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Expression of endothelin-1 is related to poor prognosis in non-small cell lung carcinoma

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Abstract

The endothelin (ET) system influences tumourigenesis and tumour progression by various mechanisms, including angiogenesis. The aim of this study was to determine whether the expression of endothelin-1 (ET-1) is related to the angiogenic phenomenon in lung cancer and whether it could be involved in its clinical behaviour. Expression of ET-1, endothelin-converting enzyme-1 (ECE-1) and endothelin-receptors ETA and ETB was examined in 201 non-small cell lung carcinoma (NSCLC) and corresponding normal tissues using real-time polymerase chain reaction (RT-PCR). Forty NSCLC were also analysed for vascular endothelial growth factor (VEGF) expression by a competitive-PCR approach to assess whether ET-1 expression was related to this angiogenic factor. A higher number of cases with ET-1, ECE-1 and ETA mRNA expression was observed in malignant lung tumours compared with normal lung tissues (45.7% *versus* 33% for ET-1 ($P < 0.0001$); 38.3% *versus* 16.5% for ECE-1 ($P = 0.004$); and 42.8% *versus* 28.5% for ETA ($P < 0.0001$)). On the other hand, ETB mRNA was higher in normal lung tissues than in tumour samples (58.5% *versus* 52.8% ($P < 0.0001$)). Immunohistochemical analysis was also performed in 78 cases, selected from among those with high ET-1 mRNA, to confirm the presence of ET-1 protein and to determine its distribution and localisation. Moreover, an interesting relationship was observed between ET-1 and VEGF mRNA levels ($P = 0.02$). At univariate analysis, clinical–pathological parameters, such as sex, nodal metastatic involvement and stage, and ET-1 expression were seen to be significant predictors of worse prognosis regarding both overall survival ($P = 0.001$, $P = 0.0003$, $P = 0.001$ and $P = 0.03$, respectively) and disease-free interval ($P = 0.0005$, $P = 0.0007$, $P = 0.001$ and $P = 0.04$, respectively). We conclude that ET-1 could be involved in angiogenic phenomena in NSCLC and may represent a further indicator of progression and poor prognosis in this type of cancer, with interesting therapeutic implications.
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Keywords: Endothelin-1; Vascular endothelial growth factor; Prognosis; NSCLC

1. Introduction

The endothelin (ET) family consists of three isoforms (ET-1, ET-2 and ET-3), all of which contain 21 amino-acid residues. The isoforms ET-2 and ET-3 differ from ET-1 by two and six amino acids, respectively, and share significant homology, especially at the carboxyterminal, with sarafotoxins. ET-1 is the most abundant isoform and has been best characterised [1].

The endothelin-1 gene, mapped to chromosome 6 [2], encodes a precursor peptide, preproendothelin-1, which is cleaved firstly by a neutral endopeptidase to form proendothelin-1, or big ET-1, and in a second step by endothelin-converting enzyme-1 (ECE-1) to ET-1 [3]. This proteolytic cleavage of a Trp-Val bond by ECE-1

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is critical to the functions of ET-1, because the precursors have negligible biological activity [4].

Increased expression of ET-1 has been shown in various human malignancies, such as ovarian, colorectal and prostate cancer [5–8], suggesting a possible involvement of ET-1 expression in tumour development [9]. The tumour-promoting activity of ET-1 is mediated through autocrine and paracrine effects on tumour-cell proliferation [10], invasion [11,12], angiogenesis and neovascularisation [6,8,11,13,14], even if several aspects of the potential role of ET remain unclear.

The endothelins exert their physiological action via two receptors, ETA and ETB, which are G-protein coupled transmembrane receptors found in both vascular and non-vascular tissue [15]. ET-1 stimulates proliferation and migration of endothelial cells through the ETB [16–19] and is a potent mitogen for vascular smooth muscle and tumour cells through the ETA [20,21]. The hypothesised modulating effect of ET-1 on angiogenesis is supported by the finding that ET-1 expression correlates positively with neovascularisation and vascular endothelial growth factor (VEGF) expression in ovarian [6], breast [22], colorectal [23] and brain tumours [24]. Thus, in a similar way, the influence of the ET system on the progression of lung carcinomas may occur through stimulation of VEGF.

Several pre-clinical and clinical studies in various malignancies suggest that ET system may represent an interesting factor in tumour progression, but conflicting data exist regarding the prognostic value of ET-1 [25,26]. At present, there are no works available linking ET-1 expression with clinical–pathological parameters and disease outcome in lung cancer.

In this study, we investigated the expression of ET-1, ETA and ETB receptors, and ECE-1 in a sample of 201 non-small cell lung carcinoma (NSCLC). We also evaluated vascular endothelial growth factor (VEGF) mRNA expression in order to assess whether ET-1 expression was related to this angiogenic factor. Moreover, the availability of extensive histopathological and clinical data for all our cases allowed us to evaluate the prognostic role of ET-1 in our series of NSCLC.

2. Materials and methods

2.1. Surgical specimens

A total of 201 NSCLC patients, who had undergone curative surgical resection at the Service of Thoracic Surgery, University of Pisa, between 1991 and 1994, were analysed. There were 181 male patients and 20 female patients (mean age 63.4 years, median 64 years, range 41–88 years). All the patients underwent a complete pre-operative staging, including a detailed history and physical examination, the evaluation of the Performance Status according to Karnofsky, a complete blood count and biochemical profile, cardiac and pulmonary function tests, chest X-ray, bronchoscopy, computed tomography of chest, the upper abdomen and brain, bone scan, bilateral bone marrow biopsy and aspiration. The most common histological type was squamous carcinoma (114 cases), followed by adenocarcinoma (69 cases), large-cell anaplastic carcinoma (12 cases) and bronchiolo-alveolar carcinoma (6 cases). According to tumour-status, there were 48 T1 (23.9%), 132 T2 (65.6%), 21 T3 (10.5%); 64 patients showed metastatic nodal involvement at the moment of diagnosis, whereas 137 did not. A total of 125 patients were clinically staged as Stage I (SI), whereas 26 and 50 patients were staged as Stage II (SII) and Stage IIIa (SIIIa), respectively. Data on clinical behaviour were available in all of the 201 cases (median follow-up 124.50 months, mean 124.54, range 107–146). Ninety of the patients were alive at the time of analysis. After resection, tumour samples were in part frozen in liquid nitrogen and stored at −80 °C for molecular studies, and in part formalin-fixed and paraffin-embedded for histological and immunohistochemical processing. Tumours were classified according to the World Health Organization Classification (1982) [27] and according to the guidelines of the American Joint Committee for Cancer Staging (1992) [28].

2.2. RNA extraction

Total RNA was extracted from frozen lung tissue samples using RNeasy Mini Kit (Qiagen, M-Medical s.r.l., Florence, Italy); the RNeasy procedure represents a novel technology which combines the selective binding properties of a silica-gel-based membrane with the speed of the microspin principle. High-quality RNA is then eluted in 30 μl of water and digested with RNase-free DNase to guarantee that RNA is completely free of DNA contamination.

2.3. RT-PCR analysis

A constant amount of total RNA (5 μg) was reverse-transcribed at 42 °C for 60 min in a total 20 μl reaction volume using First-strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostic Corporation, Indianapolis, IN, United States of America, USA). cDNA was incubated at 95 °C for 5 min to inactivate the reverse transcriptase, and served as a template DNA for amplification using the Gene Amp PCR System 2400 (Perkin–Elmer Applied Biosystem, CA, USA). PCR was performed in a standard 50 μl-reaction mixture consisting of 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3), 0.2 mM dNTPs, 50 pico-moles of each sense and antisense primer, and 2.5 U of Amplitaq DNA Polymerase (EUROBIOTAQ, Eurobio, Les

Ulis, France). As negative control the DNA template was omitted in the reaction.

PCR primers for ET-1 cDNA were 5′-TTGAGATCTGAGGAACCC-3′ and 5′-TACGGAACAACGTGCTCG-3′ (Clontech Laboratories, Inc, Palo Alto, CA, USA); amplification was performed for 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C for 30 cycles, and in an additional extension step for 10 min.

PCR primers for ECE-1 cDNA were 5′-AGTATGACAAGGACGGGAACC-3′ and 5′-CTTACCAGACTTCGCACTTGT-3′; amplification was performed for 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C for 29 cycles, and in an additional extension step for 10 min.

PCR primers for endothelin receptors cDNA were as follows: ETA 5′-TGGCCT TTTGATCACAATGACTTT-3′ and 5′-TTTGATGTGGCATTGAGCATACAGGTT-3′; ETB 5′-ACTGGCCATTTGGAGCTGAGATGT-3′ and 5′-CTGCATGCCACTTTTCTTTCTCAA-3′. Thirty cycles of amplification were performed under the following conditions: melting at 95 °C for 1 min; annealing at 57 and 58 °C, respectively, for 1 min; and extension at 72 °C for 30 s, and in an additional step for 7 min.

Amplified PCR products were run on a 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. The following bands were visualised: 354 bp for ET-1; 459 bp for ECE-1; 302 and 428 bp, respectively, for ETA and ETB.

Amplification for VEGF was performed for 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 40 cycles. Lastly, an additional extension step was performed for 2 min. PCR primers for VEGF cDNA were as follows: forward primer, 5′-TCACCGCCTTGGCTTGTCACAT-3′; reverse primer, 5′-TGGATCCATGAACTTTCTGCTGTC-3′. Three bands were detected: 452, 584 and 656 bp, corresponding to the VEGF isoforms 121, 165 and 189 [29].

The presence of a 412 PCR band, amplified with specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the same cDNAs, was used as internal control. PCR primers for GAPDH cDNA were as follows: forward primer, 5′-CGATGCTGGCGCTGAG TAC-3′; reverse primer, 5′-CGTTCAGCTCAGGGATGACC-3′. As negative control the cDNA template was omitted in the reaction.

2.4. VEGF-competitive-PCR

In order to obtain the quantification of VEGF mRNA levels in all the samples in which the material was available, we employed a technique based on a competitive-PCR approach using a non-homologous internal standard, called VEGF competitor. We constructed a 600-bp VEGF competitor, as described previously [30]: a titration assay was made to evaluate the opportune competitor dilutions to be used. Competitive-PCR reactions were then performed adding dilutions of VEGF competitor to aliquots of cDNA, derived from 8 μg of total RNA. The relative densitometric measure of the electrophoretic bands was then plotted, and the point of equal intensity between the bands of the competitor-PCR MIMIC and target gene was taken as concentration of the cDNA samples.

2.5. Immunohistochemistry

In a restricted section of 78 tumoural samples, mainly selected from among the cases with high ET-1 mRNA expression at molecular analysis, immunostaining for endothelin was performed using the Avidin Biotin Peroxidase Complex (ABC) method in formalin-fixed, paraffin-embedded tissue samples. The sections were deparaffinised and rehydrated through graded alcohols. After heating three times in a microwave oven for 5 min at 750W in citrate buffer (0.01 M, pH 6.0), the sections were incubated with 1% (v/v) hydrogen peroxidase in methanol for 30 min to block the endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS) and incubation for 20 min with 20% (v/v) normal blocking serum, the sections were incubated for 1 h at 25 °C with rabbit anti-endothelin polyclonal antibody (Chemicon, dilution 1:100). Anti-endothelin is raised against a synthetic octapeptide containing the carboxyterminal heptapeptide common to ET-1, ET-2 and ET-3. After the primary antibodies, a biotinylated secondary one was applied and followed by detection using the ABC method (Vector Laboratories, Burlingame, CA, USA); the ABC method was used by developing immunoreaction with diaminobenzidine. Light counterstaining was performed with haematoxylin. Incubation of parallel slides omitting the first antibody was performed as negative control. For each sample, endothelin expression was evaluated as percentage of positive cells in a total of at least 1000 tumour cells. A value of 1% of positive cells was used as the cut-off to distinguish negative from positive tumours. Following previous literature, cytoplasmic staining intensity was scored semi-quantitatively into different grades on an arbitrary four-tiered scale from 0 to 3. The following scoring criteria were agreed upon before the analysis: grade 0, no detectable immunostaining of tumour cells; 1+, weak staining of the majority of tumour cells; 2+ and 3+, moderate and strong staining intensity of tumour cells, respectively.

2.6. Statistical analysis

All statistical analyses were carried out using Statistica software (Stat-soft). A χ^2 test was used to analyse the associations between the different variables. The *a priori* level of significance was set at *P*-value of less than 0.05. Univariate analysis was performed by modelling

Kaplan–Meier survival curves. A multivariate analysis was performed to evaluate the independent prognostic role of ET-1 expression after accounting for other significant covariates. The Cox model was first used to select from among variables that significantly affected survival in univariate analysis, and then from among those variables whose prognostic role was independent.

3. Results

3.1. Clinical–pathological parameters and survival

Among the clinical–pathological parameters, sex, nodal metastatic involvement and advanced stage were significantly associated with a worse overall and disease-free survival (Table 1).

3.2. ET system mRNAs in NSCLC

Among the tumour samples, 45.7% of cases resulted positive for ET-1 messenger, 38.1% for ECE-1, 42.5% for ETA, 52.9% for ETB. Examples of electrophoretic runs are shown in Fig. 1. A significantly higher number of cases with ET-1, ECE-1 and ETA mRNA expression

Table 1
Univariate survival analysis

Features	Number of patients	Overall survival (P value)	Disease-free interval (P value)
Sex			
Male	181	0.001	0.0005
Female	20		
Age (years)			
⩽63.4	110	0.40	0.41
>63.4	91		
Tumour status			
T_1	48	0.06	0.02
T_2	132		
T_3	21		
Node status			
N_0	137	0.0003	0.0007
N_1	30		
N_2	34		
Stage			
S_I	125	0.001	0.001
S_{II}	26		
S_{IIIa}	50		
Histotype			
Squamous	114	0.32	0.44
Adeno-	69		
Bronchiolo-alveolar	12		
Anaplastic	6		
Endothelin-1			
Low	152	0.03	0.04
High	43		

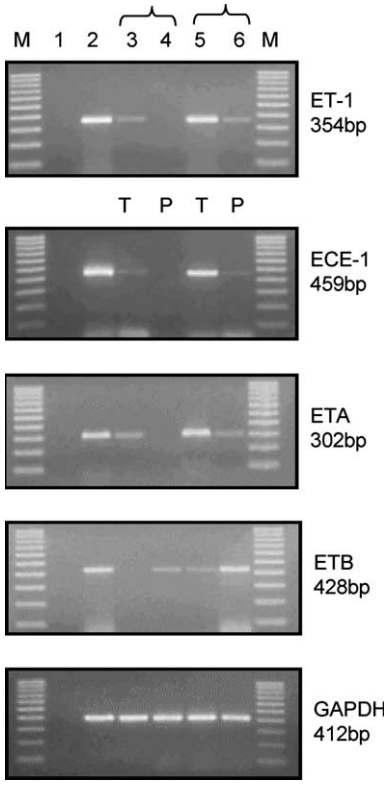

Fig. 1. Endothelin (ET) system mRNAs expression detected by reverse-transcription polymerase chain reaction (RT-PCR). M, molecular weight marker (100-bp ladder, Pharmacia); lane 1, negative control; lane 2, positive control; lanes 3–6, two couples of non-small cell lung cancer (T) and corresponding normal tissue (P). The presence of a 412 PCR band amplified with specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the same cDNA was used as an internal control.

was present in lung tumours compared with the adjacent normal lung tissue (45.7% *versus* 33% for ET-1; 38.3% *versus* 16.5% for ECE-1; 42.8% *versus* 28.5% for ETA) ($P < 0.0001$; $P = 0.004$; $P < 0.0001$, respectively). By contrast, although ETB mRNA was also expressed by tumour samples, ETB positivity was found to be higher in normal lung tissue (52.8% *versus* 58.5%) ($P < 0.0001$) (Fig. 2).

Final quantified endothelin-1 messenger levels were expressed as a ratio to GAPDH expression; assuming the median value of 1.3 of endothelin expression relative to GAPDH, we distinguished tumours with low (153 cases) from tumours with high (43 cases) ET-1 mRNA expression. In five samples there were no materials available for ET-1 mRNA analysis.

3.3. Immunohistochemistry for ET-1

Seventy-eight tumoural samples were selected, mainly from among those with high mRNA expression, for immunohistochemical analysis, in order to confirm the positivity obtained with molecular techniques and to provide accurate endothelin protein localisation. A

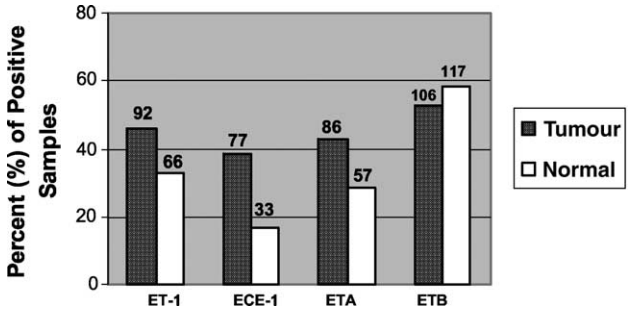

Fig. 2. Percentage of positive samples for endothelin (ET)-1, endothelin-converting enzyme (ECE)-1, ETA and ETB endothelin-receptors expression. Numbers above columns represent the absolute numbers of core samples; total numbers of core specimens analysed were $n = 201$ for tumours and $n = 200$ for adjacent normal tissue.

strong immunoreactivity with the anti-ET-1 antibody was seen in all cancer samples with high ET-1 mRNA expression (χ^2 test; $P = 0.03$), confirming the RT-PCR results. The ET-1 immunostaining was mostly localised in the cytoplasm; the pattern of staining in a significant case is shown in Fig. 3.

For each tumour sample analysed, the percentage of positive cells for ET-1 staining was evaluated, as shown in Table 2.

3.4. Relationship between ET-1 and VEGF mRNA expression

Forty NSCLC were also analysed for VEGF expression by a competitive-PCR approach in order to assess whether ET-1 expression was related to this angiogenic factor. The exact number of VEGF cDNA molecules was evaluated, and according to the median value (475 VEGF cDNA molecules; range 0–2400) we distinguished tumours with high (25 out of 40) from tumours with low VEGF mRNA expression.

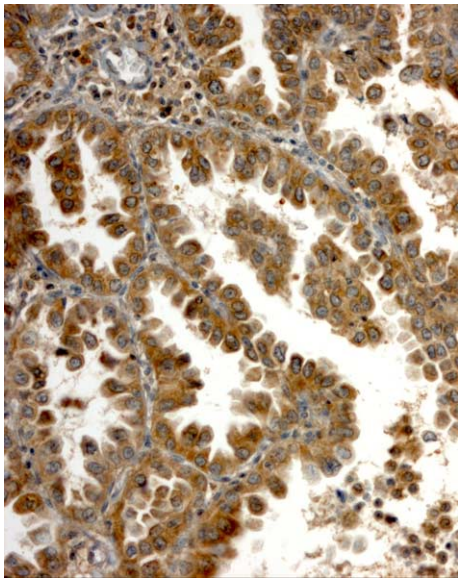

Fig. 3. Immunohistochemical expression of endothelin (ET)-1 in adenocarcinoma of the lung. Intense cytoplasmic staining is present in neoplastic cells.

Table 2
Endothelin-1 protein expression in 78 non-small cell lung cancer samples

Cases (n)	Positive cells (%)
7	0
1	5
6	10
2	20
3	30
5	40
2	50
10	70
23	80
14	90

Table 3 shows the correlation between ET-1 expression on the one hand and VEGF on the other; the seven tumours with ET-1 positivity also showed high levels of VEGF mRNA (χ^2 test; $P = 0.02$).

3.5. ET-1 expression and outcome in NSCLC

Statistical analysis of survival data revealed that elevated ET-1 expression was significantly associated with shorter survival, in terms of both overall survival and disease-free interval (respectively, $P = 0.03$ and $P = 0.04$) (Table 1). Fig. 4 shows Kaplan–Meier survival plots generated on the basis of low and high expression of ET-1 mRNA.

3.6. Multivariate analysis

All the variables that significantly affected survival in univariate analysis maintained their independent prognostic influence on overall survival in a Cox proportional-hazard model ($P = 0.001$ for sex; $P = 0.0001$ for nodal metastatic involvement; $P = 0.007$ for stage; $P = 0.04$ for ET-1 expression) (Table 4).

4. Discussion

Tumour development depends on neovascularisation with the recruitment and proliferation of endothelial cells and tissue remodelling. The upregulation or alteration of

Table 3
Correlation between vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1) mRNA expression levels

		VEGF mRNA expression level		
		Low	High	
ET-1 mRNA expression level	Low	15	18	$P = 0.02$
	High	0	7	

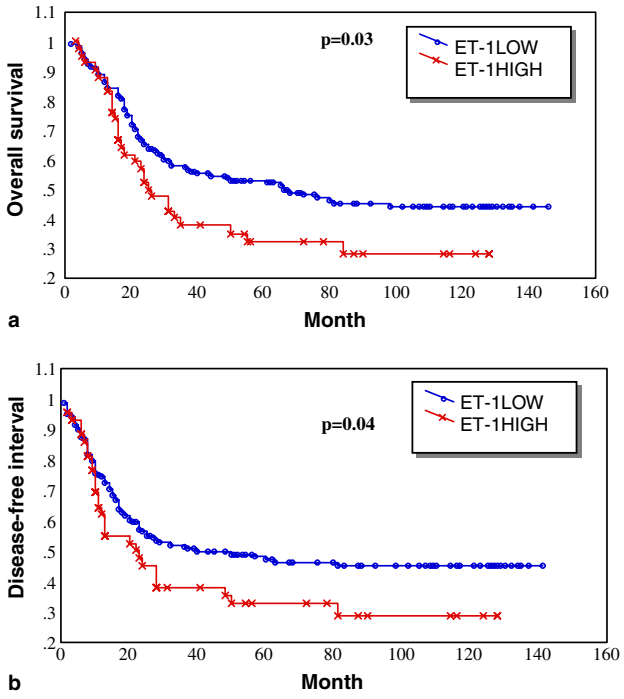

Fig. 4. Kaplan–Meier overall survival (a) and disease-free interval (b) curves in relation to endothelin (ET)-1 mRNA expression levels in non-small cell lung cancer samples.

Table 4
Cox proportional-hazard model of overall survival

Variables	Beta	Standard error of beta	*t*	*P*
Sex	0.5	0.136	3.668	0.001
Age	−0.014	0.191	−0.074	0.97
Tumour status	−0.136	0.112	−1.205	0.219
Node status	0.474	0.115	4.103	0.0001
Stage	0.345	0.122	2.809	0.007
Histotype	0.189	0.126	1.498	0.146
Endothelin (ET-1)	0.450	0.218	2.066	0.04

the physiological functions of growth factors may lead to an unbalanced growth of the cell. ET-1 is an important growth stimulator in various neoplastic diseases, including breast, ovarian and prostate cancer [8,31–33]. The discovery in 1985, and the isolation in 1988, of ET-1 has generated considerable research [34].

A large body of *in vitro* and *in vivo* studies demonstrated ET-1 over-expression in many malignancies. Engagement of the cognate receptor by ET-1 triggers tumour proliferation, angiogenesis, anti-apoptotic effect, and metastatic potential [35]. ET-1 may exert its roles in a synergistic way with other growth factors, such as VEGF, but the exact link between ET axis and angiogenesis remains unclear. Moreover, the use of ET-1, ETA and ETB as biological markers for characterisation of more aggressive tumours is open to speculation; conflicting results exist concerning the prognostic role of ET system in breast cancer [9] and little data is available regarding NSCLC [36–38].

In this study, we evaluated the expression of ET-1, ETA, ETB and ECE-1, involved in the generation of the active form of ET-1, in a series of 201 NSCLC and corresponding normal samples.

We have demonstrated that a significantly higher percentage of cases with ET-1, ECE-1 and ETA mRNA expression is present in lung cancer compared with normal tissue. ETB mRNA was also expressed in tumour samples, but a higher number of positive cases was found in normal tissue than in tumoural tissue, as reported by Drimal and colleagues [39]. We therefore postulated that ET-1 could be one of the essential autocrine growth factors involved in growth regulation of NSCLC, as previously demonstrated in ovarian cancer [32,40]. This finding was also confirmed in other human tumours, including meningiomas [41], astrocytomas [42] and prostate adenocarcinoma [8,43,44]. Interestingly, in all of these models, the normal counterparts express mainly the ETB subtype, whereas in primary and metastatic tumour cells ETA is likely to become the predominant form [40].

Recent studies suggest that endothelins and their receptors participate in tumour-associated angiogenesis. ET-1 modulates various stages of neovascularisation, including endothelial cell proliferation, migration, invasion, protease production and tube formation, and it stimulates neovascularisation *in vivo* [13]; in vascular smooth muscle cells ET-1, predominantly through ETA, enhances VEGF secretion [11]. Because the regulation of VEGF production is a critical event in tumour angiogenesis, we can postulate that, in pathological conditions such as cancer, ET-1 and VEGF may have a complementary and co-ordinated role during neovascularisation. Our data are consistent with recent findings in several malignancies [6,22–24], in which increased expression of ET-1 is associated with elevated VEGF levels, suggesting ET-axis may accelerate neovascularisation also in lung cancer. However, several of our NSCLC samples with low ET-1 positivity also showed high VEGF mRNA levels, suggesting a probable involvement of other factors; thus, to further our knowledge of the exact biochemical link between ET-1 and angiogenesis in NSCLC requires additional studies in a larger number of cases.

Moreover, the prognostic role of ET axis needs further investigation, which may lead to the identification of more aggressive tumour groups, and could therefore add significant information regarding standard prognostic variables. In breast cancer, several studies failed to find a significant correlation between ET-1 expression and clinical–pathological variables [25,26,45,46], but more recent work has suggested its influence on prognosis in just this kind of tumour [9]. Thus far, to our knowledge, there are no studies on the prognostic role of ET-1 in NSCLC.

Because extensive histopathological, clinical and follow-up data were available for all our cases, univariate and multivariate statistical analysis were performed. Among the clinical–pathological parameters, sex, nodal metastatic involvement and advanced stage were significantly associated with a worse overall and disease-free survival. We also observed that ET-1 mRNA expression was statistically related to reduced overall survival and disease-free interval. At multivariate analysis, all variables that significantly affected survival in univariate analysis maintained their independent prognostic influence on overall survival in a Cox proportional-hazard model. This data may have notable importance in terms of biological characterisation, suggesting that ET-1 may add useful information regarding the clinical evaluation of NSCLC progression, and that it may represent a potential target for an anti-angiogenic therapy using selective ET receptor antagonists.

Conflict of interest statement

None declared.

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