

# Expression of endothelin receptor subtypes in bronchial tumors

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**Abstract.** The importance of endothelin-1 (ET-1) in cell growth, migration and stimulation of angiogenesis suggests that ET-1 may play a role in tumor progression. The expression of the ET-1 receptors ETA (ET<sub>A</sub>R) and ETB (ET<sub>B</sub>R) was analyzed by immunohistochemistry in fragments of human lung carcinomas. Samples were obtained from 11 patients with adenocarcinoma (ADK), 12 with squamous cell carcinoma (SCC) and 8 patients with small cell carcinoma (SCLC). Morphologically normal airway areas adjacent to the tumors served as controls. ADK and SCC samples had ET<sub>A</sub>R and ET<sub>B</sub>R levels similar to normal tissues; however, the ET<sub>A</sub>R/ET<sub>B</sub>R ratio was higher in ADK than in SCC. We also observed the presence of endothelin receptors in SCLC, although the ET<sub>A</sub>R levels and the ratio ET<sub>A</sub>R/ET<sub>B</sub>R were lower than in normal tissue and in other carcinomas. In conclusion, both ET<sub>A</sub>R and ET<sub>B</sub>R are present in lung carcinomas but at different levels, according to the histological type of tumor.

## Introduction

The endothelin (ET) family is comprised of four homologous 21 amino acid peptides, ET-1, ET-2, ET-3 and ET-4 (1). All ET isoforms arise from post-translational processing of preprohormones. ET-1 is the most abundant and extensively studied isoform and is also the most relevant in human cardiovascular physiology and pathophysiology (1,2). Areas of ET production and binding sites for ET usually coexist, suggesting possible local effects. In the human lung, ET-1 is mainly produced by endothelial cells, airway epithelial cells

and alveolar epithelial cells (3,4) and ET-1 binding sites are found in bronchial smooth muscle cells, endothelial cells, nerves, bronchial and bronchiolar epithelial cells, including glandular cells and alveolar epithelial cells (3-5).

ET-1 exerts numerous biological effects, including vasoconstriction, cell proliferation, epithelial ion transport and cell migration (1-3,6-8). There are 2 types of ET receptors (ETR): the ETA receptors (ET<sub>A</sub>R) and the ETB receptors (ET<sub>B</sub>R). The ET-1/ET<sub>A</sub>R system stimulates the proliferation of several airway cells such as epithelial, bronchial and vascular smooth muscle cells (9-12). The ET-1/ET<sub>B</sub>R system induces bronchoconstriction in addition to the migration and proliferation of endothelial cells (8,10,13,14). Some ET-1 effects involve both receptors; for example, Sato *et al* (8) showed that blockade of both ET<sub>A</sub>R and ET<sub>B</sub>R is required for the complete inhibition of ET-1-induced vasoconstriction in the rat pulmonary circulation. On the other hand, the stimulation of ET<sub>B</sub>R may counteract some of the ET-1/ET<sub>A</sub>R system effects, for example by leading to vasodilatation or inhibition of epithelial cell proliferation (1,7,12). ET<sub>B</sub>R is also involved in the clearance of ET-1 from the circulation; therefore, the blockade of ET<sub>B</sub>R may result in enhanced ET-1 activity via ET<sub>A</sub>R (3,7,8,15,16). This clearance primarily takes place in the pulmonary circulation (16).

The importance of ET-1 in cell growth, cell migration and angiogenesis suggests that ET-1 may play a role in tumor progression. Increased expression of ET-1 has been reported in various human cancers such as ovarian, breast, colorectal, bladder, renal and prostate cancer (17-22). ET-1 expression is also increased in several lung cancer cell lines (23) and in freshly excised fragments of non-small cell lung carcinomas (24,25). ET-1 levels are also increased in the breath condensate of patients with non-small cell lung cancer and this increase may be associated with poor prognosis (25,26). In addition, elevated ET-1 concentrations in the saliva of oral cancer patients or breath condensate of lung cancer patients has been proposed as indicators to monitor patients at risk for oral or lung carcinoma (26,27). Several studies have provided evidence that ET-1 is involved in proliferation and migration of cancer cells such as prostate, melanoma, colorectal and ovarian cancer cells (18,28-31). In non-small cell lung carcinomas (NSCLC) and in breast carcinomas, a positive

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correlation has been found between ET-1 and the expression of vascular endothelial growth factor (VEGF) mRNA, suggesting that ET-1 might be involved in angiogenesis in these carcinomas (25,32).

Depending on the type of cancer, ET<sub>A</sub>R and ET<sub>B</sub>R levels may be maintained, increased or decreased, which is likely to affect the biological effects of ET-1 (20). We therefore decided to investigate by immunohistochemistry the localization and distribution of ET<sub>A</sub>R and ET<sub>B</sub>R in tumors obtained from patients suffering from various types of lung carcinomas.

## Materials and methods

**Surgical specimens.** Lung samples were obtained from patients who underwent curative surgical resection for lung cancer and gave their informed consent, in accordance with the current French legislation. The histological types of the primary tumors were squamous cell carcinoma (SCC, 12 patients, mean age 73±3 years), adenocarcinoma (ADK, 11 patients, mean age 66±3 years) and small cell lung carcinoma (SCLC, 8 patients, mean age 74±4 years). In 5 of the above patients (3 ADK and 2 SCC), samples of morphologically normal bronchi were dissected at sites distant from the tumor. One fragment of normal lung was also obtained from a smoker patient who died of head trauma and had no lung disease (21 pack-years, 45 years old). A sample of prostatic adenocarcinoma served as a positive control for ET<sub>A</sub>R antibody specificity, as it has been shown that this tissue expresses high levels of ET<sub>A</sub>R and no or low levels of ET<sub>B</sub>R (19,32). The positive control for ET<sub>B</sub>R antibody specificity was the bronchial smooth muscle of the patient without lung disease (32-34).

After resection, samples were formalin-fixed, dehydrated and paraffin-embedded for immunohistological processing.

**Immunohistochemistry.** Immunostaining was performed using polyclonal sheep antibodies raised against the N-terminus of human receptors ET<sub>A</sub>R and ET<sub>B</sub>R (Alexis Biochemicals, San Diego, CA, USA) (32,35).

Four micrometer-thick sections were mounted on Superfrost Plus Slides (Fisher Scientific, Fairlawn, NJ, USA) and processed for immunohistochemistry. Before ET<sub>A</sub>R and ET<sub>B</sub>R staining, specimens were dewaxed, rehydrated and subjected to heat-induced antigen retrieval in a water bath (95°C, pH 6.0 with 0.01 M citrate buffer, 40 min). The sections were then incubated for 30 min in Tris-buffered saline (TBS) with 0.1% bovine serum albumin. After washing with TBS and incubating for 30 min with 20% swine serum, sections were exposed for 10 min to 3% H<sub>2</sub>O<sub>2</sub> to block the endogenous peroxidase activity. After an additional wash with TBS, the sections were incubated overnight at 4°C with ET<sub>A</sub>R antibody at a dilution of 1/10 or with ET<sub>B</sub>R antibody at a dilution of 1/50.

The two antibodies were detected using the Avidin Biotin Peroxidase Complex (ABC) method. After the primary antibody incubation, a biotinylated secondary antibody was applied followed by detection using the LSAB+® system-HRP kit (ref K-0679, DakoCytomation S.A., Trappes, France). The ABC method was used by developing the immunoreaction with diaminobenzidine. Finally, the sections were rinsed in

TBS for 5 min, counterstained with hemalun solution and mounted (RCM 90, Tech-Inter, Maurepas, France).

Negative controls were performed by omitting the primary antibody or, in other slides, by replacing it by a non-specific antibody (DakoCytomation). The staining intensity of malignant cells was graded semi-quantitatively from 1 to 5 as follows: 1, no staining; 2, detectable staining; 3, 'mild' staining; 4, 'moderate' staining and 5, 'strong' staining.

**Statistical analysis.** Quantitative data were expressed as mean ± standard deviation and median; qualitative data were expressed as frequency and percent. Comparison of staining intensities between the four groups (normal, ADK, SCC and SCLC) was performed using the Kruskal-Wallis non-parametric test, and comparison of ET<sub>A</sub>R and ET<sub>B</sub>R staining intensities in each group was performed using a Wilcoxon non-parametric test. A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using SAS software v 9.2 (SAS Institute Inc, USA).

## Results

Fig. 1 displays the ET<sub>A</sub>R and ET<sub>B</sub>R immunostaining in negative and positive control tissues. Negative controls exhibited no staining (Fig. 1A and B). The staining for ET<sub>B</sub>R was of strong intensity in bronchial smooth muscle cells (Fig. 1C and E), whereas samples of prostatic adenocarcinoma displayed high ET<sub>A</sub>R staining intensity (Fig. 1D and F). These observations confirm the specificity of the primary antibodies.

**ET<sub>A</sub>R and ET<sub>B</sub>R expression in normal samples.** In the morphologically normal samples, 80 to 100% of the ciliated cells from the surface epithelium and 50% of the serous cells from the submucosal glands displayed a homogeneous cytoplasmic labelling for ET<sub>A</sub>R and ET<sub>B</sub>R (Fig. 2A and B). The staining intensity was higher (usually rated as 'moderate') for ET<sub>A</sub>R than for ET<sub>B</sub>R (usually rated as 'clear'). The basal cells usually displayed 'clear to moderate' staining for both receptors. The mucous cells located in the surface epithelium and in the glands were negative for both ET<sub>A</sub>R and ET<sub>B</sub>R.

**ET<sub>A</sub>R and ET<sub>B</sub>R expression in tumors.** When staining was present, 70 to 100% of the carcinoma cells were positive for ET<sub>A</sub>R and ET<sub>B</sub>R (Fig. 2C and H). The immunolabeling patterns of ET<sub>A</sub>R and ET<sub>B</sub>R were cytoplasmic and homogeneous in all three types of cancer. All samples of ADK displayed ET<sub>A</sub>R staining, usually with 'mild to moderate' intensity. Two out of 12 samples were negative for ET<sub>B</sub>R, while the others displayed 'detectable to mild' staining intensity (Table I). Additionally, all SCC samples exhibited ET<sub>A</sub>R and ET<sub>B</sub>R staining, usually with 'detectable to mild' intensity for both receptors. Five of the 8 SCLC samples were negative for ET<sub>A</sub>R staining, while the others displayed a 'detectable to mild' staining. Two of the SCLC samples were negative for ET<sub>B</sub>R, 3 displayed a 'detectable' staining and 2 displayed a 'mild staining'.

There was no statistically significant difference in ET<sub>A</sub>R staining intensity between normal tissue, ADK and SCC groups. In contrast, ET<sub>A</sub>R staining in SCLC samples was

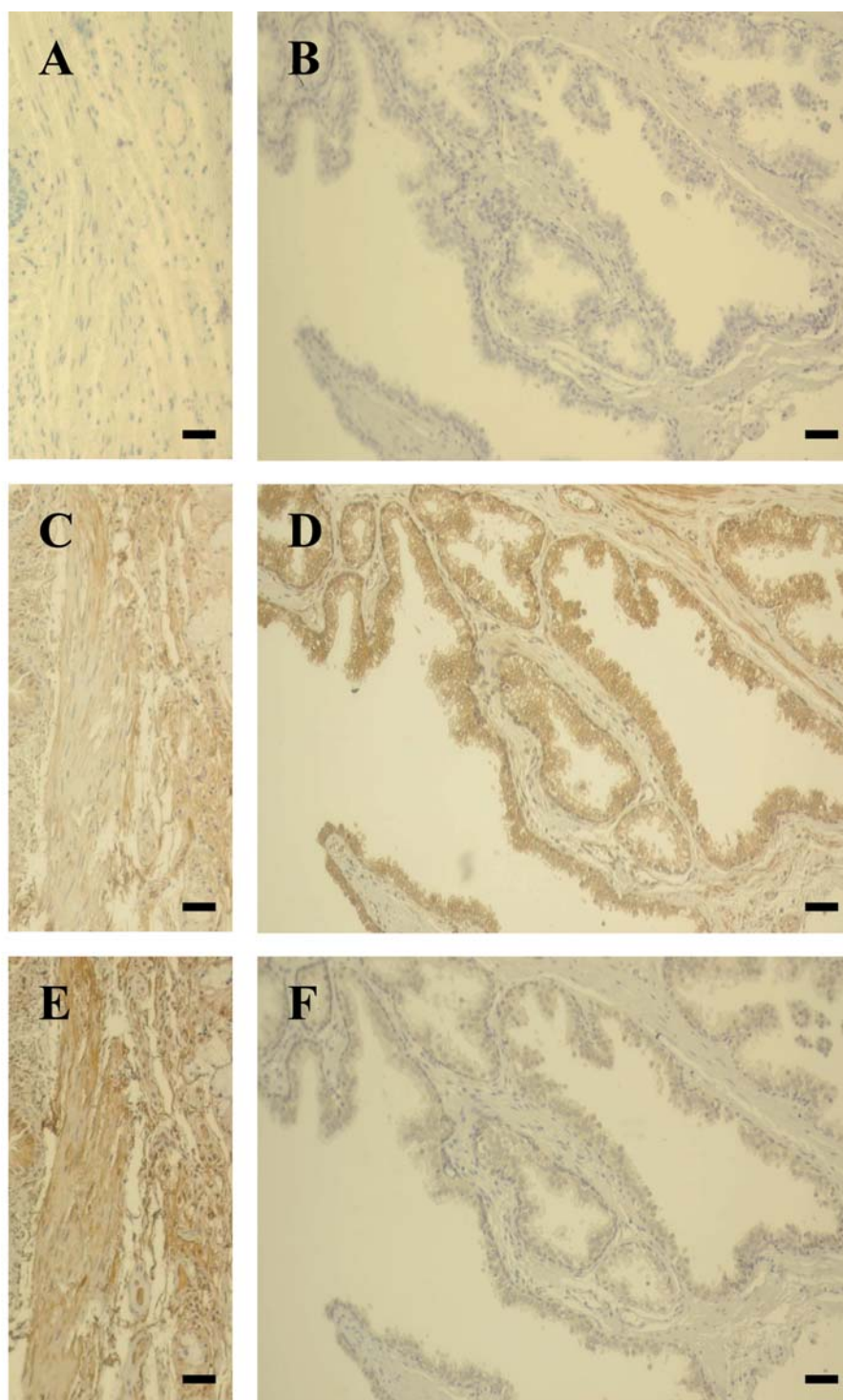


Figure 1. ET<sub>A</sub>R and ET<sub>B</sub>R immunostaining in control tissues. The immunostaining was applied to serial sections of human bronchial smooth muscle (A, C and E) and cancer prostate (B, D and F). No primary antibody was used in A and B. Other sections were exposed to antibodies raised against ET<sub>A</sub>R (C and D) or ET<sub>B</sub>R (E and F). Bars, 50  $\mu$ m.

significantly lower than in the three other groups. Additionally, there was no significant difference in ET<sub>B</sub>R staining intensity between the four groups. ET<sub>A</sub>R staining intensity was significantly higher than ET<sub>B</sub>R staining in normal tissue ( $P=0.016$ ) and in ADK samples ( $P=0.015$ ). No difference was found between ET<sub>A</sub>R and ET<sub>B</sub>R staining intensities in SCC and in SCLC samples.

Comparison of the ratios of the staining intensities of ET<sub>A</sub>R and ET<sub>B</sub>R in each sample revealed differences between the four groups (Table I): the ET<sub>A</sub>R/ET<sub>B</sub>R ratio in the SCLC group was significantly lower than in the three other groups ( $P=0.03$ ) and the ratio in the ADK group was significantly higher than in the SCC group ( $P=0.04$ ). No statistically significant difference was observed between the SCC or the ADK group and the normal group.



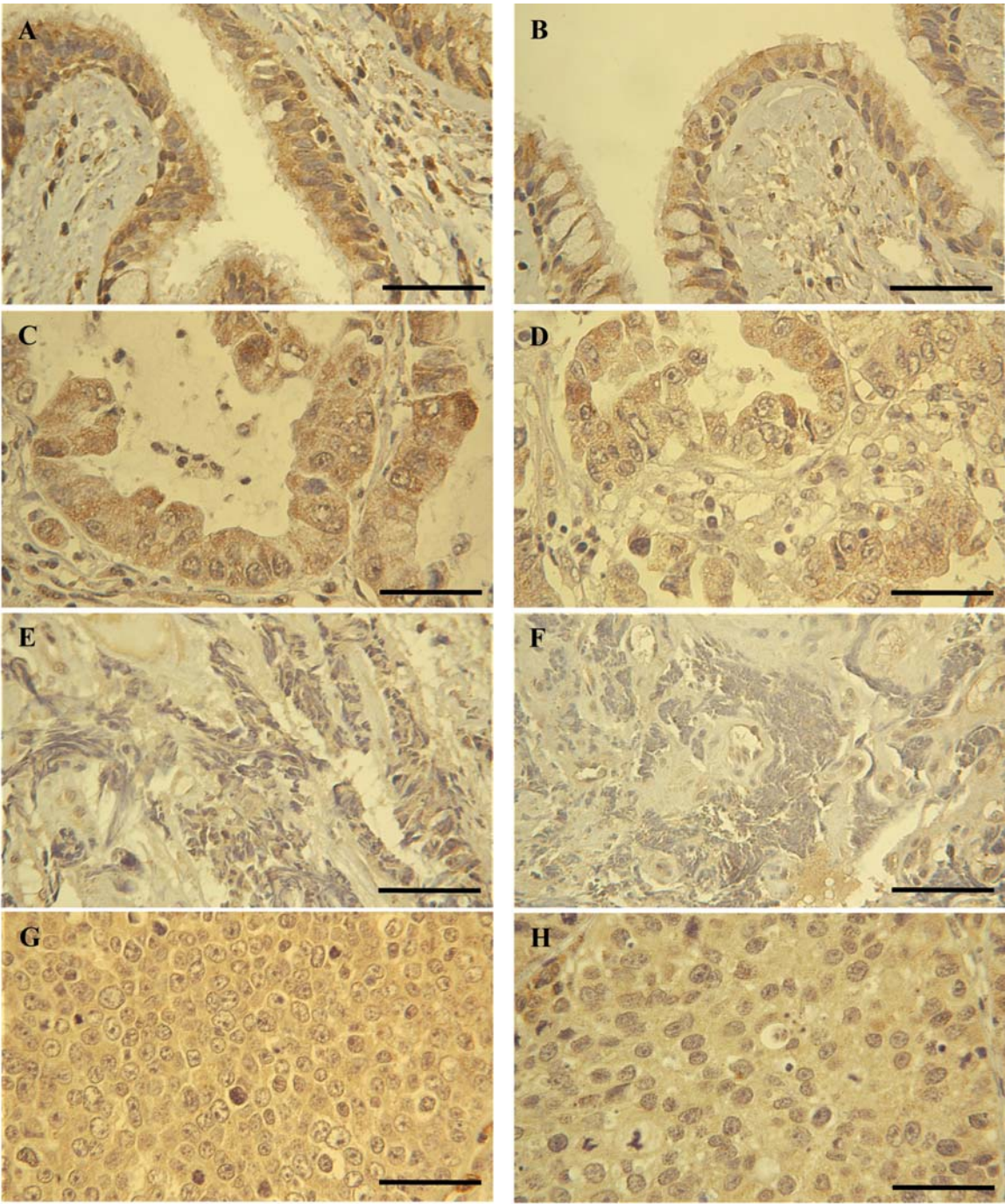


Figure 2. ET<sub>A</sub>R and ET<sub>B</sub>R immunostaining in lung cancer tissues. Areas of morphologically normal airway epithelium selected in samples from patients with adenocarcinoma (A and B), areas in adenocarcinoma (C and D), small cell carcinoma (E and F) and squamous cell carcinoma (G and H) samples were immunostained with antibodies raised against ET<sub>A</sub>R (A, C, E and G) or ET<sub>B</sub>R (B, D, F and H). Bars, 50 μm.

Table I. Semi-quantification of the ETR immunostaining intensity in normal and the 3 tumor groups.

| Group  | ET <sub>A</sub> R | ET <sub>B</sub> R | ET <sub>A</sub> R/ET <sub>B</sub> R |
|--|-------------------|-------------------|-------------------------------------|
| Normal (n=10)  | 3.4±0.7 (3.5)     | 2.6±0.8 (2.5)     | 1.3±0.3 (1.3)                       |
| ADK (n=12)   | 3.6±0.9 (4.0)     | 2.3±0.9 (2.0)     | 2.0±1.1 (1.7)                       |
| SCC (n=13)   | 2.7±0.6 (3.0)     | 2.3±0.5 (2.0)     | 1.3±0.4 (1.2)                       |
| SCLC (n=8 for ET <sub>A</sub> R and 7 for ET <sub>B</sub> R) | 1.4±0.5 (1.1)     | 1.8±0.9 (1.5)     | 0.9±0.5 (0.6)                       |

Mean ± standard deviation (median). ADK, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma; ET<sub>A</sub>R and ET<sub>B</sub>R, endothelin A and B receptors, respectively.



This study evaluated the presence of the endothelin receptors  $ET_A$  and  $ET_B$  by immunohistochemistry in biopsies and samples from patients with various types of lung carcinoma. The staining patterns in morphologically normal lung samples (widespread staining in epithelial cells for both receptors and no  $ET_A$ R staining and diffuse  $ET_B$ R staining in bronchial smooth muscle cells) were consistent with previously reported observations (35). Our data demonstrate that  $ET_A$ R and  $ET_B$ R are present in almost all samples of lung ADK and SCC, with levels similar to normal tissues; however, the  $ET_A$ R/ $ET_B$ R ratio is higher in ADK than in SCC. We also observed the presence of endothelin receptors in SCLC, although  $ET_A$ R levels and the  $ET_A$ R/ $ET_B$ R ratio are lower than in normal tissues and in other tumor types.

Previous studies on the expression of ET receptors in lung carcinomas have provided conflicting results and did not distinguish between different types of lung cancer. Boldrini *et al* (25) and Ahmed *et al* (23) studied the expression of  $ET_A$ R and  $ET_B$ R mRNA in 201 samples collected from NSCLC patients and in a panel of 7 SCLC and 4 NSCLC cell lines, respectively. Boldrini *et al* (25) found that  $ET_A$ R was expressed in 42.5% of tumor samples vs. 28.5% for the adjacent normal lung tissue. In contrast, Ahmed *et al* (23) found that  $ET_A$ R mRNA was most abundant in the control normal bronchial epithelial cells, with low expression in lung cancer cell lines. The present study revealed that  $ET_A$ R was present in nearly all samples of normal, ADK and SCC tissue. We found a similar staining intensity in ADK and SCC tumor samples compared to the adjacent normal lung tissue, suggesting that, contrary to several malignancies (20),  $ET_A$ R is not overexpressed in lung cancer. Moreover, similar to a report by Ahmed *et al* (23) we found that SCLC displayed no or a weak  $ET_A$ R signal compared to normal samples. Differences in  $ET_A$ R immunostaining between non-small cell carcinomas (ADK and SCC) and small cell carcinoma may be linked to the fact that they derive from different lung cells (epithelial and neuroendocrine, respectively). Regarding the expression of  $ET_B$ R, Boldrini *et al* (25) found that  $ET_B$ R mRNA was present in half of the lung tumor samples they tested (53%). In contrast, Ahmed *et al* (23) and the present study found that the presence of  $ET_B$ R was widespread in a panel of cell lines and in tumor samples, respectively. Recently, Knight *et al* (36) found that, due to the silencing of gene promoter by hypermethylation,  $ET_B$ R mRNA levels were reduced in freshly excised samples of NSCLC compared with normal adjacent lung tissue. In contrast, we found no difference in the  $ET_B$ R staining intensity between normal tissue and the three groups of tumors. Although decreased  $ET_B$ R levels have been observed in a majority of malignancies and  $ET_B$ R has even been reported to be absent in advanced prostate cancer (19), melanoma and colon cancer have been shown to have increased  $ET_B$ R expression (37,38). Overall, our results demonstrate that the  $ET_A$  and  $ET_B$  receptors are expressed in tumor cells in the main types of human lung carcinomas.

The effect of ET-1 is the result of its combined action on both receptors, leading to separate, overlapping or opposite effects. In tissues where both receptors are present, the comparison of the  $ET_A$ R/ $ET_B$ R ratio may be useful to determine the global effect of ET-1. It has been proposed that modifications in the relative levels of  $ET_A$ R to  $ET_B$ R in tissues, more precisely an increase in the  $ET_A$ R/ $ET_B$ R ratio, can lead to the progression of cells from a normal to a more malignant phenotype (19,39). We found that the  $ET_A$ R/ $ET_B$ R ratio differed between the lung cancer types. An increased  $ET_A$ R/ $ET_B$ R ratio has previously been reported in ovarian, prostate and breast cancers (20) and in contrast, a decreased  $ET_A$ R/ $ET_B$ R ratio has been observed in melanoma (37). If modifications of expression of ET receptors and of the  $ET_A$ R/ $ET_B$ R ratio are major determinants of the effect of ET-1 on cancer onset and progression, our results suggest that ET-1 may have a different impact on the different lung cancer types.

Our results suggest that endothelin receptor agonists and antagonists could be useful for the treatment of lung cancer. However, to date, there is no clinical assay on the effects of endothelin receptor antagonists on lung cancer. Endothelin receptor antagonists have shown promising preclinical results in prostate and ovarian cancer treatment (31,40,41). In prostate cancer, several phase II and phase III clinical trials with atrasentan (ABT-627) or ZD4054, two highly  $ET_A$ R selective antagonists, did not demonstrate beneficial effects on time to progression (40,42-44). However, a small but significant positive effect has been observed on survival (44). Another therapeutic strategy is the combination of one endothelin receptor agonist or antagonist with an established anticancer drug. For example, the efficacy of paclitaxel on breast tumor bearing rats was improved by stimulating  $ET_B$ R (45). In this study, pretreatment with the  $ET_B$ R agonist IRL 1620 induced the delivery of increased amounts of paclitaxel to the tumor by increasing the blood flow, which caused a significant reduction in tumor volume and in some cases complete remission. Similarly, in a model of nude mice with nasopharyngeal carcinoma cell xenografts,  $ET_A$ R blockade by atrasentan inhibited the growth of tumor cells, and combined treatment of ABT-627 with the cytotoxic drug cisplatin or 5-fluorouracil produced additive antitumor effects (46). However, a phase I/phase II clinical trial on the effect of atrasentan plus carboplatin and paclitaxel in NSCLC demonstrated the treatment is safe, but has no significant benefit on efficacy and survival (47).

In conclusion, this study shows that  $ET_A$ R and  $ET_B$ R are found in nearly all samples from patients with lung carcinoma and that the different types of lung cancer have unique expression patterns. Further investigations are needed to evaluate i) the role of ET-1,  $ET_A$ R and  $ET_B$ R expression on lung cancer prognosis and clinical outcome and ii) the relevance of ETR agonists and antagonists in therapeutic strategies in lung cancer. Our results suggest that the differences in expression of the ET receptors in the various types of lung cancer may impact the efficacy of these molecules.



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