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TTF-1 Gene Expression in Human Lung Tumours

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Tissue-specific transcription factors control cell determination and differentiation. TTF-1 is a tissue-specific transcription factor expressed in the thyroid and lung. We investigated the expression of TTF-1 in normal human lung, and in various histopathological types of lung cancers by immunohistochemistry. In normal lung, TTF-1 expression was restricted to bronchial and alveolar epithelial cells. TTF-1 expression was found in 7 of the 29 cases of non-small cell lung carcinomas. In these tumours, the expression of TTF-1 did not correlate with the histological degree of differentiation. Results obtained using RNase protection assay confirmed that TTF-1 was expressed only in a subset of non-small cell carcinomas. TTF-1, as expected, was not expressed in neoplasms having a neuroendocrine cell origin, such as carcinoids. Interestingly, TTF-1 was always expressed in small cell lung carcinomas. These findings indicate that: (i) small cell lung carcinomas could originate from the endothermal cell lineage and (ii) dedifferentiation processes that operate in these neoplasms do not affect molecular mechanisms necessary for *TTF-1* gene expression.

Key words: lung cancers, gene expression, transcription factors, SCLC, NSCLC, immunohistochemistry

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INTRODUCTION

TISSUE-SPECIFIC transcription factors contribute to the transcriptional activation of genes expressed in particular cell types [1–3]. Tissue-specific transcription factors play a crucial role in the determination of cell fate during development and in the expression of differentiated phenotypes [4, 5]. TTF-1 is a tissue-specific transcription factor expressed in the thyroid, lung and in some areas of the brain during development [6]. Due to its early appearance during development, TTF-1 could play an important role in cell differentiation and morphogenesis of both the thyroid and lung [7].

Lung cancer is one of the most common causes of death [8] and is classified into two groups: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) which includes squamous cell, adenocarcinoma and large cell carcinoma [9]. In order to improve the diagnostic and prognostic value, this histopathological classification could be complemented by the use of molecular markers. Therefore, since the expression of TTF-1 is an early event during lung differentiation, the evaluation of TTF-1 expression in lung tumours may have an impact in clinical research and in the management of these neoplasms. TTF-1 activates transcription of thyroglobulin (*Tg*) and thyroperoxidase (*TPO*) genes in the follicular thyroid cell, but not in lung epithelial cells. Conversely, TTF-1 activates transcription of the surfactant B protein (*SBP*) gene in lung epithelial cells, but not in follicular thyroid cells [10]. The ability of TTF-1 to activate transcription of different sets of genes in the thyroid and lung remains unknown. The study of *TTF-1* gene expression in human lung cancers could also be of great benefit in beginning to understand the molecular mechanisms determining the tissue-specific restriction of TTF-1 transcriptional activity on different sets of genes.

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MATERIALS AND METHODS

Tissue collection and RNA extraction

Lung tumour specimens were obtained from 47 patients undergoing surgery for clinical indications. Immediately after surgery, pieces were cut into fragments of about 1 cm^3 and frozen in liquid nitrogen. Histological diagnosis was obtained for all tissues. Fetal lungs were taken from 16–18-week-old spontaneous abortions (3 cases). Total RNA from frozen tissues and cells was prepared by the guanidium-thiocyanate acid-phenol procedure [11].

Immunohistochemistry

Immunohistochemical detection of TTF-1 was performed on lung sections using an antiserum to TTF-1 raised in rabbit. The specificity of this antibody in immunohistochemical stud-

ies has been previously demonstrated [7]. Specimens of normal and neoplastic tissues from surgical lobectomy were fixed in 4% buffered formaldehyde and routinely embedded in paraffin. The sections were immunostained for TTF-1 using the avidin-biotinylated peroxidase (ABC) technique. Briefly, after rehydration and inhibition of endogenous peroxidase, the sections were incubated with 2% normal goat serum. Subsequent incubations were as follows (all at room temperature): antiserum to TTF-1 (1:200 diluted) for 2 h, biotin-labelled goat antirabbit for 30 min, avidin-biotinylated peroxidase complex (Vector Lab., Burlingame, California, U.S.A.) for 30 min, incubation in substrate solution containing 0.05% diaminobenzidine and 0.01% H_2O_2. All incubations with antibodies were performed in phosphate-buffered saline (PBS) solutions. After each step, sections were washed with PBS. The sections were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted in Permount. Positive and negative controls were carried out in each assay. In order to exclude false negative results due to variability of the staining efficiency, tumour sections used for TTF-1 staining included normal adjacent tissue. For each case, the extent of TTF-1 positivity was evaluated by determining the percentage of positive nuclei within the tumour. A minimum of 5000 cells, in different areas of each section was counted using a 400× magnification objective. A double staining for TTF-1 and chromogranin was performed in sections from fetal lungs using a monoclonal antibody to chromogranin A (Dako, Denmark) with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique.

RNase protection assay

Due to the low recovery of RNA from neoplastic samples, only a subset of the cases studied by using immunohistochemistry was also investigated with the RNase protection assay. A 73 bp Bgl II/Pst I DNA fragment of the coding sequence of human *TTF-1* was cloned in Bluescript and used as a template to transcribe the antisense RNA probe to be used in the RNase mapping assay. The DNA template was linearised using Sac I, treated with proteinase K, extracted with phenol/chloroform, ethanol precipitated and dissolved to 0.4 μg/ml. 0.2 μg of DNA were then incubated in 5 μl of *in vitro* transcription mixture (400 μM each ATP, GTP, CTP, 20 μM UTP, 0.03 mM DTT, 0.5 μl RNAsin, 1.5 μl α-^{32}P-UTP and 2.5 units of T3 RNA polymerase) at 37°C for 30 min. DNA was removed by digestion with RQ-DNaseI in 50 μl of digestion mix (40 mM Tris–Cl pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 10 μg tRNA, 1 unit RQ-DNaseI) at 37°C for 30 mins. In the RNase protection assay, 20 μg of total RNA and 50 000 cpm of probe were vacuum dried, resuspended in 10 μl of hybridisation buffer (0.05 M Pipes pH 6.8, 0.4 M NaCl, 1 mM EDTA, 80% formammide), heated at 96°C for 2 mins and incubated for 12–16 h at 45°C. Unhybridised RNA was digested by adding 300 μl of RNase mixture (40 μg/ml RNase YA, 2 μg/ml RNase T1, 0.01 M Tris–Cl pH 7.5, 0.3 M NaCl, 5 mM EDTA) and incubating for 60 min at 37°C. The mixture was then phenol/chloroform extracted and ethanol precipitated. Aliquots were loaded on a 6% polyacrylamide denaturing gel. The plasmid pAT153 cut with Hinf I and 5′ labelled using γ-^{32}P-ATP and polynucleotide kinase was used as a molecular weight marker. Quantitation of *TTF-1* mRNAs was performed by densitometric scanning of the autoradiograms using a LKB laser densitometer.

RESULTS

TTF-1 expression in normal lung

TTF-1 expression in normal lung tissues was investigated by using immunohistochemistry. A few epithelial cells of the bronchial wall and most of the epithelial cells of the alveolar structures showed a nuclear staining, indicating the presence of TTF-1 protein (Figure 1a). Neuroendocrine cells scattered along the bronchial wall could be identified by chromogranin staining in fetal lungs. In TTF-1-chromogranin double-stained sections, the chromogranin-positive cells did not express TTF-1 (Figure 1b). These results indicate that TTF-1 is expressed only in epithelial cells of the lung and therefore, could be used as a molecular marker of differentiation for neoplasms arising from this cell type.

TTF-1 expression in lung tumours

When used on sections from rat embryos, TTF-1 staining appeared to be specific [7]. However, in order to evaluate the possibility of obtaining false positive or false negative results in human samples, the staining efficiency of TTF-1 was tested both in TTF-1-positive and TTF-1-negative control tissues. Sections from positive controls (normal human lung, 10 samples and normal human thyroid, 10 samples) were always positive for TTF-1 staining in bronchial and alveolar cells and

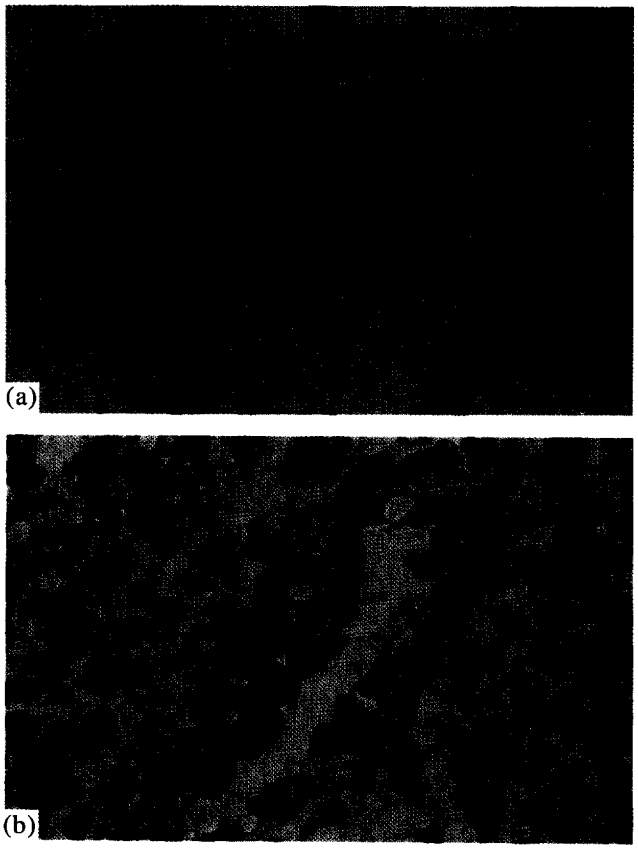

Figure 1. Immunohistochemical detection of TTF-1 in normal human lungs. (a) Normal adult lung: many epithelial cells lining alveolar walls show nuclear immunoreactivity for TTF-1 (immunoperoxidase-haematoxylin, ×400). (b) Fetal lung: the nuclei of epithelial cells are positive for TTF-1. A neuroendocrine cell, identified by chromogranin staining (red), is negative for TTF-1 (immunoperoxidase-APAAP-haematoxylin, ×1000).

in follicular cells (data not shown). In contrast, sections from negative controls (parathyroid adenoma, three samples; normal salivary gland, three samples; normal skeletal muscle, two samples and normal lymph node, two samples) were always negative for TTF-1 staining (data not shown). Therefore, the possibility of false negative or false positive results in performing TTF-1 staining in human tissue appears slight. TTF-1 expression was investigated in 29 NSCLC (13 squamous cell carcinomas, 15 adenocarcinomas, including four of the bronchioalveolar variant, and one large cell carcinoma), 10 SCLC and eight carcinoids, using immunohistochemistry (Table 1). Representative cases are shown in Figure 2. All SCLC tested were TTF-1 positive. As expected, all carcinoids (originating from neuroendocrine cells) were TTF-1 negative. Among NSCLC, a heterogeneous pattern was observed. In fact, only three of the 13 squamous cell carcinomas and four of the 15 adenocarcinomas showed the presence of TTF-1. No correlation was seen between TTF-1 expression and either histological degree of differentiation or size and nodal metastases (data not shown).

TTF-1 *mRNA expression*

Using the immunohistochemistry technique, only seven of the 29 NSCLC were positive for TTF-1. Surprisingly, several well differentiated adenocarcinomas and bronchioalveolar carcinomas did not express TTF-1. In order to corroborate results obtained by the immunohistochemistry technique, several samples of lung carcinomas were studied by using the RNase protection assay. This is a sensitive assay to measure mRNA levels. In fact, by this assay, we were able to measure the low amounts of *TTF-1* mRNA present in redifferentiated thyroid cells, not detectable by Northern blot (Lonigro and associates, unpublished data). As for the immunohistochemical detection of TTF-1, the possibility of obtaining false negative and (particularly, due to the sensitivity of the assay) false positive results was evaluated by using a panel of negative and positive control RNAs. In all positive controls tested (normal thyroid, three samples and normal lung, two samples), a TTF-1-specific signal was detected and, in contrast, negative controls (Hela and HepG2 cell lines) do not show TTF-1-specific signals (data not shown). *TTF-1* mRNAs of several lung cancers are shown in Figure 3. Table 2 shows results obtained by both RNase protection and immunohistochemistry. A good correlation was found when results of both methods were compared. In fact, in eight of the 14 tumours, results were concordant between RNase

Table 1. *TTF-1 protein expression in human lung tumours*

Diagnosis	*TTF-1* expression Positive/ total	%	Median value (range) of stained cells in positive samples
SCC	3/13	23	66 (7–98)
AC	3/11	27	85 (77–95)
BAC	1/4	25	34
CT	0/8	0	—
SCLC	10/10	100	79 (54–97)
LCC	0/1	0	—

SCC, squamous cell carcinoma; AC, adenocarcinoma; BAC, bronchioalveolar carcinoma; CT, carcinoid tumour; SCLC, small cell lung carcinoma; LCC, large cell carcinoma.

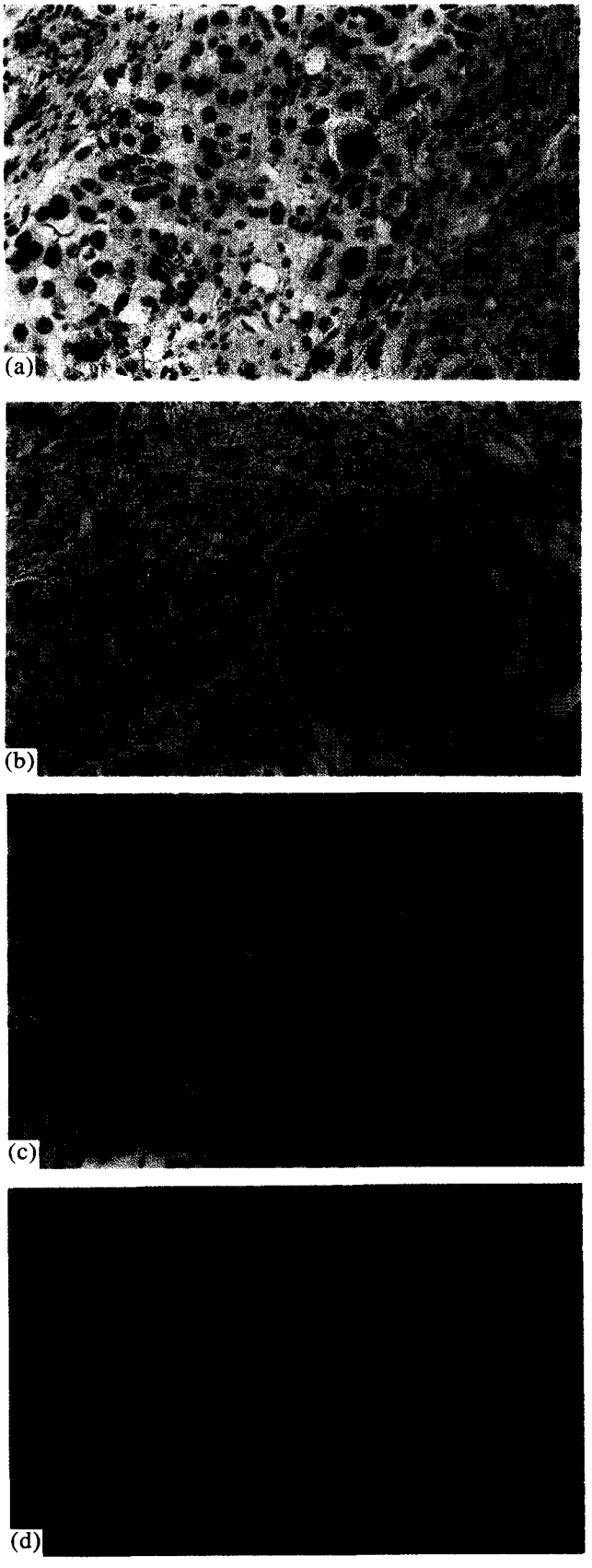

Figure 2. Different patterns of TTF-1 expression in lung tumours. (a) TTF-1 positive squamous cell carcinoma. (b) TTF-1 positive adenocarcinoma. (c) Small cell carcinoma showing intense immunoreactivity. (d) No TTF-1 staining is observed in a typical carcinoid. Immunoperoxidase-haematoxylin, ×400.

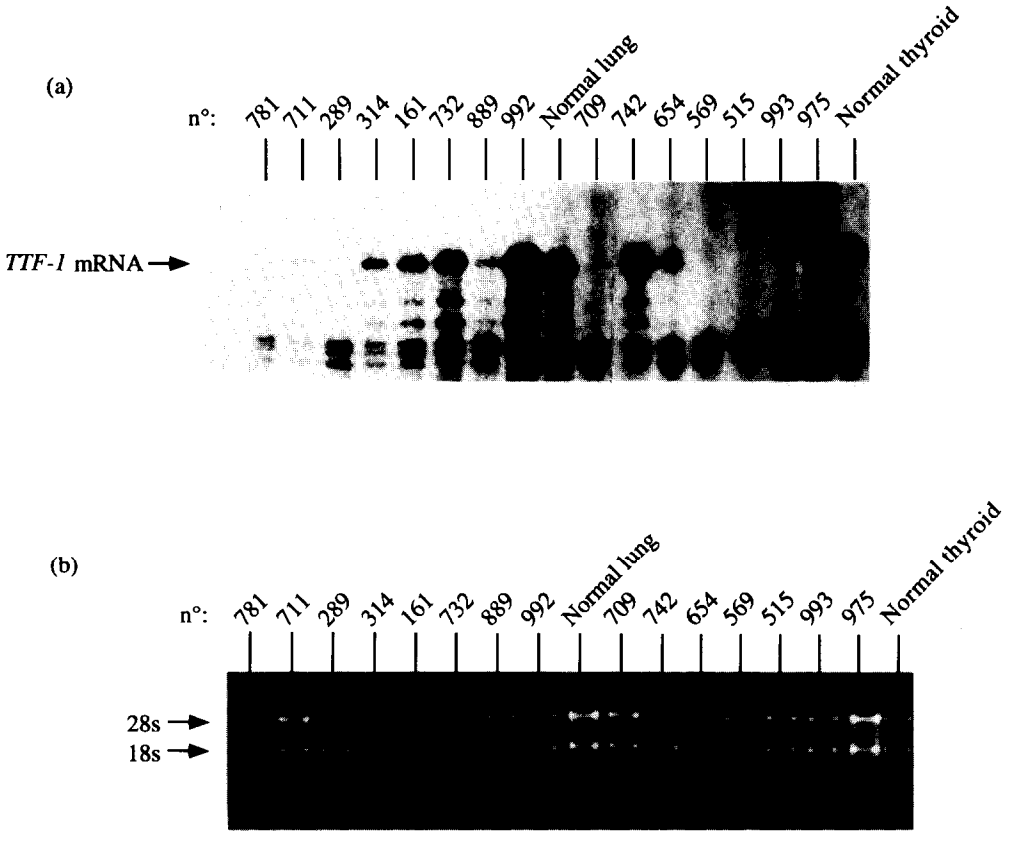

Figure 3. Detection of *TTF-1* mRNA in lung tumours by RNase protection. (a) Autoradiograms showing *TTF-1* mRNA detected by RNase protection assay. (b) Agarose gels showing the RNAs used in the RNase protection assay. In both, numbers above each lane indicate the case number.

Table 2. Relationship between TTF-1 *mRNA and protein in lung cancers*

Case no.	Diagnosis	*TTF-1* mRNA levels	TTF-1 protein (% of positive cells)
711	AC	3	Negative
161	AC	41	Positive (82%)
975	AC	96	Negative
889	SCC	6	Negative
781	SCC	2	Negative
314	SCC	9	Negative
654	SCC	27	Positive (7%)
992	SCC	177	Positive (93%)
515	SCC	0	Negative
569	SCC	0	Negative
709	CT	0	Negative
993	CT	0	Negative
742	SCLC	148	Positive (97%)
732	LCC	94	Negative

The diagnoses are indicated as in Table 1. *TTF-1* mRNA and TTF-1 protein levels were evaluated by RNase protection and immunohistochemistry, respectively. The values expressing *TTF-1* mRNA levels were obtained by densitometric scanning of autoradiograms.

protection and immunohistochemistry dialyses. In only two cases was a discrepancy evident between the two techniques (732 and 975). In one adenocarcinoma (711) and three squamous cell carcinomas (889, 781 and 314), very low levels of *TTF-1* mRNA were detected while TTF-1 protein was absent. Previous findings on control tissues exclude the possibility that these discrepancies are due to false positive or false negative results. A more plausible explanation is that the positivity detected by RNase protection was due to contaminating normal tissue. In fact, non-cancerous cell contamination is often observed in NSCLC [12, 13] and a careful observation of sections of our cases revealed the presence of normal cells trapped between aggregates of neoplastic cells (Figure 4). In cases 732 and 975, the absence of TTF-1 protein was associated with the presence of a high amount of the corresponding mRNA, and therefore an alternative

Figure 4. Squamous cell carcinoma: tumour cells are negative for TTF-1 but residual alveolar cells within the tumour show nuclear positivity. Immunoperoxidase-haematoxylin, ×400.

explanation could be that neoplastic cells of these tumours produced *TTF-1* mRNA, but either a specific block of *TTF-1* mRNA translocation or a rapid degradation of the translated protein occurred.

DISCUSSION

The study of transcription factors in human tumours appears to be a promising approach in understanding the molecular events of neoplastic transformation [14, 16]. The expression of TTF-1 has been investigated both in thyroid and lung neoplasms ([17] and this research). In differentiated thyroid tumours (originating from the follicular cell) TTF-1 is always present. In contrast to this finding, some lung tumours of epithelial origin, histologically having a differentiated phenotype, do not express TTF-1. The reasons for the discrepancy between thyroid and lung tumours are not evident and future studies are needed to determine whether the molecular mechanisms underlying *TTF-1* gene expression are different in thyroid and lung, and only the latter are sensitive to neoplastic transformations or whether the pathogenetic events driving neoplastic transformation are different in thyroid and lung, and those present in lung tumors are more effective in preventing *TTF-1* gene expression.

A second interesting finding of this study was that TTF-1 was expressed in all SCLC. Most of these tumours are composed of cells with a neuroendocrine phenotype, marked by high levels of L-dopa decarboxylase, neuron-specific enolase and neuropeptides [18]. These characteristics resemble those of endocrine cells found in normal bronchial mucosa. Therefore, it has been proposed that SCLC are derived from neuroendocrine cells [19–21]. This view is supported by the finding that the nervous system-specific transcription factor N-Oct 3 is present in SCLC cells [22]. In contrast, NSCLC appear to have an endodermal origin [18] and thus, SCLC and NSCLC probably originate from different cell lineages. However, a contradicting hypothesis considers that SCLC and NSCLC originate from a common stem cell. This view is based on several observations: (i) clinical evidence of transition between the different tumours, and mixed tumours with both SCLC and NSCLC phenotypes; (ii) development of NSCLC in patients after chemotherapy for SCLC; and (iii) induction of a NSCLC phenotype when SCLC cell lines are transfected with several oncogenes [23]. Our data demonstrate the presence of TTF-1 in SCLC, but not in neuroendocrine cells of the normal lung or in tumours having a clear neuroendocrine origins, such as lung carcinoids and thyroid medullary carcinomas. These findings support the view that SCLC tumours develop from an endodermal precursor and, therefore, SCLC and NSCLC appear to develop from a common epithelial stem cell [24]. Further studies on expression of transcription factors specific for epithelial cells of the lung (such as HFH-4 or HFH-8) [25] or for neuroendocrine cells (such as isl-1) [26] in SCLC and NSCLC could definitely demonstrate this point.

At this stage, our findings suggest only a limited clinical usefulness of TTF-1 detection. However, the observation that all SCLC examined show TTF-1 positivity suggests a potential use of this marker for clinical purposes. TTF-1 detection could distinguish SCLC from some atypical carcinoids showing histological characteristics similar to SCLC [27]. In addition, the evaluation of TTF-1 may help to indicate the origin of metastatic cells when the location of the primary tumour is unknown (up to 5% of SCLC patients) [28]. TTF-1 detection could also be useful for detecting recurrences of SCLC after achieving a complete remission. However, the positivity of normal lung tissue may preclude using this test as there may be too many false positives (due to TTF-1 present in normal cells). Further studies are required before the use of TTF-1 detection in clinical practice can be recommended. Between RNAse protection and immunostaining, we provide evidence that results obtained using the former method could be biased by the presence of normal tissue, so TTF-1 immunohistochemical detection is probably the better method for extensive clinical trials.

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