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Expression of c-*ets*-1, Collagenase 1, and Urokinase-Type Plasminogen Activator Genes in Lung Carcinomas

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The c-ets-1 transcription factor has been involved in the* in vitro *transactivation of matrix-degrading protease genes that might play an important role in tumor invasion. Using* in situ *hybridization, we analyzed serial frozen sections for c-ets-1, collagenase 1, and urokinase-type plasminogen activator gene expression in 54 lung carcinomas including 34 non-neuroendocrine carcinomas (18 squamous carcinomas, 10 adenocarcinomas, 3 large cell carcinomas, and 3 basaloids) and 20 neuroendocrine carcinomas (7 small cell lung carcinomas, 4 large cell neuroendocrine carcinomas, 4 well differentiated neuroendocrine carcinomas, and 5 carcinoids). c-ets-1 gene was expressed in stromal cells in 44/54 lung carcinomas including one metastasizing carcinoid. c-ets-1 transcripts were also detected in cancer cells more frequently in neuroendocrine than in non-neuroendocrine carcinomas (P = 0.0059) and in stages III and IV and metastasis more frequently than in stages I and II (P = 0.0065). Collagenase 1 gene was expressed in 16/34 non-neuroendocrine tumors and in 1/20 neuroendocrine tumors, either in stromal (12/17) or in cancer cells (6/17). Urokinase-type plasminogen activator mRNAs were expressed in 45/54 lung carcinomas in stromal and/or cancer cells. In non-neuroendocrine tumors, c-ets-1 and collagenase 1 gene expressions in stromal cells were correlated. These results demonstrate that the transcription factor c-ets-1, collagenase 1, and urokinase-type plasminogen activator are involved in lung cancer invasion and suggest that c-ets-1 protein might transactivate collagenase 1 gene during tumor invasion. (Am J Pathol 1995, 147:1298–1310)

Lung carcinomas represent a heterogeneous group of tumors usually divided on the basis of histological, biological, clinical, and therapeutic aspects into small cell lung carcinomas (SCLCs) and non-small cell lung carcinomas (NSCLCs) including squamous carcinomas, adenocarcinomas, and large cell carcinomas.[1] None of the genetic alterations involved in lung cancer development, including c-*myc* gene family activation and tumor suppressor gene inactivation, have been proven to be valuable predictors for the metastatic spread of the tumor.[2–5] Thus, it is impossible to predict the invasive potential of the majority of lung carcinomas, excluding the highly aggressive SCLCs and basaloid carcinomas,[6] and the rather benign carcinoids.

Cancer invasion and metastasis are multistep processes involving angiogenesis of the primary tumor, neoplastic cells intravasation, systemic dissemination via the blood circulation, and extravasation into the target organ. Thus the degradation of extracellular matrix, which allows cell migration and is accomplished by secreted proteases such as serine proteases and metalloproteinases, is a crucial step in the metastatic cascade.

In vivo, increased urokinase-type plasminogen activator (u-PA) gene expression was found in extracts of most malignant tumors (see ref. 7 for review), including lung carcinomas,[8–11] when compared with their non-neoplastic counterparts. Although a corre-

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lation between u-PA mRNA content evaluated by Northern blot analysis and metastasis has been observed in one study on human lung carcinomas,[8] there is no consensus when u-PA protein content is compared with clinicopathological parameters.[9-11] These discrepancies can perhaps be explained by the variable proportion of u-PA-negative cells in tumor extracts. Immunohistochemical localization of u-PA reveals its presence in cancer cells of human lung carcinomas,[10,12] and a high u-PA level was associated with poor prognosis in pulmonary adenocarcinomas.[12] u-PA gene expression has not yet been studied using *in situ* hybridization in lung carcinomas. So, which cell types were responsible for u-PA mRNA production and whether *in situ* u-PA mRNA levels were correlated with tumor invasion and eventual metastasis in a large panel of lung tumors remained to be established.

Several *in vitro* studies have suggested interstitial collagenase (collagenase 1) involvement in tumor invasion.[13-15] Elevated levels of collagenase 1, which is a representative member of a larger family of matrix metalloproteinases, have been detected *in vivo* on sections of several types of human carcinomas (skin, head and neck, colon, and breast tumors)[16-23]; mRNAs and proteins were mainly observed in the stroma. Collagenase 1 mRNAs were also detected in primary lung carcinomas by Northern blot analysis[24,25] but the possible significance of this expression has not yet been established. To our knowledge, no studies have dealt with collagenase 1 gene expression *in situ* in a large number of lung carcinomas.

It has been suggested that c-ets-1 proteins may regulate matrix-degrading protease gene expression during tumor invasion.[26] The c-*ets*-1 proto-oncogene is the cellular progenitor of the viral oncogene v-*ets* of the avian acute leukemia virus E26.[27] Recently the c-*ets*-1 gene, which encodes a transcription factor,[28] has been involved in the *in vitro* transactivation of two metalloproteinase gene promoters collagenase 1[29] and stromelysin 1,[30] as well as of the u-PA promoter.[31,32] Interestingly, the c-*ets*-1 gene was found to be expressed within endothelial cells of well vascularized tumors and in stromal fibroblasts of invasive tumors including three lung carcinomas.[26,33] c-ets-1 transcripts have also been detected in various other processes that require extensive extracellular matrix remodeling, such as vasculogenesis, migration of neural crest cells, feather formation in chick embryos,[34] and angiogenesis during wound healing in humans.[26]

Using *in situ* hybridization, we investigated the involvement of c-*ets*-1 gene during tumor invasion by a detailed description of its expression in a series of 54 lung tumors comprising 5 carcinoids. Working on serial sections, we compared the pattern of expression of c-*ets*-1, collagenase 1, and u-PA genes. We report that c-*ets*-1, collagenase 1, and u-PA genes are mainly expressed in the stroma of lung tumors. We also provide the first evidence that c-*ets*-1 gene can be expressed in cancer cells of neuroendocrine (NE) tumors including not only SCLCs but also well differentiated NE carcinomas (WDNEC) and large cell NE carcinomas (LCNEC). Finally, these results suggest that c-ets-1 protein is involved in the transcriptional regulation of the collagenase 1 gene during lung tumor invasion.

Table 1. *Histological Classification, Clinical Stages and Sources of Tumors Studied*

Histology	Primary tumors			
	Number	I and II Stage	III and IV Stage	Metastasis
Carcinoid	5	5	0	0
WDNEC	4	3	1	0
LCNEC	4	3	1	0
SCLC	7	0	0	7
Squamous carcinoma	18	9	7	2
Adenocarcinoma	10	6	4	0
Large cell carcinoma	3	0	1	2
Basaloid carcinoma	3	3	0	0
Total	54	29	14	11

Materials and Methods

Tissue Samples

Tumor tissue was obtained at the primary site at lung resection in 43 patients and from the lymph node metastatic site in 7 SCLCs and 4 NSCLCs. According to the tumor, node, metastasis (TNM) classification,[35] 29 patients were at stages I and II (early stages) and 14 patients at stages III and IV (late stages) (Table 1). Representative samples of the fresh tumor tissue were fixed in formalin for histological observation. A part of the fresh material was directly frozen in isopentan and stored at −80°C.

The tumors consisted of 34 non-NE carcinomas (NSCLCs) including 18 squamous carcinomas, 10 adenocarcinomas, 3 large cell undifferentiated carcinomas according to the 1981 World Health Organization classification[1] and three basaloid carcinomas according to Brambilla et al,[6] and of 20 NE carcinomas including 7 SCLCs, 5 carcinoids,[35] 4 WDNEC (atypical carcinoids) according to Warren et al,[36] and 4 LCNEC according to Travis et al[37] (Table

1). Two normal lung tissues and two lymph nodes were also examined.

Preparation of Radioactive RNA Probes

RNA probes were prepared from the following expression vectors. The 820-bp c-*ets*-1 cDNA fragment cloned in the plasmids pSP64 and pSP65 (Promega Biotec, Madison, WI) was kindly provided by Dr. D. Stéhelin (Institut Pasteur, Lille, France). This fragment encompasses sequences encoding the central region of the human c-ets-1 protein (nucleotides 260-1086) and does not cross-hybridize with c-*ets*-2 mRNAs.[26] The plasmid pX7, which contains the complete human interstitial type I collagenase cDNA cloned in pSP64, was provided by Dr. P. Angel (Institut für Genetik und Toxicologie, Karlsruhe, Germany). The pSP64 u-PA plasmid harboring a 600-bp *Pst*I-*Eco*RI fragment of human u-PA cDNA was a generous gift of D. Belin (Faculté de médecine, Geneva, Switzerland). ^{35}S-labeled antisense and control sense RNA probes were transcribed from linearized plasmids as described in Vandenbunder et al[38] with (α-^{35}S)CTP (1200 Ci/mmol, Amersham Corp., Arlington Heights, IL).

In Situ *Hybridization*

In situ hybridization was carried out on cryostat sections of frozen samples. Six μm thick sections were transferred to aminopropyltriethoxy-silane-coated slides (Aldrich Chemical Co., Milwaukee, WI). We also used deparaffinized tissue sections from formalin-fixed materials for the *in situ* hybridization protocol, which has been previously published,[38] to compare with results obtained on frozen tissue sections. Hybridization signal was better on frozen sections because of a very weak background and probably because of an optimal mRNA preservation.

Cryostat sections were immediately transferred to dry ice for 30 minutes, then fixed in formaldehyde (Formal-fixx, Shandon Lipshaw, Inc., Pittsburgh, PA) for 15 minutes at 4°C, washed twice in phosphate-buffered saline, and dehydrated in graded alcohols. RNA probes were applied (20,000 to 30,000 cpm/μl) in a solution of deionized formamide (50%), dextran sulfate (10%), tRNA (0.5 mg/ml), Denhardts 1X, 100 mmol/L dithiothreitol (DTT), 0.3 mol/L NaCl, 5 mmol/L EDTA, and 20 mmol/L Tris-HCL. Sections were covered by siliconized, autoclaved coverslips and hybridized at 65°C overnight (16 to 18 hours) in a chamber humidified with 80 ml of a mixture similar to the hybridization mixture (formamide 50%, 4X SSC). After hybridization, slides were washed twice in 4X SSC, 10 mmol/L DTT for 30 minutes and one hour. Sections were then washed in stringent mixture (0.15 mol/L NaCl, 20 mmol/L Tris-HCL (pH 8.0), 5 mmol/L EDTA) with 50% formamide and 100 mmol/L DTT at 65°C for 30 minutes, and treated with RNAse A (20 μg/ml) in 0.4 mol/L NaCl, 10 mmol/L Tris-HCL (pH 7.5), 50 mmol/L EDTA at 37°C for 30 minutes. This was followed by washing in 2X SSC at 60°C for 15 minutes and in 0.1X SSC at 60°C for 15 minutes. Sections were then dehydrated and air dried.

Emulsion Autoradiography and Staining

Slides were dipped in autoradiographic emulsion NTB2 (Eastman Kodak), stored in black air-tight boxes at 4°C and exposed for 10 to 15 days. After exposure and development, labeling with bisbenzimide (Hoechst dye 33258) was done to visualize the nuclei.[38] Sections were examined under dark-field and epifluorescence illumination. The hybridization signal was scored (0 to 300) corresponding to the mean intensity of cellular staining (+ to +++) multiplied by the percent of signal-expressing cells (0 to 100%) (eg, 80% stromal cells with staining intensity of 1+ gave a score of 80). The two investigators who scored the cases (IB, EB) were unaware of the clinical information and outcome. In parallel with antisense probes, we always used sense control probes on serial sections, which never gave any signal. In addition, hematoxylin-eosin-safran staining and antivimentin (Boehringer Mannheim, Meylan, France) and anti-CD34 (Dakopatts, Glostrup, Denmark) indirect immunoperoxidase technique, carried out on adjacent sections, contributed to the precise identification of the different cell populations expressing a positive hybridization signal.

Results

c-ets-*1 Gene Expression in Stromal Cells*

Out of 49 human lung carcinomas excluding carcinoids, 43 (88%) expressed c-*ets*-1 gene within stromal cells. In contrast, only one out of five carcinoids displayed detectable levels of c-*ets*-1 mRNA in the stroma (Table 2). Sections of normal lung tissues as well as uninvolved lung tissues adjacent to tumoral areas were always negative for c-*ets*-1.

Fibroblastic expression of c-*ets*-1 gene was detected in all positive cases. However, intensity of the hybridization signal varied according to each tumor examined, and generally only a weak signal for c-*ets*-1 could be observed within fibroblasts. In addition, over the tumor section, expression of c-*ets*-1

Table 2. *Results of c-*ets*-1 Probe Hybridization in Stromal or Cancer Cells According to Histological Classes*

Histology	Number of cases	c-*ets*-1$^+$ cases	c-ets-1-positive compartment	
			Stromal cells	Cancer cells*
Carcinoid	5	1	1	0
WDNEC	4	2	1	1
LCNEC	4	4	2	2
SCLC	7	7	2	5
Non-NE carcinoma (non-SCLC)	34	30	28	2
Total	54	44 (81%)	34	10

*A simultaneous expression of c-*ets*-1 mRNA was found in cancer and in stromal cells.

was found restricted to some stromal areas. A generalized diffuse staining was observed in these areas (Figure 1A) except in six cases (four squamous carcinomas, one adenocarcinoma, one large cell carcinoma) where a strong signal restricted to fibroblastic cells surrounding tumor cell islands was noted (Figure 1B). Furthermore, in the 44 positive tumors c-*ets*-1 transcripts were also detected within endothelial cells of stromal vessels as determined by immunostaining of CD34 on a serial section. A strong labeling, above the level of the stroma, of almost all the vessels was noted in six cases: three SCLCs (Figure 1, C and D), one WDNEC, one adenocarcinoma, and one carcinoid. Mature lymphocytes located in the lymph nodes as well as in stroma and in normal lung tissues were always positive for c-*ets*-1 and were used as positive controls. It should be pointed out that in some tumors showing a lymphoid stroma, it was difficult to appreciate the part of the signal that belonged to fibroblastic cells.

c-ets-*1 Gene Expression in Cancer Cells*

In addition, in 10 cases among the 54 human lung carcinomas investigated we clearly observed c-ets-1 transcripts located in carcinoma cells. Of these 10 cases, 8 were NE carcinomas and only 2 were non-NE carcinomas (1/3 large cell carcinomas, 1/18 squamous carcinomas). Thus, in these 10 cases c-*ets*-1 gene expression occurred simultaneously in the stromal and tumoral compartments (Table 2). In the eight positive NE carcinomas, the amount of c-ets-1 transcripts was considerably lower in cancer cells than in stromal cells (Figure 1, E–G). The proportion of positive cancer cells was elevated (between 50 and 100% of the cancer cells). No specific distribution of the signal was noted. It should be pointed out that c-*ets*-1 expression in cancer cells was significantly more frequent in NE than in non-NE carcinomas ($x^2 = 7.58$, $P = 0.0059$) (Table 3). This striking correlation will be discussed below. Furthermore, the proportion of NE carcinomas labeled in cancer cells with c-*ets*-1 probe increased when the grade of differentiation of the tumors decreased (Table 3). In the two non-NE carcinomas, the hybridization signal for c-*ets*-1 in cancer cells was strong and involved all the neoplastic cells of the large cell carcinoma and only the more differentiated epithelial cells underlying the keratinized layer of the well differentiated squamous carcinoma.

Collagenase 1 and u-PA Gene Expression

Collagenase 1 mRNA expression was observed in 16 out of 34 non-NE lung carcinomas and in one out of 20 NE tumors (Table 4). This NE tumor positive for collagenase 1 mRNA expression was an SCLC, and transcripts were detected in cancer cells of this tumor. In non-NE carcinomas, collagenase 1 mRNAs were preferentially seen in fibroblastic cells. This labeling was heterogeneous and restricted only to some stromal section areas. In two cases, fibroblasts located just beneath tumor cells exhibited a strong hybridization signal with collagenase 1 probe, whereas in the other cases only occasional fibroblasts in the deeper stroma were positive. Collagenase 1 expression could also be detected within cancer cells and was more intense at the periphery of tumor lobules in two NSCLCs. Simultaneous expression in stromal and tumoral compartments of collagenase 1 gene occurred in only one squamous carcinoma. This restricted pattern of expression of collagenase 1 to stromal or cancer cells was observed in all histological types.

u-PA gene was expressed at varying levels in 44 out of 49 lung carcinomas excluding carcinoids (Table 4). u-PA mRNAs were detected more frequently within stromal cells, located widely in the deeper stroma (Figure 2A), and only in one case restricted to the tumor-stroma interface. Stromal cells expressing u-PA gene were fibroblasts, perivascular cells, and inflammatory cells such as macrophages and neutrophils. In addition, u-PA gene was also expressed within cancer cells, in non-NE carcinomas exclu-

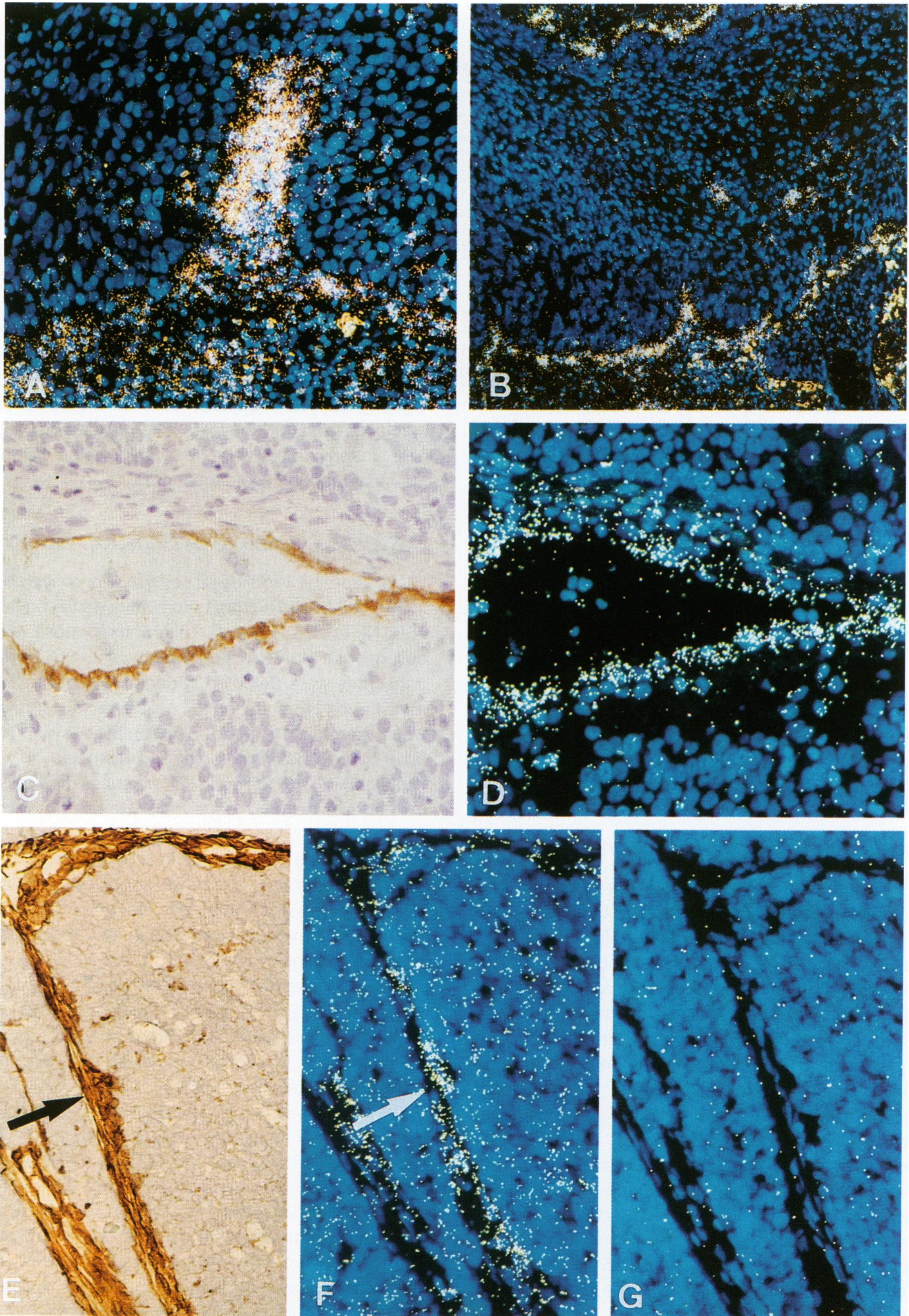
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Table 3. *c-ets-1 mRNA Positivity in Cancer Cells According to NE Phenotype, Stage, and NE Differentiation of the Tumor*

Variables	c-ets-1 mRNAs in cancer cells: Number	Cases	*P* value
NE histology			
NE carcinomas	20	8	*P* = 0.0059
Non-NE carcinomas	34	2	
NE differentiation			
Carcinoid	5	0	
WDNEC	4	1	
LCNEC	4	2	
SCLC	7	5	
Stage			
I and II	29	1	*P* = 0.0065
III and IV and metastasis	25	9	

sively, and more intensely along the peripheral part of the tumor lobule (Figure 2B). In one squamous carcinoma, areas of disruption of epithelial perilobular palisading were intensively labeled (Figure 2, C and D). In four squamous carcinomas, the labeling for u-PA was also detected in cancer cells located at the central part of the lobule, either surrounding necrosis (two cases) or adjacent to keratinized layer (two well differentiated carcinomas).

The five carcinoids showed no expression of collagenase 1 gene. Only one carcinoid was weakly stained in stromal cells with u-PA probe.

We never found collagenase 1 and u-PA gene expression in normal lung tissues and in lymph nodes. In addition, endothelial cells and lymphocytes of tumor stroma were always negative for these two genes.

*Comparison of c-*ets*-1, Collagenase 1, and u-PA mRNA Distribution*

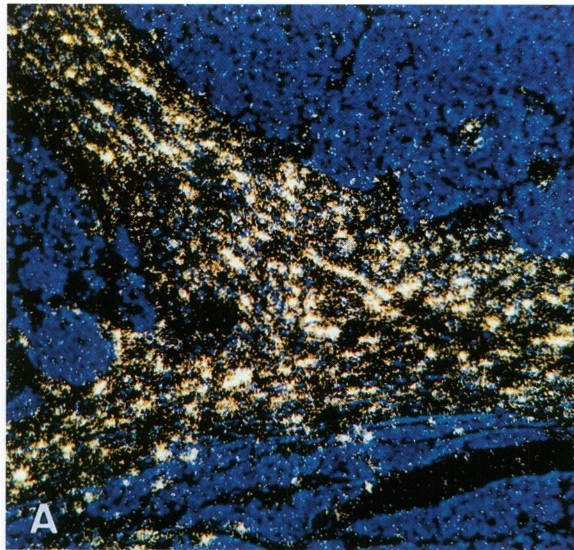
The pattern of expression of c-*ets*-1, collagenase 1, and u-PA genes was studied on serial sections. In the stromal compartments of the 34 non-NE carcinomas, c-*ets*-1 and u-PA genes were co-expressed in 25 cases, and c-*ets*-1 and collagenase 1 genes in 11 cases. We noted that c-*ets*-1 and collagenase 1 gene expression were mainly restricted to the same stromal areas (Figure 2, E and F), whereas u-PA gene was more widely expressed. Moreover, in stromal cells, the levels of c-*ets*-1 and collagenase 1 gene expression were significantly correlated ($P = 0.028$) (Table 5). However, in some occasional stromal areas collagenase 1 transcripts were not superimposed with those of c-*ets*-1, and collagenase 1 mRNAs were never detected in endothelial cells where c-*ets*-1 mRNAs were otherwise abundant.

In comparing c-*ets*-1, collagenase 1, and u-PA gene expression in the 20 NE carcinomas, the results were quite different. Indeed, 14/20 NE tumors (7 SCLCs, 2 WDNEC, 4 LCNEC, 1 carcinoid) that showed a strong hybridization signal in stromal cells with c-*ets*-1 probe exhibited no signal with collagenase 1 probe. Moreover, in 8/20 cases, NE lung neoplastic cells showed a detectable hybridization signal for c-*ets*-1 but lacked the expression of mRNAs for collagenase 1 and u-PA genes.

Table 4. *Results of Collagenase 1 and u-PA Probes Hybridization in Stromal (S) and/or Cancer (C) Cells According to Histological Classes*

Histology	Collagenase 1: Number positive	S	C	S + C	u-PA: Number positive	S	C	S + C
Carcinoid	0/5	0	0	0	1/5	1	0	0
WDNEC	0/4	0	0	0	1/4	1	0	0
LCNEC	0/4	0	0	0	4/4	4	2	2
SCLC	1/7	0	1	0	6/7	6	1	1
Non NE carcinoma (non SCLC)	16/34	12	5	1	33/34	27	21	15
Total	31%	71%	35%	6%	83%	87%	53%	40%

Figure 1. In situ *hybridization of c-*ets*-1 mRNA on frozen sections of primary lung carcinomas.* (**A**, **B**, **D**, **F**, **G**) *Darkfield photomicrographs of section counterstained with Hoechst 33258 after* in situ *hybridization with ^{35}S-labeled antisense* (**A**, **B**, **D**, **F**) *or sense* (**G**) *RNA probes.* (**E**) *Brightfield photomicrographs after indirect immunoperoxydase technique* (**C**, **E**). (**A**, **B**) *Sections of epidermoid carcinomas.* (**A**) *Diffuse and intense staining with c-*ets*-1 probe of fibroblasts infiltrating a tumoral lobule* (×*150*). (**B**) *c-ets-1 transcripts are detected in fibroblasts immediately underlying the tumoral lobule* (×*80*). (**C**, **D**) *Section of a stromal vessel in an SCLC. Endothelial cells stained with anti-CD34 antibody* (**C**) *exhibit a strong hybridization signal with c-*ets*-1 probe* (**D**) (×*150*). (**E**, **F**, **G**) *Serial sections of a SCLC* (×*200*). (**E**) *Vimentin immunostaining to discriminate stromal cells from carcinoma cells.* (**F**) *c-*ets*-1 expression in carcinoma cells; hybridization signal with c-*ets*-1 probe within carcinoma cells was diffuse and lower than in stromal cells* (**arrow**) *but significantly higher than the background on the adjacent sense section* (**G**).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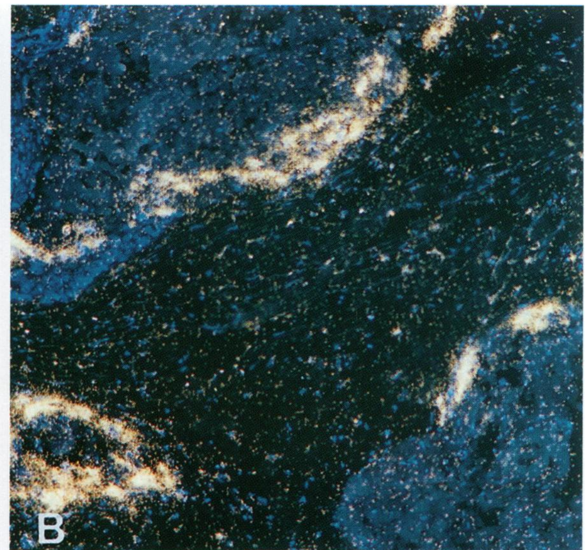
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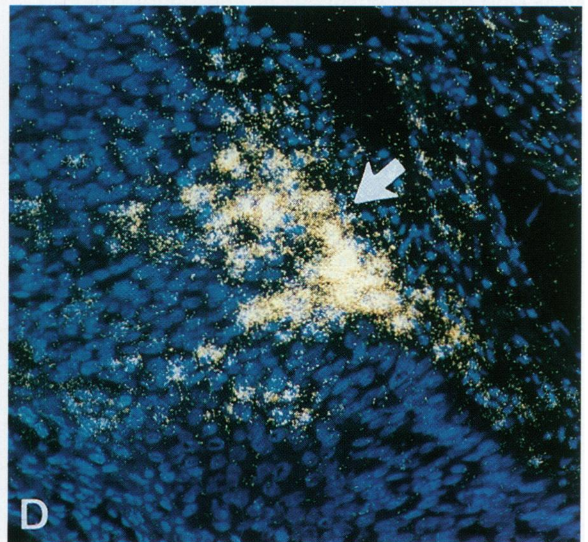
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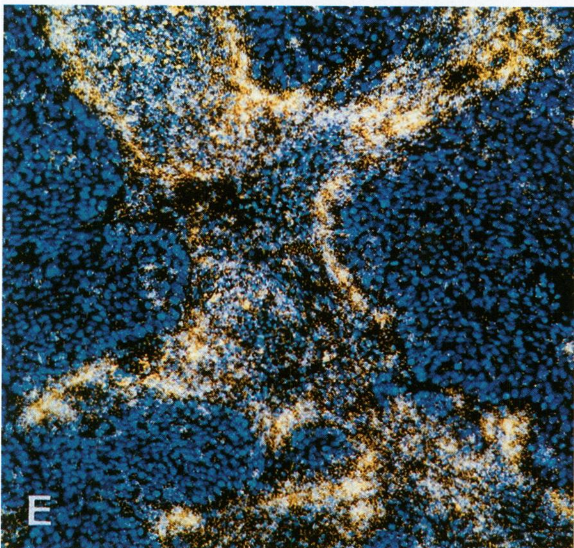
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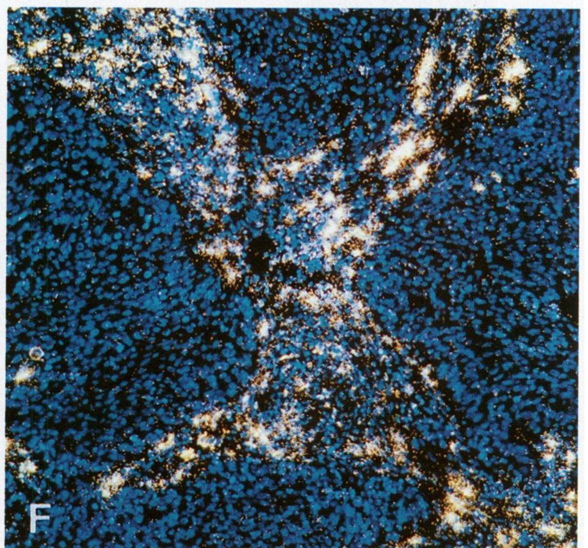
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Table 5. *Correlation Between c-ets-1 and Collagenase 1 mRNA Score in Stromal Cells of non-NE Lung Carcinomas*

Collagenase 1 mRNA score	c-*ets*-1 mRNA score		
	0 to 40	40 to 150	>150
0	12	7	3
<40	4	2	1
>40	0	1	4

mRNA levels were scored as described in Materials and Methods. Three c-*ets*-1 mRNA score classes were arbitrarily defined (0 to 40, 40 to 150, and >150). Collagenase mRNA scores were classified as undetectable (0), <40, and >40. The levels of c-*ets*-1 and collagenase 1 gene expression in stromal cells of non-NE lung carcinomas were significantly correlated (χ^2 = 10.9, P = 0.028).

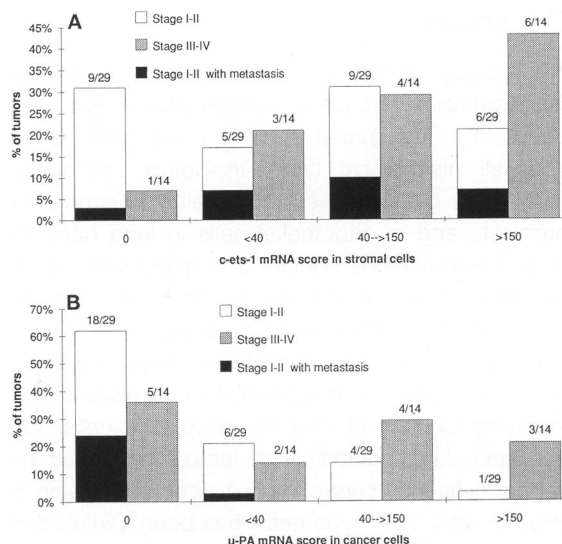

Figure 3. *Distribution of c-ets-1 mRNA levels in stromal cells* (A) *and u-PA mRNA levels in cancer cells* (B) *in stage I and II* (white bar) *and stage III and IV* (gray bar) *tumors. mRNA levels were scored as described in Materials and Methods and classified as undetectable (0) or detectable using in the latter case three RNA classes arbitrarily defined as score 40 or less, score between 40 and 150, and score 150 or more.* (black bar) *Stage I and II tumors corresponding to patients followed for >2 years and who have shown a metastatic recurrence. The numbers above the bars correspond to the number of tumors in each class of mRNA to the total number of stage I and II or stage III and IV tumors.* (A) *Stage III and IV tumors were more frequently c-ets-1 positive than negative* (P = *0.03*). (B) *Stage I and II tumors exhibited more frequently low u-PA mRNA scores (<40) in cancer cells than high u-PA mRNA scores (>40)* (P = *0.03*).

*Correlations Between c-*ets*-1, Collagenase 1 and u-PA mRNA Levels in Stromal or Cancer Cells with Tumor Stage and Metastatic Recurrence*

When scores of c-*ets*-1 expression in stromal cells were compared with tumor stage, stage I and II tumors were evenly distributed on the score scale, whereas the proportion of stage III and IV tumors gradually increased with c-*ets*-1 mRNA score (Figure 3A). Stage III and IV tumors were more frequently c-*ets*-1 positive in stromal cells than negative (P = 0.03). Moreover, the frequency of c-*ets*-1 gene expression in NE and non-NE cancer cells was significantly higher in advanced clinical stages (stages III and IV and metastasis) as compared with stages I and II (x^2 = 7.39, P = 0.0065) (Table 3). Conversely, the frequency of stage I and II tumors decreased when the level of u-PA mRNA in tumor cells increased; 24/29 stage I and II tumors exhibited low u-PA mRNA scores in cancer cells and 5/29 high u-PA mRNA scores, and this difference was significant (P = 0.03); stage III and IV tumors were evenly distributed on u-PA mRNA score scale (Figure 3B). Among the 29 patients who underwent stage I and II tumor resection and were followed for >2 years, 8 showed a metastatic evolution, 18 showed no metastatic recurrence, and three were not evaluable for metastatic recurrence. C-*ets*-1, collagenase 1, and u-PA mRNA levels in the stage I and II tumors that metastasized were compared with cases without recurrence. c-*ets*-1 gene was expressed only in one carcinoid, and the follow-up of this patient showed further metastatic spread clinically detected 3 years after the tumor resection, whereas the four other patients with carcinoid were found free of disease. Thus, considering only the carcinoids, metastatic spread was obviously correlated with c-*ets*-1 expression in stromal cells. No correlation was found between u-PA mRNA levels in stromal cells or collagenase 1 mRNA levels with stage or metastatic recurrence (data not shown). In stage I and II tumors the frequency of metastasis was significantly higher in cases with positive c-*ets*-1 stromal expression combined with negative u-PA cancer cell expression, than in any other phenotypes (P = 0.016) (Figure 3, A and B).

Figure 2. (A–D) In situ *hybridization of u-PA mRNA on frozen sections of primary lung carcinomas.* (A) *Diffuse and intense staining of stromal area of an SCLC with u-PA antisense probe* (×75). (B–F) *Well differentiated epidermoid carcinoma.* (B) *Only the cancer cells located at the lobular periphery underlying the stroma showed a strong expression of u-PA transcripts* (×75)*; and, at a higher magnification* (D)*, u-PA transcripts were detected within cancer cells exhibiting signs of disorganization* (arrow) *as shown on the epifluorescence photomicrograph of the same section* (C) (×150). *On serial sections, c-ets-1* (E) *and collagenase 1* (F) *transcripts were observed in the same stromal area* (×75). (A–F) *Darkfield photomicrographs of sections counterstained with Hoechst 33258 after* in situ *hybridization with* ^{35}*S-labeled antisense RNA probes.*

Discussion

In this study we used *in situ* hybridization to analyze the expression of c-*ets*-1, collagenase 1, and u-PA genes in a large panel of human lung carcinomas from all histological types including carcinoids. mRNAs for c-*ets*-1 were found mainly in stromal fibroblasts and in endothelial cells in lung carcinomas. These findings confirm and generalize, on an extended series, recent reports obtained by others on a restricted number of carcinomas.[26,33]

In some cases, the level of c-*ets*-1 gene expression was higher in or restricted to fibroblasts surrounding clusters of invasive neoplastic cells. We can speculate that diffusible factors produced by cancer cells may control c-*ets*-1 gene expression in stromal cells. This hypothesis has been explored in malignant keratinocyte-dermal fibroblast co-cultures experiments showing that c-*ets*-1 induction in fibroblasts is dependent on a combination of cell-cell contact and of a tumor cell-derived soluble factor.[39] In addition it has been shown that cytokines such as tumor necrosis factor-α (TNF-α) and basic fibroblast growth factor (bFGF) can stimulate *in vitro* the expression of c-*ets*-1 gene in fibroblasts.[26,33]

Our study provides the first evidence of *in vivo* expression of c-*ets*-1 gene within carcinoma cells. Statistical analysis shows that the frequency of c-*ets*-1 gene expression within cancer cells was significantly higher in NE than in non-NE carcinomas ($P = 0.0059$) and in advanced clinical stages than in stages I and II ($P = 0.0065$). As c-*ets*-1 gene expression in cancer cells occurred mainly in stage III and IV or in metastasizing carcinomas it should be a sign of tumoral aggressiveness. In agreement with this observation the frequency of tumors expressing c-*ets*-1 mRNA in cancer cells increased when the level of NE differentiation decreased; c-*ets*-1 gene was maximally expressed in SCLCs, which are poorly differentiated NE carcinoma (Table 3). Of particular interest is the finding that LCNECs are closely related to SCLC in c-*ets*-1 expression in stromal and cancer cells as well as absence of collagenase 1 expression. This supports the new classification of NE tumors proposed by Travis et al.[37] c-*ets*-1 gene expression within cancer cells has already been observed *in vitro* within cells of neuroblastoma and neuroepithelioma, two tumors of neural crest origin, and in cells of Ewing's sarcoma, which is a tumor of bone and soft tissue[40] presently classified as a peripheric neuroectodermic tumor. Neuroblastoma and neuroepithelioma arises in cells from the embryonal neural crest, and Ewing's sarcoma cells appear to be ontogenetically related to tumors derived from the neural crest.[40] It has been shown that c-*ets*-1 gene was transiently expressed in neural crest cells at the onset of their migration.[34] Thus we can speculate that c-*ets*-1 gene is expressed *in vivo* in neoplastic cells derived from the neuroectoderm. Although NE lung tumors were previously expected to arise from the neuroectoderm, it is now usually admitted that they derive from the endodermic lung epithelium by dedifferentiation followed by NE differentiation of preexisting epithelial cells.[41] So c-*ets*-1 expression could be linked to the adoption of an amine precursor uptake and decarboxylase (APUD) differentiation program.

In this study, c-*ets*-1 gene expression was detected in endothelial cells in almost all lung carcinomas, suggesting that this gene is involved in angiogenesis. This agrees with the observation that c-*ets*-1 gene is highly expressed in endothelial cells in developing vessels of chick, mouse, and human embryos,[26,34] in granulation tissue and in stromal vessels of several well vascularized tumors, whereas this gene was not expressed in mature vessels in adult tissues.[26,33] Angiogenesis is a crucial step in the first stage of tumoral invasion.[42] Indeed, the number of microvessels in human biopsies has been correlated with metastasis in cutaneous melanoma,[43] in breast carcinoma,[44] in prostate carcinoma,[45] and in NSCLCs.[46] However, It could be pointed out that all the carcinoids are well vascularized tumors, which generally exhibit a rather benign clinical course. Among the five carcinoids studied, c-ets-1 transcripts were detected in endothelial cells only in the metastasizing one. Probably these c-*ets*-1 expressing-vessels can be more easily penetrated by the neoplastic cells, thereby increasing the chance for metastases.

In contrast to c-*ets*-1 gene, collagenase 1 and u-PA gene expression were never observed within endothelial cells, although in some cases u-PA transcripts were detected in perivascular cells. These findings are in agreement with previous observations on various carcinomas in which authors failed to detect collagenase 1 mRNAs[17] and u-PA mRNAs[33] within endothelial cells. Strikingly, *in vitro* studies demonstrate that bFGF and transforming growth factor-β (TGF-β) could induce u-PA gene expression,[47] and vascular endothelial growth factor could induce collagenase 1 gene expression[48] in cultured endothelial cells. However, it is probable that cultured cells may not be representative of the cells involved in a tumoral community in which many cellular interactions certainly occur.

Consistent with previous reports on lung tumor extracts,[8,10,24,25] this *in situ* hybridization study dem-

onstrated an overexpression of collagenase 1 and u-PA mRNAs in lung carcinomas when compared with their non-neoplastic counterparts. Collagenase 1 gene expression was tissue-specific because it was restricted to non-NE tumors. Nevertheless, a common feature of NE lung carcinoma cells is the constitutive expression of the adhesion molecule NCAM.[49] Given that *in vitro* study showed that NCAM transfection of a rat glioma cell line induces down-regulation of collagenase 1 gene expression,[50] the lack of expression of collagenase 1 mRNAs in NE lung cancer cells is consistent with this finding. However, the absence of collagenase 1 gene expression in stromal cells of NE lung carcinomas remains unexplained. This is the first report on *in situ* hybridization study of u-PA gene expression in lung carcinoma. u-PA mRNAs were predominantly expressed in stromal cells. However, this localization of u-PA mRNAs provides no information about the cellular localization and the enzymatic activity of the u-PA protein. Indeed, secretion of u-PA and its interaction with a specific cellular binding site[51] could influence immunolocalization of u-PA. However, because this study deals mainly with the relationship between the expression of the c-ets-1 transcription factor and the transcription of its putative target gene, we have not investigated yet the presence and activities of the u-PA or collagenase 1 protein.

Studies on the regulation of proteinase gene expression suggest that cytokines, growth factors, and oncogenes may control their transcription. In cultured fibroblasts, interleukin-1, platelet-derived growth factor, epidermal growth factor (EGF), TNF-α, and bFGF could induce collagenase 1 gene expression;[52] and EGF, TGF-α, TGF-β could induce urokinase gene expression.[53] In the present study, mRNAs both for collagenase 1 and urokinase genes were mainly detected in the stroma and sometimes in cells adjacent to malignant tumor cells, suggesting a paracrine-mediated activation of expression of these genes. However, the transcriptional pathway involved in the regulation of matrix protease gene expression remains unclear. In co-transfection experiments, c-*ets*-1 has been found to transactivate collagenase 1[29] and u-PA[31] gene promoters. We demonstrate here that in several lung tumors c-*ets*-1 and collagenase 1 gene expression in fibroblasts were topographically and quantitatively correlated, suggesting that c-*ets*-1 might transactivate *in vivo* the collagenase 1 gene. Indeed, this co-localization was not generalized and not observed within cancer cells. On the other hand, c-*ets*-1 and u-PA gene expression were seldom superimposed. Both collagenase 1 and u-PA promoters contain a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element that binds the transcription factor AP1 (*fos-jun*).[54,55] Since c-*ets*-1 can cooperate with the transcription factors c-*fos*/c-*jun*[28] for transcriptional activation, it is likely that a combination of several factors is necessary to transactivate protease genes. At the same time, it has been shown that binding sites both for c-*ets*-1 and AP1 are required for u-PA gene transactivation.[32] The lack of transcription factors cooperating with c-*ets*-1 could explain the non-superimposed expression of c-*ets*-1 and u-PA genes in the stroma of lung carcinomas. Furthermore, in NE cancer cells no expression of collagenase 1 and u-PA genes was noted, whereas c-ets-1 transcripts were detected. As c-*ets*-1 did not transactivate protease genes in this cellular compartment, the target genes of the c-ets-1 transcription factor in neoplastic cells remain to be established.

Concerning the role of c-*ets*-1 in tumor aggressiveness, the lack of expression of c-*ets*-1 gene in carcinoids is in agreement with the lack of expression of this gene in several benign and non-invasive tumors (eg, colon, breast, and ovary) and in nontumoral lesions observed in a previous study.[33] Strikingly, only the metastasizing carcinoid exhibited a strong hybridization signal with the c-*ets*-1 probe. Moreover, we found that advanced clinical stage tumors were more frequently c-*ets*-1 positive than negative in stromal cells ($P = 0.03$) as well as in cancer cells ($P = 0.0065$). Altogether, these results suggest that the presence of c-*ets*-1 could be used as a prognostic marker to discriminate subpopulations of aggressive tumors.

Stage I and II tumors exhibited low u-PA mRNA scores in cancer cells more frequently than high scores ($P = 0.03$), but the frequency of metastatic recurrence was significantly higher in phenotypes simultaneously positive for c-*ets*-1 mRNA in stromal cell and negative for u-PA mRNA in cancer cells ($P = 0.016$). This inverse correlation of u-PA mRNA levels in cancer cells with metastatic recurrence is not in agreement with a previous report obtained, using immunohistochemistry, on a large series of lung adenocarcinoma.[12] Again, mRNA may not reflect protein level, and immunohistochemistry does not discriminate synthesis from interaction with cell receptors. We are currently generalizing these results in a larger number of tumors to correlate the score of expression of c-*ets*-1 and u-PA genes with metastatic recurrence and also with survival.

In conclusion, we demonstrate in a large panel of lung carcinomas that c-*ets*-1 oncogene expression is a frequent event in tumors and we confirm its

involvement in angiogenesis. In this study, c-*ets*-1 transcripts were strikingly detected in NE carcinoma cells, but the role of this transcription factor in this cellular compartment remains unknown. The good concordance between c-*ets*-1 and collagenase 1 transcripts localization confirms *in vivo* the possibility that c-*ets*-1 might transactivate collagenase 1 gene.

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References

1. Sobin LH, Yesner R: Histological typing of lung tumors. International Histological Classification of Tumors. Geneva, World Health Organization, 1981.
2. Gazzeri S, Brambilla E, Jacrot M, Chauvin C, Benabid AL, Brambilla C: Activation of *myc* gene family in human lung carcinomas and during heterotransplantation into nude mice. Cancer Res 1991, 51:2566–2571
3. Gazzeri S, Brambilla E, Caron De Fromentel C, Gouyer V, Jacrot M, Brambilla C: Abnormalities of *p53* gene and expression in human lung carcinomas. Int J Cancer 1994, 58:24–32
4. Brambilla E, Gazzeri S, Moro D, Caron De Fromentel C, Gouyer V, Jacrot M, Brambilla C: Immunohistochemical study of p53 in human lung carcinomas. Am J Pathol 1993, 143:199–210
5. Gouyer V, Gazzeri S, Brambilla E, Bolon I, Moro D, Perron P, Benabid AL, Brambilla C: Loss of heterozygosity at the Rb locus correlates with loss of Rb protein in primary malignant neuroendocrine lung carcinomas. Int J Cancer, 1994, 58:818–824
6. Brambilla E, Moro D, Veale D, Brichon PY, Stoebner P, Paramelle B, Brambilla C: Basaloid carcinomas of lung a new phenotypic entity with prognosis significance. Hum Pathol 1992, 23:993–1003
7. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L: Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985, 44:140–239
8. Sappino AP, Busso N, Belin D, Vassali JD: Increase of urokinase-type plasminogen activator gene expression in human lung and breast carcinomas. Cancer Res 1987, 47:4043–4046
9. Markus G, Takita H, Camiolo S, Corasanti J, Evers J, Hobika GH: Content and characterization of plasminogen activators in human lung tumors and normal lung tissue. Cancer Res 1980, 40:841–848
10. Nagayama M, Sato A, Hayakawa H, Urano T, Takada Y, Takada A: Plasminogen activators and their inhibitors in non-small cell lung cancer. Cancer 1994, 73:1398–1405
11. Pedersen H, Grondahl-Hansen J, Francis D, Osterlind K, Hansen HH, Dano K, Brünner N: Urokinase and plasminogen activator inhibitor type I in pulmonary adenocarcinoma. Cancer Res 1994, 54:120–123
12. Oka T, Ishida T, Nishino T, Sugimachi K: Immunohistochemical evidence of urokinase-type plasminogen activator in primary and metastatic tumors of pulmonary adenocarcinoma. Cancer Res 1991, 51:3522–3525
13. Mignatti P, Robbins E, Rifkin DB: Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 1986, 47:487–498
14. Brown PD, Levy AT, Margulies IMK, Liotta LA, Stetler-Stevenson WG: Independent expression and cellular processing of Mr 72.000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res 1990, 50:6184–6191
15. Ossowski L: Invasion of connective tissue by human carcinoma cell lines: requirement for urokinase, urokinase receptor, and interstitial collagenase. Cancer Res 1992, 52:6754–6760
16. Bauer E, Gordon J, Reddick M, Eisen A: Quantitation and immunocytochemical localization of human skin collagenase in basal cell carcinoma. J Invest Dermatol 1977, 69:363–367
17. Polette M, Clavel C, Muller D, Abecassis J, Binninger I, Birembaut P: Detection of mRNAs encoding collagenase 1 and stromelysin 2 in carcinomas of the head and neck by *in situ* hybridization. Invasion & Metastasis 1991, 11:76–83
18. Gray ST, Wilkins RJ, Yun K: Interstitial collagenase gene expression in oral squamous cell carcinoma. Am J Pathol 1992, 141:301–306
19. Gray ST, Yun K, Motoori T, Kuys YM: Interstitial collagenase gene expression in colonic neoplasia. Am J Pathol 1993, 143:663–671
20. Hewitt RE, Leach IH, Powe DG, Clark IM, Cawston TE, Turner DR: Distribution of collagenase and tissue inhibitor of metalloproteinases (TIMP) in colorectal tumours. Int J Cancer 1991, 49:666–672
21. Wolf C, Rouyer N, Lutz Y, Adida C, Loriot M, Bellock JP, Chambon P, Basset P: Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells, and possibly implicated in tumor progression: Proc Natl Acad Sci USA 1993, 90:1843–1847
22. Muller D, Wolf C, Abecassis J, Millon R, Engelmann A, Bronner G, Rouyer N, Rio MC, Eber M, Methlin G, Chambon P, Basset P: Increased stromelysin 3 expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. Cancer Res 1993, 53:165–169
23. Polette M, Clavel C, Cockett M, Girod-de-Bentzmann S, Murphy G, Birembaut P: Detection and localization of

mRNAs encoding matrix metalloproteinases and their tissue inhibitor in human breast pathology. Invasion & Metastasis 1993, 13:31–37
24. Urbanski SJ, Edwards DR, Maitland A, Leco KJ, Watson A, Kossakowska AE: Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. Br J Cancer 1992, 66:1188–1194
25. Muller D, Breathnach R, Engelmann A, Millon R, Bronner G, Flesch H, Dumont P, Eber M, Abecassis J: Expression of collagenase-related metalloproteinase genes in human lung or head and neck tumours. Int J Cancer 1991, 48:550–556
26. Wernert N, Raes MB, Lassalle PH, Dehouck MP, Gosselin B, Vandenbunder B, Stéhelin D: c-*ets*-1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. Am J Pathol 1992, 140:119–127
27. Leprince D, Gegonne A, Coll J, De Taisne C, Schneeberger A, Lagrou C, Stéhelin D: A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature 1983, 306:395–397
28. Wasylyk B, Wasylyk C, Flores P, Begue A, Leprince D, Stéhelin D: The c-ets proto-oncogenes encode transcription factors that cooperate with c-*fos* and c-*jun* for transcriptional activation. Nature 1990, 346:191–193
29. Gutman A, Wasylyk B: The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J 1990, 9:2241–2246
30. Wasylyk C, Gutman A, Nicholson R, Wasylyk B: The c-ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J 1991, 10:1127–1134
31. Stacey KJ, Fowles LF, Colman MS, Ostrowski MC, Hume DA: Regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor. Mol Cell Biol 1995, 15:3430–3441
32. Nerlov C, Rorth P, Blasi F, Johnsen M: Essential AP-1, and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. Oncogene 1991, 6:1583–1592
33. Wernert N, Gilles F, Fafeur V, Bouali F, Raes MB, Pyke C, Dupressoir T, Seitz G, Vandenbunder B, Stéhelin D: Stromal expression of c-ets1 transcription factor correlates with tumor invasion. Cancer Res 1994, 54:5683–5688
34. Vandenbunder B, Queva C, Desbiens X, Wernert N, Stéhelin D: Expression of the transcription factor c-*ets*-1 correlates with the occurrence of invasive processes during normal and pathological development. Invasion & Metastasis 1995, 14:198–209
35. Mountain CF: A new international staging system for lung cancer. Chest 1986, 89:225–233
36. Warren WA, Memoli VA, Gould VE: Well-differentiated and small cell neuroendocrine carcinomas of the lung: two related but distinct clinico-pathologic entities. Virchows Arch B 1988, 55:299–310
37. Travis WD, Linnoila RI, Tsokos MG, Hitchcock CL, Cutler GB, Nieman L, Chrousos G, Pass H, Doppman J: Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. Am J Surg Pathol 1991, 15:529–553
38. Vandenbunder B, Pardanaud L, Jaffredo T, Mirabel MA, Stéhelin D: Complementary patterns of expression of c-*ets*-1, c-*myb* and c-*myc* in the blood forming system of the chick embryo. Development 1989, 107:265–274
39. Borchers AH, Powell MB, Fusenig NE, Tim Bowden G: Paracrine factor and cell-cell contact-mediated induction of protease and c-*ets* gene expression in malignant keratinocyte/dermal fibroblast cocultures. Exp Cell Res 1994, 213:143–147
40. Sacchi N, Wendtner CM, Thiele CJ: Single-cell detection of ets-1 transcripts in human neuroectodermal cells. Oncogene 1991, 6:2149–2154
41. Le Douarin NM, Teillet MA: The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol 1973, 30:31–48
42. Folkman J: The role of angiogenesis in tumor growth. Semin Cancer Biol 1992, 3:65–71
43. Srivastava A, Laidler P, Davies R, Horgan K, Hughes LE: The prognostic significance of tumour vascularity in intermediate-thickness (0.76–4 mm thick) skin melanoma. Am J Pathol 1988, 133:419–423
44. 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
45. 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
46. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA: Relation of neovascularization to metastasis of non-small-cell lung cancer. Lancet 1992, 340: 145–146
47. Pepper MS, Belin D, Montesano R, Orci L, Vassali JD: Transforming growth factor-β1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells *in vitro*. J Cell Biol 1990, 111:743–755
48. Unemori EN, Ferrara N, Bauer EA, Amento EP: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 1992, 153:557–562
49. Brambilla E, Veale D, Moro D, Morel F, Dubois F, Brambilla C: Neuroendocrine phenotype in lung cancers. Comparison of immunohistochemistry with biochemical determination of enolase isoenzymes. Am J Clin Pathol 1992, 98:88–97
50. Edvardsen K, Chen W, Rucklidge G, Walsh FS, Obrink B, Bock E: Transmembrane neural cell-adhesion molecule (NCAM), but not glycosyl-phosphatidylinositol-anchored NCAM, down-regulates secretion of matrix metalloproteinases. Proc Natl Acad Sci USA 1993, 90: 11463–11467

51. Vassali JD, Baccino D, Belin D: A cellular binding site for the Mr 55.000 form of the human plasminogen activator, urokinase. J Cell Biol 1985, 100:86–92
52. Mauviel A: Cytokine regulation of metalloproteinase gene expression. J Cell Biochem 1993, 53:288–295
53. Laiho M, Keski-Oja J: Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res 1989, 49:2533–2553
54. Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P: 12-*O*-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol Cell Biol 1987, 7:2256–2266
55. Cassady AI, Stacey KJ, Nimmo KA, Murphy KM: Von der Ahe D, Pearson D, Botteri FM, Nagamine Y, Hume DA: Constitutive expression of the urokinase plasminogen activator gene in murine RAW264 macrophages involves distal and 5′ non-coding sequences that are conserved between mouse and pig. Nucleic Acids Res 1991, 19:6839–6847