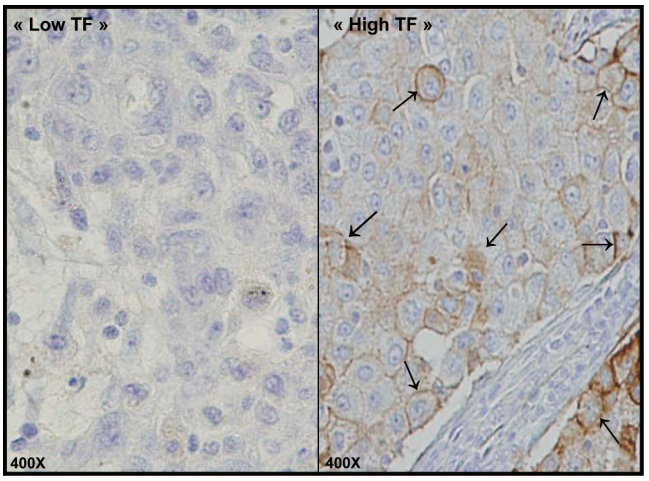

FIGURE 2. Immunohistochemical staining for TF in lung tumors. Cancerous cells positive for TF (→) were only seen in samples with high TF expression.

histologic type, and differentiation or lymph node status) (Table 2).

VEGF Expression and Microvascular Density in NSCLC Tumors

$VEGF_{165}$ and $VEGF_{189}$ gene expression was evaluated in NSCLC tumors and paired nonaffected lungs by specific RT-PCR and was variable from one patient to another. $VEGF_{165}$ gene expression was not significantly different in either tissue type ($p = 0.158$) (Figure 1). In contrast, $VEGF_{189}$ mRNA levels were lower in tumors than in corresponding nonaffected lungs ($p < 0.0001$) (Figure 1).

On the other hand, $VEGF_{165}$ gene expression was 5- to 10-fold lower than those of $VEGF_{189}$, but the numbers of transcripts of these two genes were strongly correlated in both categories of lung tissue ($p < 0.0001$; Table 3). In contrast, $VEGF_{165}$ mRNA levels were not correlated with those of TF.

TABLE 3. Spearman Correlation Matrix to Evaluate TF, VEGF, and VWF Gene Coexpression in Nonaffected Lungs and NSCLC Tumors (*p* Values in Brackets)

	TF	$VEGF_{189}$	$VEGF_{165}$
Nonaffected lungs			
TF	1		
VEGF189	r = 0.29 (0.01)	1	
VEGF165	r = −0.038 (0.39)	r = 0.43 (<0.0001)	1
VWF	r = 0.32 (0.005)	r = 0.316 (0.006)	r = 0.002 (0.49)
NSCLC tumors			
TF	1		
VEGF189	r = 0.23 (0.03)	1	
VEGF165	r = −0.035 (0.42)	r = 0.64 (<0.0001)	1
VWF	r = 0.05 (0.34)	r = 0.20 (0.06)	r = 0.32 (0.005)

A weak correlation was only found between the expression of TF and $VEGF_{189}$ genes in both nonaffected lungs and NSCLC tumors (Table 3).

$VEGF_{165}$ and $VEGF_{189}$ transcripts were also measured in 12 pulmonary cell lines (Figure 1) and no correlation with TF gene expression was evidenced.

VEGF protein expression was also evaluated by conventional immunohistochemical analysis ($n = 22$) and with TMAs ($n = 64$) in the lung tumors using a monoclonal antibody that recognized both $VEGF_{165}$ and $VEGF_{189}$ isoforms. Significant staining was specifically visualized in bronchial epithelial cells (apical pole), in a few alveolar macrophages and in stroma cells (fibroblasts). Twenty-six samples (40.6%) were classified in the "High VEGF" group according to the percentage of positive cells and 38 (60.4%) in the "Low VEGF" group, but this was not related to TF protein expression ($p = 0.26$).

Tumor MVD was evaluated by measuring VWF gene expression in lung biopsies of the 64 patients using a specific

RT-PCR method. Results were expressed in equivalent endothelial cells for 10^7 copies of 18S RNA using standard curves established by measuring VWF mRNA levels in HUVEC. VWF gene expression was significantly lower in NSCLC tumors ($p < 0.0001$), reflecting a striking reduction in the number of endothelial cells in cancerous tissues compared with nonaffected lungs (Figure 3*A*).

Moreover, VWF and TF mRNA levels were only correlated in noncancerous lung tissues ($p = 0.005$), but not in NSCLC tumors ($p = 0.34$) (Table 3).

MVD was also studied by TMA in all tumors and by conventional immunohistochemistry in both nonaffected and cancerous lungs of 22 patients.

The CD34 staining score evaluated using TMA was well correlated with the number of endothelial cells estimated by measuring VWF gene expression (r = 0.67; $p = 0.001$). On the other hand, it was significantly lower in NSCLC tumors (median score = 287, range: 65–585) than in paired nonaffected lungs (450, 75–735; $p < 0.0001$) and this result also demonstrated that MVD was considerably reduced in cancerous tissues. The most frequent picture visualized in conventional immunohistochemistry was in fact characterized by the presence of cancer cells that filled alveoli and pushed away pre-existing vessels (Figure 3*B*). In addition, MVD was not significantly different in "Low TF" and "High TF" tumors (median = 264 versus 245, $p = 0.85$).

Finally, there was no relationship between tumor VEGF expression or MVD and the clinicopathological features of NSCLC according to the multivariate analysis performed.

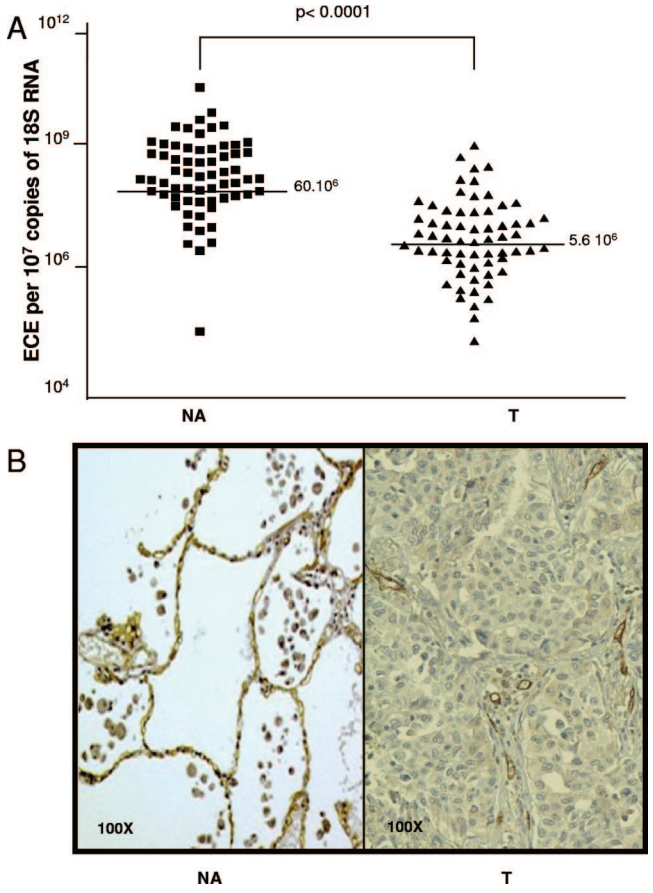

FIGURE 3. Microvascular density (MVD) evaluated by RT-PCR specific for VWF gene (*A*) and immunohistochemical staining of endothelial cells (*B*). (1) Results of RT-PCR expressed as ECE (Endothelial Cell Equivalent) per 10^7 copies of 18S RNA (control gene) in nonaffected lungs (NA) and tumors (T) of 64 patients. (2) CD34 staining of lung tissues from one patient with adenocarcinoma. Endothelial cells and vessels (→) were less numerous and less frequently visualized in the tumor (T) than in nonaffected lung tissue (NA).

Codon 12 K-*ras* Mutation and Relationship with TF, $VEGF_{189}$, $VEGF_{165}$, and VWF Expression

The presence of codon 12 K-*ras* gene mutation was investigated in genomic DNA extracted from 40 tumors using an enriched PCR-restriction fragment length analysis. This mutation was found in eight tumors (20%), which were all adenocarcinomas, and in none of the nonaffected lung samples. Importantly, TF and $VEGF_{189}$ mRNA levels were higher in K-*ras* mutated tumors ($p < 0.0001$), but not those of $VEGF_{165}$ and VWF (Table 4). Moreover, a significant correlation between TF and $VEGF_{189}$ gene expression was found in mutated tissues (Spearman rank correlation test; r = 0.68, $p = 0.03$) but not in lung tumors without K-*ras* mutation (r = 0.27).

On the other hand, the codon 12 K-*ras* gene mutation was present in 4 of 11 pulmonary cancer cell lines (A549, H358, Calu1, and H23). TF and $VEGF_{189}$ gene expression

TABLE 4. Median TF, $VEGF_{165}$, $VEGF_{189}$, and VWF mRNA Levels in NSCLC Tumors and in Cancerous Pulmonary Cell Lines According to Codon 12 K-*ras* Status [i.e., Wild Type (WT) and Mutated] (Ranges in Brackets)

	n	TF	$VEGF_{165}$	$VEGF_{189}$	VWF
NSCLC					
Wild type K-*ras*	32	11,045 (50–309,550)	2880 (445–13,187)	5935 (50–37,1480)	6548 (239–40,123)
Mutated K-*ras*	8	48480[a] (760–154,830)	1250 (16–12,594)	9150[a] (1166–286,430)	5048 (560–21,985)
Pulmonary cell lines					
Wild type K-*ras*	7	1122 (4–20,866)	1702 (126–11,960)	131 (5–4873)	NA
Mutated K-*ras*	4	6549 (224–57,250)	6062 (2–21,460)	916 (15–14,776)	NA

[a] $p < 0.0001$.

also seemed higher in mutated cells (Table 4) but no statistical analysis was performed because of the low number of cell lines studied.

DISCUSSION

In this study, the expression of TF and $VEGF_{189}$ genes was found to be significantly decreased in NSCLC tumors compared with nonaffected lungs and MVD was considerably reduced. TF mRNA levels were assayed by a specific and sensitive molecular method and the results obtained correlated well with TF protein levels measured by enzyme-linked immunosorbent assay. On the other hand, TF expression was variable from one tumor to another, and such variation was also found with cancerous pulmonary cell lines. This variability in TF expression has already been reported in two studies, which had also demonstrated that TF was more expressed in pulmonary cell lines derived from metastases and in cancerous tissues from patients with advanced lung cancer.[8,33] Our results were in agreement with these findings, because TF mRNA levels were higher in tumors from patients with the most severe stages of NSCLC, i.e., those with T3–T4 tumor status ($p = 0.04$) and stages III–IV ($p = 0.03$). In addition, strong TF protein staining was more frequently observed in cancerous cells in patients with T3–T4 tumor status ($p = 0.01$).

Previous studies in patients with colorectal and breast cancer have shown that TF expression in tumors is correlated with clinical progression.[34,35] In addition, it was also recently demonstrated that TF-VIIa signaling was a major contributor to tumor growth in breast cancer.[36]

TF expression was first studied in human NSCLC by immunohistochemistry by Koomagi and Volm who also found that only some tumors synthesized TF (65 of 191; 32%).[37] Kaplan-Meier survival analysis also revealed that survival times were longer in patients with TF-negative tumors than in patients with TF-positive tumors. In addition, a relationship between TF and metastasis has been demonstrated in patients with NSCLC.[8] Altogether these results and our data support that tumor TF could be a prognostic factor in lung cancer.

Mechanisms that regulated TF expression in cancer cells remained unknown. TF synthesis may be particularly controlled by several tumor-related events, including activation of oncogenes, inactivation of tumor suppressor genes and changes in the tumor microenvironment such as hypoxia or inflammation.[38]

A link between TF expression and K-*ras* has recently been reported in colorectal cancer cells,[21] and we therefore looked for the presence of a single mutation in codon-12 of this oncogene which has been shown to be relatively frequent in lung cancer.[39] This point mutation was present in 20% of the tumors studied, which were all adenocarcinomas, and was absent in noncancerous lung tissues sampled in the same patients. Interestingly, both TF and $VEGF_{189}$ mRNA levels were higher in NSCLC tumors exhibiting the K-*ras* mutation. In addition, a significant correlation between the levels of TF and $VEGF_{189}$ transcripts could be evidenced only in mutated tissues. A common axis of regulation may therefore exists between TF and $VEGF_{189}$ involving the K-*ras* pathway in NSCLC. However, other oncogenes such as *EGFR* and tumor suppressor genes such as *P53* and *PTEN* may also regulate TF gene expression in NSCLC, as in other types of cancer.[38]

TF has been identified as an important regulator of angiogenesis and mainly acts through up-regulation of VEGF.[40] According to our findings, $VEGF_{189}$ is the major VEGF isoform synthesized in NSCLC tumors, with five times higher mRNA levels measured compared with $VEGF_{165}$. However, the expression of $VEGF_{165}$ and$VEGF_{189}$ genes was highly correlated in lung tissues. These findings showing an over expression of $VEGF_{189}$ in lung tumors are in agreement with those of a previous study which also demonstrated the impact of this vascular growth factor on the prognosis of NSCLC.[41] VEGF isoforms probably have different functions in cancer tissues. $VEGF_{165}$ is mainly secreted and freely diffusible whereas $VEGF_{189}$, which is more basic and binds to heparin, is cell-associated and almost completely sequestered in the extracellular matrix.[13] Both $VEGF_{165}$ and $VEGF_{189}$ are proangiogenic but only $VEGF_{189}$ has been identified to be directly involved in tumor growth and this effect could involve non angiogenic processes such as cell adhesion.[42,43] In our study, although $VEGF_{189}$ mRNA levels were correlated with those of TF in lung tumors, but only when K-*ras* was mutated and no relationship was found between TF and VEGF protein levels evaluated by immunohistochemistry. This apparent discrepancy could in part be because of the use of an antibody that binds both to $VEGF_{165}$ and $VEGF_{189}$ isoforms. On the other hand, no apparent relationship could be found between the expression of TF and $VEGF_{165}$ genes in lung tissues, whether cancerous or not. Such a relationship has previously been demonstrated in melanoma,[3] but such tumors are highly vascularized. In contrast, NSCLC tumors contain fewer vessels than noncancerous lungs, as shown by our evaluation of MVD.

MVD was evaluated in lung tumors by two different methods, one based on the use of a CD34-specific antibody that is very sensitive for staining endothelial cells in human lungs[30] and the other being a PCR method measuring tissue VWF mRNA levels developed to estimate lung MVD more precisely. VWF is a highly specific marker for vascular endothelial cells and many authors have studied the localization of the VWF antigen by immunohistochemistry to evaluate MVD in cancerous tissues.[44] VWF mRNA levels were 10-times lower in tumors compared with noncancerous lung tissues, correlated well with CD34-specific staining scores, and demonstrated that MVD is reduced in NSCLC tumors. These results are in agreement with those of Eberhard et al., who also found that MVD counts were higher in normal lungs (348%) than in carcinomas and that new proliferating endothelial cells were rare in tumors.[45] This low MVD also explains why TF expression is lower in tumors than in nonaffected tissues. The noncancerous lung is indeed highly vascularized and TF gene is likely over-expressed in many patients at risk for cancer such as heavy smokers.[46]

The impact on the clinical prognosis of tumor MVD and angiogenesis in NSCLC has been documented by several studies.[47] However, we did not find any correlation between

VWF gene expression in tumors and NSCLC stage. In fact, tumor MVD might not necessarily be such a good indicator of angiogenesis in most NSCLC as in other human cancers.[48] Several studies have shown clearly that at least half NSCLC grow by exploiting pre-existing vessels and without morphologic evidence of neo-angiogenesis.[49–51] On the other hand, MVD has previously been shown to decrease from the invading front to the inner part of NSCLC tumors.[52] This feature might also have contributed to our results since the biopsies analyzed were taken only from the inner parts of lung tumors, in which the cancerous cells filled the alveoli in most cases and therefore the only vessels present were those trapped in the alveolar septa.

In conclusion, this study demonstrates that TF gene expression and MVD are reduced in NSCLC tumors compared with nonaffected lung tissue. However, the TF gene is more highly expressed in tumors when the K-*ras* oncogene is mutated and in advanced stages of NSCLC. Taken together, our results support the hypothesis that an axis involving TF/$VEGF_{189}$ and the K-*ras* pathway has a role in the progression of lung carcinoma.

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REFERENCES

1. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* 1990;87:6934–6938.
2. Osterud B, Bjorklid E. Sources of tissue factor. *Semin Thromb Hemost* 2006;32:11–23.
3. Abe K, Shoji M, Chen J, et al. Regulation of vascular endothelial growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. *Proc Natl Acad Sci USA* 1999;96:8663–8668.
4. Ollivier V, Bentolila S, Chabbat J, Hakim J, de Prost D. Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII. *Blood* 1998;91: 2698–2703.
5. Rickles FR, Levine MN. Epidemiology of thrombosis in cancer. *Acta Haematol* 2001;106:6–12.
6. Kakkar AK, Lemoine NR, Scully MF, Tebbutt S, Williamson RC. Tissue factor expression correlates with histological grade in human pancreatic cancer. *Br J Surg* 1995;82:1101–1104.
7. Bromberg ME, Sundaram R, Homer RJ, Garen A, Konigsberg WH. Role of tissue factor in metastasis: functions of the cytoplasmic and extracellular domains of the molecule. *Thromb Haemost* 1999;82:88–92.
8. Sawada M, Miyake S, Ohdama S, et al. Expression of tissue factor in non-small-cell lung cancers and its relationship to metastasis. *Br J Cancer* 1999;79:472–477.
9. Zhang Y, Deng Y, Luther T, et al. Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. *J Clin Invest* 1994;94:1320–1327.
10. Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. *J Biol Chem* 1999;274:23969–23976.
11. Tsopanoglou NE, Maragoudakis ME. Role of thrombin in angiogenesis and tumor progression. *Semin Thromb Hemost* 2004;30:63–69.
12. Belting M, Dorrell MI, Sandgren S, et al. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 2004;10:502–509.
13. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669–676.
14. Mechtcheriakova D, Wlachos A, Holzmuller H, Binder BR, Hofer E. Vascular endothelial cell growth factor-induced tissue factor expression in endothelial cells is mediated by EGR-1. *Blood* 1999;93:3811–3823.
15. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1–8.
16. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993;143:401–409.
17. Gorski DH, Leal AD, Goydos JS. Differential expression of vascular endothelial growth factor-A isoforms at different stages of melanoma progression. *J Am Coll Surg* 2003;197:408–418.
18. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005;23:1011–1027.
19. White RH, Chew HK, Zhou H, et al. Incidence of venous thromboembolism in the year before the diagnosis of cancer in 528,693 adults. *Arch Intern Med* 2005;165:1782–1787.
20. Slebos RJ, Kibbelaar RE, Dalesio O, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 1990;323:561–565.
21. Yu JL, May L, Lhotak V, et al. Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. *Blood* 2005;105:1734–1741.
22. Beasley MB, Brambilla E, Travis WD. The 2004 World Health Organization classification of lung tumors. *Semin Roentgenol* 2005;40: 90–97.
23. Sobin LH. Lung and pleural tumours. In Sobin LH, Wittekind C. (Eds.), TNM Classification of Malignant Tumours. New York: Wiley-Liss, 2002. Pp. 97–108.
24. Reverdiau P, Jarousseau AC, Thibault G, et al. Tissue factor activity of syncytiotrophoblast plasma membranes and tumoral trophoblast cells in culture. *Thromb Haemost* 1995;73:49–54.
25. Rollin J, Iochmann S, Blechet C, et al. Expression and methylation status of tissue factor pathway inhibitor-2 gene in non-small-cell lung cancer. *Br J Cancer* 2005;92:775–783.
26. Kerr R, Stirling D, Rae M, White AR, Ludlam CA. A novel method for quantitation of human von Willebrand factor messenger RNA. *Thromb Res* 2002;106:237–241.
27. Wang KK, Liu N, Radulovich N, et al. Novel candidate tumor marker genes for lung adenocarcinoma. *Oncogene* 2002;21:7598–7604.
28. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; 4:844–847.
29. Khorana AA, Ahrendt SA, Ryan CK, et al. Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. *Clin Cancer Res* 2007;13:2870–2875.
30. Yano T, Tanikawa S, Fujie T, Masutani M, Horie T. Vascular endothelial growth factor expression and neovascularisation in non-small cell lung cancer. *Eur J Cancer* 2000;36:601–609.
31. Urban T, Ricci S, Danel C, et al. Detection of codon 12 K-ras mutations in non-neoplastic mucosa from bronchial carina in patients with lung adenocarcinomas. *Br J Cancer* 2000;82:412–417.
32. de Monte M, Nonnenmacher H, Brignon N, Ullmann M, Martin JP. A multivariate statistical analysis to follow the course of disease after infection of cats with different strains of the feline immunodeficiency virus (FIV). *J Virol Methods* 2002;103:157–170.
33. Keller T, Salge U, Konig H, Dodt J, Heiden M, Seitz R. Tissue factor is the only activator of coagulation in cultured human lung cancer cells. *Lung Cancer* 2001;31:171–179.
34. Shigemori C, Wada H, Matsumoto K, Shiku H, Nakamura S, Suzuki H. Tissue factor expression and metastatic potential of colorectal cancer. *Thromb Haemost* 1998;80:894–898.
35. Ueno T, Toi M, Koike M, Nakamura S, Tominaga T. Tissue factor expression in breast cancer tissues: its correlation with prognosis and plasma concentration. *Br J Cancer* 2000;83:164–170.
36. Versteeg HH, Schaffner F, Kerver M, et al. Inhibition of tissue factor signaling suppresses tumor growth. *Blood* 2008;111:190–199.
37. Koomagi R, Volm M. Tissue-factor expression in human non-small-cell lung carcinoma measured by immunohistochemistry: correlation between tissue factor and angiogenesis. *Int J Cancer* 1998;79:19–22.
38. Rak J, Klement P, Yu J. Genetic determinants of cancer coagulopathy,

angiogenesis and disease progression. *Vnitr Lek* 2006;52(suppl 1): 135–138.
39. Richardson GE, Johnson BE. The biology of lung cancer. *Semin Oncol* 1993;20:105–127.
40. Belting M, Ahamed J, Ruf W. Signaling of the tissue factor coagulation pathway in angiogenesis and cancer. *Arterioscler Thromb Vasc Biol* 2005;25:1545–1550.
41. Oshika Y, Nakamura M, Tokunaga T, et al. Expression of cell-associated isoform of vascular endothelial growth factor 189 and its prognostic relevance in non-small cell lung cancer. *Int J Oncol* 1998;12:541–544.
42. Tomii Y, Yamazaki H, Sawa N, et al. Unique properties of 189 amino acid isoform of vascular endothelial growth factor in tumorigenesis. *Int J Oncol* 2002;21:1251–1257.
43. Herve MA, Buteau-Lozano H, Vassy R, et al. Overexpression of vascular endothelial growth factor 189 in breast cancer cells leads to delayed tumor uptake with dilated intratumoral vessels. *Am J Pathol* 2008;172:167–178.
44. Lackner C, Jukic Z, Tsybrovskyy O, et al. Prognostic relevance of tumour-associated macrophages and von Willebrand factor-positive microvessels in colorectal cancer. *Virchows Arch* 2004;445:160–167.
45. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res* 2000;60:1388–1393.
46. Cirillo P, DE Rosa S, Pacileo M, et al. Nicotine induces tissue factor expression in cultured endothelial and smooth muscle cells. *J Thromb Haemost* 2006;4:453–458.
47. Meert AP, Paesmans M, Martin B, et al. The role of microvessel density on the survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2002;87:694–701.
48. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002;94:883–893.
49. Offersen BV, Pfeiffer P, Hamilton-Dutoit S, Overgaard J. Patterns of angiogenesis in nonsmall-cell lung carcinoma. *Cancer* 2001;91:1500–1509.
50. Pezzella F, Pastorino U, Tagliabue E, et al. Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. *Am J Pathol* 1997;151:1417–1423.
51. Adighibe O, Micklem K, Campo L, et al. Is nonangiogenesis a novel pathway for cancer progression? A study using 3-dimensional tumour reconstructions. *Br J Cancer* 2006;94:1176–1179.
52. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000;60:3088–3095.

