Detection of epithelial- and neural type of intermediate filament proteins in human lung tumors

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With 4 figures and 2 tables in the text

Introduction

Human lung cancer is a form of malignancy with a very high mortality rate and, furthermore, with an increase in tumor incidence, especially among women in western countries. Individual lung tumors are often characterized by a high degree of heterogeneity in their tumor cell population. Based upon histological and cytological criteria, tumors can be divided into two major groups. These are the non-small cell lung cancers (NSCLC), which can be subdivided into adenocarcinomas, large cell carcinomas, and small cell lung cancers (SCLC), which can be subdivided into pure SCLC and morphological/biochemical variants of these which have been designated variant SCLC (Carney et al. 1985). At the moment each category has its own specific therapeutical approach. Small cell lung cancers, for example, are primarily subjected to nonsurgical procedures.

Therefore, pathological diagnosis is of great importance and requires well defined criteria. However diagnosis is rather difficult and hampered by the availability of only a limited number of diagnostic tools, which, furthermore, are mostly of histological and cytological origin. At the moment, pathological diagnosis of lung tumors can be summarized as follows. Although on first sight the terms "small cell lung cancer" and "non-small cell lung cancer" might suggest that differentiation between these two groups can easily be performed by light microscopy on basis of cell-size, the continuous spectrum of this morphological determinant between the smallest and largest carcinoma cells confronts the pathologist with considerable problems in determining the tumor type (Hirsch et al. 1983, Vollmer et al. 1985). Problems are especially pronounced in cases where the diagnosis must be made on basis of small and mechanically damaged biopsies. Sputum cytology, bronchial brushes or lung aspirations are often of little help.

A major problem in lung cancer diagnosis is the heterogeneity of the tumor cell population, especially because tumor cell heterogeneity appears to be variable. The WHO-classification of lung tumors (WHO, 1982) is based upon histological and cytological criteria and distinguishes between three subtypes of SCLC, namely oat-cell type, intermediate cell type, and a mixed oat-cell type consisting of oat-cell carcinoma cells mixed with non-SCLC cells. Clinicopathologically the distinction between oat-cell carcinoma and the intermediate cell type carcinoma appears to be of minor significance (Hirsch et al. 1988, Radice et al. 1982) and very difficult to perform (Vollmer et al. 1985). More important, however, appears to be the distinction between the small cell lung cancers containing large cells on the one hand and the "pure"

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SCLC on the other (Radice et al. 1982, Hirsh et al. 1983). As mentioned above, the 
mixed small cell/large cell type of SCLC appears to represent a distinct pathological 
variant of SCLC and is therefore called variant SCLC. Variant SCLCs differ from the 
true SCLC subtypes in a number of characteristics, including lower therapeutic 
response rates and shorter survival periods. Hence, a proper identification of the 
different subpopulations within SCLC is of great prognostic and therapeutic value. 
Differences in response to cytostatics are expected to increase in the future with the 
introduction of new therapeutic modalities.

Mixed lung tumors of both epidermoid (squamous cell carcinoma) and adenocarcinomatous composition have generally been considered a rare occurrence. However, 
after including other than routine histological characteristics, such as ultrastructural 
features observed in the electron microscope, in the diagnosis of "pure" pulmonary 
squamous cell carcinomas or "pure" pulmonary adenocarcinomas, Trump et al. (1982) 
concluded that 49% of all lung carcinomas examined are of a mixed squamous cell 
carcinoma/adenocarcinoma composition. It is important to precisely define the relative 
proportion of each component since the overall composition may influence prognosis 
and especially the choice and effect of post-surgical treatment.

The origin of bronchial carcinoids and particular subtypes of small cell lung cancer 
is thought to be similar. Histologically and cytologically, the morphology of carcinoids 
and small cell lung cancers of the intermediate cell type can look alike with a con-
tinuum of expression of features from the two types of carcinoma ranging from true 
carcinoids through atypical carcinoids to small cell lung cancer. The differential 
diagnosis between these neoplasms is very important, since small cell lung cancer is 
a more aggressive tumor which may be treated by a combination of radiation and 
chemotherapy, while bronchial carcinoids are usually treated by surgical resection.

Differential diagnosis of pleural malignant mesothelioma and pulmonary adenocarcinoma is often difficult, not only when the diagnosis has to be made on cytological 
preparations, but also on pleural biopsy material. The exact diagnosis is also important 
in this case, because of the different therapeutic approaches. Since a few years, 
malignant mesotheliomas are treated chemotherapeutically.

From the data mentioned above, it may be clear that a variety of highly specific 
diagnostic tools are required to differentiate between the various types of lung tumor 
cells and to help disentangle the complicated pathways which are followed in their 
differentiating or dedifferentiating course.

Several authors have stressed the importance of the development of biochemical 
markers and their application in immunocytochemistry of lung tumors. Amongst 
others (CEA, beta-HCG, EMA, alpha-fetoprotein and neuron-specific enolase) inter-
mediate filament proteins, which form a considerable part of the cytoskeleton and 
occuring in virtually all cell-types, have been shown to be very useful markers in 
lung tumors antibodies to intermediate filament proteins have given rather contra-
dictory results, especially when small cell lung tumors and their cell lines were tested.

Table 1 gives an overview of data published on small cell lung cancer and lung carci-

inoids in the recent literature. Lehto et al. (1983), for example, showed that antibo-
dies to neurofilament proteins might be used to distinguish between oat-cell carcino-

ma (6 cases studied) and other carcinomas of the lung. These authors did not find 
reactivity of small cell lung carcinomas with their cytokeratin antibody. In another 
manuscript Lehto et al. (1984) describe also bronchial carcinoids to be positive for 
nurofilaments but not for cytokeratins. These results are in accordance with findings 
of Gustavson et al. (1982) and Espinosa and Azar (1982) who find that only part of the 
small cell carcinomas of the lung are positive for cytokeratins. The data from Lehto 
et al. (1983, 1984), however, were adjusted in a more recent paper (Lehto et al. 1986), 
showing that carcinoids may co-express cytokeratins and neurofilaments. Van Muijen
Table 1. Expression of intermediate filament proteins in SCLC, in SCLC cell lines, and in lung carcinoids as described in the recent literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cytokeratins</th>
<th>Neurofilaments</th>
</tr>
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<tbody>
<tr>
<td><strong>Solid SCLC tumors</strong></td>
<td></td>
<td></td>
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<tr>
<td>Gusterson et al. (1982)</td>
<td>2/15</td>
<td>n.t.</td>
</tr>
<tr>
<td>Lehto et al. (1983)</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Saba et al. (1983)</td>
<td>8/8</td>
<td>n.t.</td>
</tr>
<tr>
<td>Said et al. (1983)</td>
<td>2/16</td>
<td>n.t.</td>
</tr>
<tr>
<td>Sappino et al. (1983)</td>
<td>6/15</td>
<td>n.t.</td>
</tr>
<tr>
<td>van Muijen et al. (1984)</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Blobel et al. (1985)</td>
<td>6/0</td>
<td>0/6</td>
</tr>
<tr>
<td>Broers et al. (1985a)</td>
<td>8/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Gatter et al. (1985)</td>
<td>2/3</td>
<td>n.t.</td>
</tr>
<tr>
<td><strong>SCLC cell lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bergh et al. (1984)</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Banks-Schlegel et al. (1985)</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Broers et al. (1985b)</td>
<td>7/13</td>
<td>3/13</td>
</tr>
<tr>
<td>de Leij et al. (1985b)</td>
<td>2/4</td>
<td>1/4</td>
</tr>
<tr>
<td><strong>Lung carcinoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kahn et al. (1983)</td>
<td>13/13</td>
<td>n.t.</td>
</tr>
<tr>
<td>Said et al. (1983)</td>
<td>0/11</td>
<td>n.t.</td>
</tr>
<tr>
<td>Altmannsberger et al. (1984)</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Lehto et al. (1984)</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Lehto et al. (1985)</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Blobel et al. (1985)</td>
<td>4/4</td>
<td>2/4 (1)</td>
</tr>
<tr>
<td>Broers et al. (1985a)</td>
<td>4/4</td>
<td>0/4</td>
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et al. (1984), Blobel et al. (1985) and Broers et al. (1985a) have recently demonstrated the expression of cytokeratins in SCLC. These authors were unable to show neurofilament expression in this type of lung cancer.

When SCLC cell cultures were examined with intermediate filament antibodies, similar observations were made. Bergh et al. (1984) could show only neurofilaments in SCLC cell lines, while Banks-Schlegel et al. (1985) have found both cytokeratins and neurofilaments in such cultures. Broers et al. (1985b) could differentiate between classic- and variant-type SCLC cell lines, with classic cell lines expressing cytokeratin, but not neurofilaments, and the variant cell lines partly containing neurofilaments but not cytokeratins.

Cytokeratin positive reactions have also been found in squamous cell carcinomas and adenocarcinomas of the lung. Gusterson et al. (1982) furthermore state that the heterogeneity of phenotypic expression in lung tumors is not recognisable without the use of immunohistochemical techniques, in this case, the use of cytokeratin antibodies. Furthermore, Cosson and Pinkus (1982) use strong cytokeratin staining of mesothelioma and weak cytokeratin reaction of pulmonary adenocarcinomas in paraffin sections as a distinguishing feature for the differential diagnosis of these two types of tumors.

In the underlying report we have summarized our findings on lung cancers using both polyclonal and monoclonal antibodies to different types of intermediate filament proteins.
Materials and methods

For this study we have used both frozen section material of fresh tumor material next to paraffin
sections of routinely processed formalin fixed tissue blocks. Table 2 summarizes the different
types and numbers of lung tumors examined. Antibodies to epithelial-, neuroendocrine-, and neural
marker proteins were used to identify lung tumors and to characterize their constituent tissue
types.

Table 2. Reactivity pattern(1) of the different types of lung tumors with antibodies to intermediate
filament proteins

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>pKer</th>
<th>RCK102</th>
<th>PKK1</th>
<th>RGE53</th>
<th>RKSE50</th>
<th>NF</th>
<th>VIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-differentiated squamous cell carcinoma</td>
<td>45/45</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>40/45</td>
<td>0/45</td>
<td>0/45</td>
</tr>
<tr>
<td>Poorly differentiated squamous cell carcinoma</td>
<td>38/40</td>
<td>5/5</td>
<td>5/5</td>
<td>40/40</td>
<td>0/40</td>
<td>6/40</td>
<td>0/40</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>16/17</td>
<td>9/11</td>
<td>5/5</td>
<td>17/17</td>
<td>3/17</td>
<td>0/17</td>
<td>0/17</td>
</tr>
<tr>
<td>SCLC</td>
<td>18/22</td>
<td>10/16</td>
<td>5/5</td>
<td>16/10</td>
<td>0/10</td>
<td>3/24</td>
<td>0/10</td>
</tr>
<tr>
<td>Lung carcinoid</td>
<td>4/4</td>
<td>4/4</td>
<td>—</td>
<td>4/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Adenoid cystic carcinoma</td>
<td>—</td>
<td>—</td>
<td>5/5</td>
<td>—</td>
<td>—</td>
<td>5/5</td>
<td>—</td>
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</table>

(1) Number of positive tumors over number of tested tumors.
(2) Small areas of keratinizing cells are stained.

For detection of epithelial differentiation we have used different types of cytokeratin
antibodies.

1. A polyclonal antiserum pKer directed against skin keratins stains virtually all types of
epithelia including bronchial basal and cuboidal epithelial cells (Ramaekers et al. 1983b).
2. A monoclonal antibody RCK102 reacting with several cytokeratin polypeptides and recog-
nizing virtually all epithelia.
3. The monoclonal antibody PKK1 (kindly provided by Dr. I. Virtanen, Helsinki) reacting in
a similar manner as RCK 102 and recognizing virtually all epithelia (Holthöfer et al. 1983).
Next to these broadly cross-reacting antibodies monoclonal antibodies to specific subtypes of
cytokeratins, which react in a more restricted manner have been applied.
4. The monoclonal antibodies RGE53 and CK18-2 recognize mainly glandular epithelial tissues
and tumors, but do in general not react with squamous epithelial cells or squamous cell carcinomas
(Ramaekers et al. 1983b).
5. The monoclonal antibody RKSE50, on the other hand, stains only keratinizing stratified
squamous epithelia and keratinizing areas within tumors (Ramaekers et al. 1983b). Next to these
cytokeratin antibodies monoclonal and polyclonal vimentin antibodies were used (Ramaekers et al.
1983b).

The neural and neuroendocrine markers that we have used include polyclonal and monoclonal
antibodies to neurofilaments which have been described in detail elsewhere (Kilk et al. 1983,
Nakazato et al. 1984). These antibodies are shown to react only with neural tissues such as nerve
cells and with tumors and tissues of neural origin. Also the monoclonal antibody MOC-1, which
has been described by de Leij et al. (1985a) was used. This antibody appears to recognize a neuro-
endocrine differentiation antigen and in our hands this antibody reacts strongly with lung carcinoids
and small cell lung carcinomas.

The indirect immunofluorescence technique and immunoperoxidase technique have been
described extensively before (Ramaekers et al. 1983b).

Antibodies pKer, RCK 102, the polyclonal antiserum to vimentin and the neurofilament anti-
bodies could successfully be applied to paraffin embedded material.
Results

When squamous cell carcinomas of different degrees of differentiation were examined with cytokeratin antibodies, it was obvious that all tumors reacted with the general antibodies pKer, RCK 102 and PKK1 (Fig. 1A and Fig. 2A). Keratinizing cells could only be detected in highly differentiated tumors or tumor areas with the monoclonal antibody RKSE 60 (Fig. 2B).

When these neoplasms were investigated with the monoclonal antibody specific for glandular epithelium and adenocarcinomas (RGE 53), it was striking to notice a variable number of cells to be reactive with this antibody (Fig. 2C, D). Highly differentiated areas were negative however (Fig. 1B). In such fields of tumor cells alveolar pneumocytes, which are entrapped in the malignancy, were positive with RGE 53.

In some cases of poorly differentiated squamous cell carcinomas (Fig. 3) complete areas or complete tumor samples appeared to show this adenocarcinomatous marker (Fig. 3B). When these poorly differentiated tumors were incubated with the MOC-1 antibody it was obvious that in some cases certain areas showed a clear neuroendocrine differentiation (Fig. 3C). And even more striking was the observation that within tumors, diagnosed as poorly differentiated squamous cell carcinoma, variable numbers of cells could be detected which were reactive with the antibodies to neurofilaments (Fig. 3D—F).

Pulmonary adenocarcinomas were diffusely positive with the broadly cross-reacting cytokeratin antibodies, as well as with the more restricted antibody RGE 53 (Fig. 1D). No reaction was found with antibody RKSE 60 or with any of the neurofilament antibodies.

Of the small cell lung carcinomas that we have examined in frozen sections and paraffin sections virtually all could be stained with the cytokeratin antibodies pKer (Fig. 1E), RCK 102, PKK1 and RGE 53 (Fig. 1F). The distribution of cytokeratins in SCLC is rather typical in that in most cases a speckled thin rim around the nucleus is stained. None of the SCLCs that we have examined showed large areas of neurofilament positive cells. However, in three of twenty-four cases examined scattered neurofilament positive cells were found.

Also carcinoids could be shown to react positive with several cytokeratin antibodies (see Table 2 and Fig. 4A, B). Furthermore one of the cases examined could be shown to co-express neurofilament and cytokeratin proteins in frozen sections and in paraffin material (Fig. 4D—F). Using monoclonal and polyclonal antibodies to the different subunits it became clear that especially an antiseraum exclusively recognizing the 100 kD neurofilament protein gave a strong reaction with virtually all cells of this tumor. An antiseraum to the 200 kD neurofilament protein and the monoclonal neurofilament antibody stained much lesser cells.

Adenoid cystic carcinomas of the lung, examined as frozen sections (4 cases) or present in a thin needle aspiration, could be shown to co-express cytokeratin 18 and vimentin. This tumor-type does not seem to express neurofilament proteins.

Discussion

The results described in the underlying paper demonstrate that most, if not all lung carcinomas can be stained by cytokeratin antibodies, which exhibit a general cross-reactivity between epithelia.

Specific monoclonal antibodies to subtypes of cytokeratins can recognize certain directions of epithelial differentiation, i.e. adenocarcinomatous (RGE 53), or keratinizing squamous cell differentiation (RKSE 60). In epidermoid lung cancers, especially

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Fig. 1. Pulmonary squamous cell carcinomas (A, B), adenocarcinomas (C, D) and small cell lung carcinomas (E, F) incubated with several cytokeratin antibodies. A) RCK102; B) CK18-2; C) pKer; D) RGE53; E) pKer; F) RGE53. Indirect immunoperoxidase technique on frozen sections.
Fig. 2. Well-differentiated squamous cell carcinomas of the lung incubated with the cytokeratin antibody pKer (A), RKSE 60 (B), and RGE 83 (C, D). Indirect immunoperoxidase (A, B, D) and indirect immunofluorescence (C) technique on frozen tissue sections.

poorly differentiated squamous cell carcinomas a profound heterogeneity was observed with these antisera, in that such tumors which had been diagnosed as real squamous cell carcinomas exhibited low to high numbers of cells with adenocarcinomatous differentiation.

Furthermore, antibodies to neuroendocrine and neural marker proteins (neurofilament proteins) showed that some of these poorly differentiated squamous cell carcinomas can contain neuroendocrine components and even cells with neural characteristics. With respect to this latter finding it should be kept in mind that variant-type SCLC cell cultures can express neurofilaments next to cytokeratins (Broens et al. 1985b) and that such cell lines are derived from SCLCs containing large cell components (Carney et al. 1980). Whether or not the neurofilament-positive cells in poorly differentiated squamous cell carcinomas represent variant SCLC cells remains to be examined.

Our results furthermore show that all SCLCs and lung carcinoids express cytokeratins, which can be detected both in frozen sections and in paraffin sections (although in a lesser amount) when proper cytokeratin antibodies are applied. These results are in accord with several reports in the literature (see Table 1). We have found only few SCLCs and one carcinoid which expressed (few scattered) neurofilament positive cells next to a diffuse cytokeratin staining reaction. Therefore, we cannot sustain results described by Lehto et al. (1983), who found extensive staining with a neurofilament antibody in all SCLC tumors examined, but no reactivity with their cytokeratin antibody. Co-expression of cytokeratins and neurofilament proteins seems to be restricted to a limited number of several types of neuroendocrine tumors. Neurofilaments have been found next to cytokeratins in neuroendocrine skin carcinomas (Merkel cell tumors; Gould et al. 1985, Miettinen et al. 1983, Sibley et al. 1985, van Muijen et al. 1985) and in some pancreatic islet tumors (insulinomas; Miettinen et al. 1985). The possible cell of origin for these tumors, (Merkel cells in skin and
Fig. 3. Poorly differentiated squamous cell carcinoma of the lung incubated with cytokeratin antibodies pKar (A), and RGE 53 (B), with the neuroendocrine marker MOC-1 (C), and the monoclonal neurofilament antibody (D) and the polyclonal antiserum to the 180 kD neurofilament polypeptide (E) and the 200 kD neurofilament protein (F). Indirect immunoperoxidase technique on frozen sections (A—D) and PAP-technique on paraffin sections (E, F).
Fig. 4. Pulmonary carcinoid incubated with cytokeratin antibodies RCK 102 (A), and RGE 83 (B), the neuroendocrine marker MOC-1 (C), the monoclonal neurofilament antibody (D), and the two polyclonal neurofilament antisera directed against the 160 kD (E) and the 200 kD (F) neurofilament polypeptides. Indirect immunoperoxidase technique on frozen sections (A—D) and PAP-technique on paraffin sections. Note the perinuclear dot-like staining reaction of cytokeratin- and neurofilament antibodies, not easily distinguishable in black- and white illustrations.
pancreatic islet cells respectively) express only cytokeratins and do not contain neurofilaments. Therefore it would be of interest to study the intermediate filament expression in Kulchitsky cells, a possible candidate for the cell type from which small cell lung carcinomas and lung carcinoids arise. In general it seems that neuroendocrine cells have the potential to express neurofilaments as a result of malignant growth or neoplasia.

Summary

Five different types of lung cancers, i.e. squamous cell carcinomas, adenocarcinomas, small cell lung carcinomas, carcinoids and adenosquamous carcinomas were examined for their intermediate filament constituents, with special emphasis on the different cytokeratin polypeptides and neurofilament proteins. Polyclonal as well as monoclonal antibodies to these proteins were used in immunocytochemical techniques applied to both tumor frozen sections and paraffin sections.

Squamous cell carcinomas and adenocarcinomas could be shown to contain cytokeratins, which could be detected in both frozen sections and paraffin sections. Also small cell lung carcinomas (SCLC) and carcinoid lung tumors showed a positive staining reaction with polyclonal and monoclonal (cyto)keratin antibodies, but were negative with neurofilament antibodies, with the exception of one case of lung carcinoid, which co-expressed neurofilaments and cytokeratins. We have used antibodies to cytokeratin polypeptides, to neurofilament proteins and to a neuroendocrine related membrane antigen (MOC-1) to further subclassify heterogeneously composed squamous cell carcinomas. Using a monoclonal antibody to cytokeratin 18, normally present in glandular tissues and adenocarcinomas, we observed that more than 90 % of the squamous cell carcinomas examined can be stained with this antibody. The percentage of tumor cells, however, positive for cytokeratin 18 varied between 1 and 100 %. In these same tumors a monoclonal antibody to skin keratins, which is known to react specifically with keratinizing cells, also stained variable numbers of tumor cells. This finding confirms the presence of (keratinizing) squamous cell carcinoma elements in these tumors. Our data show that most lung tumors, heretofore considered pure squamous cell carcinomas, should be considered biologically adenosquamous carcinomas. Also areas positive with MOC-1 were found in these tumors, suggesting the presence of squamous cell carcinomas with neuroendocrine differentiation. Furthermore, in some poorly differentiated squamous cell carcinomas areas with neurofilament positive cells were detected, suggesting a neural differentiation within these neoplasms. Adenoid cystic carcinomas are shown to co-express cytokeratins and vimentin in the tumor cells. This phenomenon can be used to identify such tumors and to distinguish them from other lung tumors.

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References


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