Immunocytochemical Detection of Human Lung Cancer Heterogeneity Using Antibodies to Epithelial, Neuronal, and Neuroendocrine Antigens


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ABSTRACT

Lung cancers were investigated for their heterogeneity as expressed by their immunoreactivity for cytokeratins and neurofilament proteins, as well as for the neuroendocrine differentiation antigen MOC-1. Using broadly cross-reacting antibodies, cytokeratins were detected in nearly all cases of lung carcinomas. Keratinization could be detected only in cases of moderately to well-differentiated squamous cell carcinoma (SQC) using a monoclonal antibody to cytokeratin 10, while a monoclonal antibody reactive with cytokeratin 18, and specific for glandular epithelia, reacted with adenocarcinomas, small cell lung carcinomas (SCLC), and lung carcinoids. In SQC this antibody could detect non-squamous cell differentiation, showing increasing numbers of positive cells with decrease of histologically detectable SQC differentiation. Cells positive for neurofilaments were demonstrated in some of the poorly differentiated SQCs and in some of the cases of SCLC, possibly representing the variant type of SCLC. Also in some of the lung carcinoids neurofilament proteins were present, colocalizing with cytokeratins. MOC-1 was present in all SCLC and lung carcinoids. This antibody could also detect neuroendocrine differentiation in all combined small cell carcinomas, in one poorly differentiated adenocarcinoma, and in about 30% of the poorly differentiated SQCs. Therefore, lung cancer heterogeneity can be detected using a panel of well-defined antibodies to intermediate filaments in combination with the MOC-1 antibody. The use of these antibodies in diagnosis can have prognostic significance and can lead to a more selective therapeutic approach.

INTRODUCTION

Human lung cancer is the main cause of cancer death in western countries and its occurrence is especially rapidly growing among women (1). Different subtypes of lung cancer can be classified according to their histological appearance. The four major subtypes are SCLC, large cell carcinoma, SQC, and adenocarcinoma (2). The three latter subtypes are often referred to as non-SCLC. Individual lung tumors may show a profound heterogeneous composition. For instance, according to the work of Hess et al. (3) in cytological specimens about 50% of all lung tumors can be classified as admixtures of epidermoid and adenocarcinomatous subtypes, while also at the electron microscopic level up to 50% of lung tumors classified as SQC on the basis of routine histology appear to contain characteristics of adenocarcinomatous differentiation (4). Another admixture of different tumor subtypes can be found in combined small cell carcinoma (5), an entity of lung cancer in which SCLC and non-SCLC differentiation are found simultaneously. Also in tumors which are histologically indistinguishable from non-SCLC, (neuro)endocrine features, such as the production of peptide hormones or the presence of marker enzymes, can be detected (6, 7). Because of differences in treatment and prognosis, differentiation between SCLC and non-SCLC is of utmost clinical importance (8). SCLC express neuroendocrine features, among which is the production of neurosecretory granules detectable in the electron microscope (9). Also the production of neurotransmitters, neuropeptides, amine precursor uptake and decarboxylation-related enzymes, and cell surface markers such as those detected by the monoclonal antibody specific for neural and neuroendocrine cells MOC-1 (10) indicate this type of differentiation. Another indicator of the neuroendocrine nature of tumor cells is the coexpression of both cytokeratins and neurofilament proteins, intermediate filament proteins generally specific for epithelial and neuronal cells, respectively (11–16).

SCLC can be subdivided into two different tumor types, namely the classic and the variant type of SCLC (5). The variant type of SCLC, occurring in about 6–15% of all SCLC at first diagnosis (17, 18), consists of a mixture of small cell and large cell components and occurs in up to 30% of all SCLC after chemotherapy and/or radiotherapy (19). Within SCLC cell lines the classic and variant subtypes can be distinguished from each other on the basis of the presence of cytokeratins and the absence of neurofilaments in the classic subtype and the absence of cytokeratins in the variant cell lines, which may contain neurofilaments (20, 21). Also coexpression of cytokeratins and neurofilaments in some SCLC cell lines has been suggested (22, 23).

In order to investigate the degree of heterogeneity in lung tumors and to detect the two different subtypes of SCLC we have applied immunocytochemical techniques in a large series of lung tumor specimens. We have tested for the presence of different subtypes of cytokeratins, the presence of neurofilaments, and the occurrence of the MOC-1 antigen (10). Our results are discussed in the light of lung cancer histogenesis and differentiation, and their significance for refinement of clinicopathological diagnosis is addressed.

MATERIALS AND METHODS

Normal Lung. Snap frozen specimens from normal human lung tissue from two different patients were stored in liquid nitrogen and 4–7-μm frozen sections were used in the immunoperoxidase technique. The frozen sections were fixed in acetone (–20°C, 5 min). Normal tissue material was resected with tumor material.

Lung Tumors. Fresh surgical specimens of 218 cases of lung cancer were snap frozen and stored in liquid nitrogen. Next paraffin sections of 76 routinely processed lung cancers from the Pathology Departments in Nijmegen and Nieuwegein, partly overlapping with the frozen section material, were used. In addition, 93 cases of paraffin-embedded lung tumors were obtained from Dr. J. Wilde, Zentralklinik für Herz- und Lungenkrankheiten, Bad Berka, G.D.R. Autopsy material (61 specimens in total) from primary tumors and metastases of up to 12 different body sites from 6 patients with a histological diagnosis of SCLC were investigated for the presence of cytokeratins and neurofilaments.

All lung tumors were classified on the basis of routine histological
hematoxylin-eosin staining according to the latest WHO classification (2).

Antibodies Used in This Study. The antibodies used in this study are summarized in Table 1 and have been described extensively before (10, 14, 20, 21, 24-32). Antibodies pKer, RCK102, RGE53, RKSEP60, MNP, and MOC-1 are available from Euro-Diagnostics B. V. (Apeldoorn, The Netherlands), CAM5.2 was from Becton and Dickinson (Eremboedgeum, Belgium), and pNF160 was a generous gift from Dr. Y. Nakazato (Gunma, Japan).

Immunofluorescence and Immunoperoxidase Technique for Frozen Sections. The indirect immunofluorescence technique and immunoperoxidase technique for fresh tissue sections, fixed in acetone (−20°C, 5 min), have been described extensively before (21). Dilutions of the primary antibodies were made in PBS (see Table 1).

As second antibodies in the indirect immunofluorescence technique either FITC-conjugated goat anti-rabbit IgG (1:25; Nordic, Tilburg, The Netherlands) or FITC-conjugated rabbit anti-mouse IgG (1:25; Nordic) were used. In control experiments PBS was used instead of the primary antibody.

In the immunoperoxidase technique swine anti-rabbit immunoglobulin conjugated to peroxidase (DAKOPatts, Glostrup, Denmark) diluted 1:25 in PBS containing 10% normal goat serum or rabbit anti-mouse immunoglobulin conjugated to peroxidase (DAKOPatts) diluted 1:25 in PBS with 5% human AB serum were used in the second step.

In order to detect coexpression of different types of intermediate filament proteins we have used the double immunofluorescence technique as described before (33) using the mouse monoclonal cytokeratin antibody RGE53 or RCK102 in combination with the rabbit neurofilament antibody pNF160. As second antibodies a mixture of FITC-conjugated goat anti-rabbit IgG (diluted 1:25; Nordic) and Texas Red-conjugated sheep anti-mouse immunoglobulin (diluted 1:50; New England Nuclear, Boston, MA) was used.

Paraffin-embedded Specimens. Cytokeratin antibodies pKer, RCK102, and CAM5.2 and the neurofilament antibodies MNP and pNF160 are the only antibodies of those summarized in Table 1 that can be applied successfully to formalin-fixed and paraffin-embedded material using the PAP method (34) or the avidin-biotin labeling technique (35).

PAP Technique (34). Paraffin-embedded tumor material was cut into 6-μm-thick sections and fixed onto glass slides using Ortho tissue adhesive (Ortho Diagnostic, Raritan, NJ). After deparaffinizing in xylene and a descending alcohol series, sections were incubated for 30 min in 100% methanol containing 2% hydrogen peroxide.

Sections used for the detection of cytokeratins were pretreated with 0.1% Pronase (Sigma Chemical Co., St. Louis, MO) in 0.05 M Tris-NaCl buffer, pH 7.6, for 15 min at 37°C. Pronase activity was terminated by washing in Tris-NaCl at 4°C for 30 min. Then, after a washing with PBS the sections were preincubated for 30 min with 10% normal swine serum in PBS and incubated overnight with the primary antibody at 4°C. Antibody dilutions were made in PBS containing 10% normal swine serum (see Table 1).

After washing in PBS, sections which were incubated with a monoclonal antibody in the first step were incubated with rabbit anti-mouse immunoglobulin (DAKOPatts) diluted 1:200 in PBS containing 10% normal swine serum for 30 min. After another washing step in PBS all sections were incubated with swine anti-rabbit immunoglobulin (DAKOPatts) diluted 1:30 in PBS with 10% normal swine serum for 30 min, washed again, and incubated with the rabbit PAP complex (DAKOPatts) diluted 1:100 in PBS with 10% normal swine serum for 30 min. Peroxidase activity was detected for 5 min using 500 mg/liter 3,3’-diaminobenzidine dissolved in 0.05 n Tris-NaCl containing 0.65% imidazole (Merck, Darmstadt, West Germany), and 0.015% hydrogen peroxide as a substrate. Cells were counterstained for 30 s with Harris’ hematoxylin (Merck) and after dehydration were embedded with Permount (Fisher Scientific Co., Springfield, NJ).

ABC Technique (35). Labeling with the ABC technique was performed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). After deparaffinizing and preincubation with 10% normal goat serum or 10% normal horse serum for 60 min, primary antibodies were applied overnight at 4°C using the same dilutions as for the PAP staining method. After a washing with PBS followed by a preincubation step with PBS containing 10% normal goat serum or 10% normal horse serum for 15 min the following conjugates were applied; biotinylated goat anti-rabbit IgG diluted 1:250 in PBS containing 10% normal goat serum for 30 min; or biotinylated horse anti-mouse IgG diluted 1:200 in PBS containing 10% normal horse serum for 30 min. After a second washing and preincubation step avidin-biotinylated peroxidase complex in PBS containing 10% normal horse serum or 10% normal goat serum was added for 30 min at an avidin-biotinylated horseradish peroxidase:PBS ratio of 1:1:100. Detection of peroxidase activity was performed as described for the PAP technique.

RESULTS

Normal Lung Tissue

The reactivity patterns of the antibodies described above in normal lung tissue are summarized in Table 2. Antibodies pKer, RCK102, and CAM5.2 react with all epithelial elements but not with nonepithelial components. RGE53 reacts with the columnar epithelial cells but not with basal cells of the bronchial lining. Also a positive staining reaction can be observed with this antibody in seromucous glands of the bronchi and in type I and type II pneumocytes within the alveoli. RKSEP60 does not react with any of the epithelial cells of the normal lung. MNP and pNF160 react only with nerve fibers. The MOC-1 antibody reacts with nerve fibers and with bronchial cells of the dispersed neuroendocrine system, the so-called Kulchitsky cells (10). These Kulchitsky cells are also positive with the cytokeratin 18 antibody RGE53 (36).

Squamous Cell Carcinomas

All SQCs show a diffuse reaction pattern (meaning a reaction in virtually all tumor cells) with the broadly cross-reacting cytokeratin antibodies pKer (Fig. 1a), RCK102, and CAM5.2 in both frozen and paraffin sections (Tables 3 and 4). In well-differentiated SQC a focal staining reaction (a reaction in part of the tumor cells) is seen with the monoclonal antibody RGE53, which is reactive only in frozen sections. The percentage of cells positive for RGE53 varied from zero (in two cases)

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### Table 1. Specificity of antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunoglobulin subclass (species)</th>
<th>Antigen recognized</th>
<th>Tissue specificity</th>
<th>Reactivity in tissue sections</th>
</tr>
</thead>
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<tr>
<td>pKer</td>
<td>Polyclonal (rabbit)</td>
<td>Several cytokeratins</td>
<td>Most epithelial tissues</td>
<td>Frozen (dilution): + (1:10) Paraffin (dilution): + (1:40) Ref.: 24</td>
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<td>RCK102</td>
<td>IgG1 (mouse)</td>
<td>Cytokeratin 5 and 8</td>
<td>Nearly all epithelial tissues</td>
<td>+ (1:5) + (1:10) 21</td>
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<td>CAM5.2</td>
<td>IgG2a (mouse)</td>
<td>Several cytokeratins</td>
<td>Nearly all epithelial tissues</td>
<td>+ (undiluted) + (1:5) 25</td>
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<td>RGE53</td>
<td>IgG1 (mouse)</td>
<td>Cytokeratin 18</td>
<td>Glandular epithelium, not with squamous epithelium</td>
<td>+ (undiluted) + (1:10) 27-29</td>
</tr>
<tr>
<td>RKSEP60</td>
<td>IgG1 (mouse)</td>
<td>Cytokeratin 10</td>
<td>Keratinizing epithelial cells</td>
<td>+ (1:5) + (1:10) 14, 20, 30, 31</td>
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<td>MNP</td>
<td>IgG1 (mouse)</td>
<td>Neurofilaments, M, 68,000 and 200,000</td>
<td>Neurofibers and neuronal tissues</td>
<td>+ (1:500) + (1:2000) 21, 32</td>
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<td>Cell surface</td>
<td>Neurofibers and neuronal tissues</td>
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<td>IgG1 (mouse)</td>
<td>Neurofilaments, M, 160,000</td>
<td>Neuronal and neuroendocrine tissues</td>
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</table>

3226
Table 2 Reactivity pattern of different antibodies to cytokeratins, neurofilaments, and surface proteins in frozen sections of normal lung tissues

<table>
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<tr>
<th>Tissue type</th>
<th>pKer.</th>
<th>RCK102 and CAM5.2</th>
<th>RGE53</th>
<th>RKSE60</th>
<th>MNF and pNF160</th>
<th>MOC-1</th>
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<td>-</td>
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<td>Columnar-cuboidal epithelium</td>
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<tr>
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<tr>
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<td>Mucous glands</td>
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<td>All stromal elements</td>
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<td>Nerve fibers</td>
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* Data from report of De Leij et al. (36).

Well-differentiated SQCs tested in frozen sections (13 of 15 [Table 3]) keratinizing areas can be demonstrated using the monoclonal antibody RKSE60 (Fig. 1c), while no reaction was found with MOC-1 or with neurofilament antibodies. The moderately well-differentiated SQC contained keratinizing areas in about one-half of the cases of frozen sections as detected by RKSE60 (44 of 85 [Table 3]). However, in frozen sections also many tumors (23 of 85) react diffusely with RGE53 (Table 3), while the presence of the MOC-1 antigen (13 of 77 [Table 3]) and neurofilaments could be demonstrated (5 of 77 [Table 3]) also, albeit in a lower number of cases as compared to poorly differentiated SQCs (17% versus 28% for MOC-1 and 6% versus 13% for neurofilaments; see below).

In contrast to the well-differentiated SQCs, in a great number of histologically poorly differentiated SQCs tested in frozen sections a diffuse staining reaction with RGE53 can be observed (14 of 27 [Table 3; Fig. 1d]), while in only a minor number of these latter tumors (8 of 28 [Table 3]) keratinizing areas or single keratinizing cells could be detected with RKSE60. Also the presence of the MOC-1 antigen was demonstrated in 7 of 25 cases of frozen sections of these poorly differentiated SQCs.

Fig. 1. Peroxidase anti-peroxidase staining on a paraffin section (a) and immunoperoxidase (b–f) and immunofluorescence (g, h) staining patterns in frozen sections of SQCs. a, cytokeratins detected in paraffin sections with the polyclonal cytokeratin antibody pKer. × 275. b, focal staining reaction of a small part of the tumor cells with the monoclonal antibody to cytokeratin 18, RGE53, in a well-differentiated SQC. × 290. c, RKSE60 stains only keratinizing cells within these well-differentiated SQC. × 170. In poorly differentiated SQC (d, × 220), while in some of these poorly differentiated tumors areas reacting with the MOC-1 antibody (e, × 175) or with the neurofilament antibody MNF (f, × 275) can be observed. g, h, double-label immunofluorescence technique in frozen sections demonstrating coexpression of cytokeratins (g, × 275) and neurofilaments (h, × 275) in some tumor cells of poorly differentiated SQC, while many tumor cells contain only cytokeratins and no neurofilaments.
NEUROENDOCRINE COMPONENTS IN LUNG CANCER

Table 3 Expression of cytokeratins, neurofilaments, and the MOC-1 antigen in frozen sections of human lung tumors

<table>
<thead>
<tr>
<th>Squamous cell carcinoma</th>
<th>Well-differentiated</th>
<th>Moderately well-differentiated</th>
<th>Poorly differentiated</th>
<th>Adenosquamous cell carcinomas</th>
<th>Adeno-carcinoma</th>
<th>SCLC</th>
<th>SCLC-squamous</th>
<th>Large cell carcinoma</th>
<th>Carcinoid</th>
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<td></td>
</tr>
<tr>
<td>F+</td>
<td>0/15</td>
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* F+, focal reaction; D+, reaction in virtually all cells.

Table 4 Patterns of intermediate filament protein expression of different types of lung cancer in paraffin sections

<table>
<thead>
<tr>
<th>Non-SCLC</th>
<th>Neuroendocrine tumors</th>
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<td>Squamous cell carcinoma</td>
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<td>25/32</td>
</tr>
<tr>
<td>RCK102</td>
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<tr>
<td>F+</td>
<td>16/27</td>
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<tr>
<td>D+</td>
<td>10/27</td>
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<tr>
<td>CAM5.2</td>
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<tr>
<td>F+</td>
<td>2/5</td>
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<td>D+</td>
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<td>MNF</td>
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<td>D+</td>
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<td>pNF160</td>
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<tr>
<td>F+</td>
<td>6/14</td>
</tr>
<tr>
<td>D+</td>
<td>0/14</td>
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* F+, focal reaction; D+, reaction in virtually all tumor cells.

* Only selected cases of poorly differentiated tumors were tested.

(Fig. 1e; Table 3). Strikingly, the presence of neurofilaments could also be observed in 3 of 23 cases (Table 3; Fig. 1f), but no overlap was found between those cases staining for MOC-1 and neurofilaments, except for one case in which a coexpression of MOC-1 and neurofilaments in the same tumor occurred. In most tumors only a focal reaction with these two antisera was observed. Also in paraffin sections the presence of neurofilaments could be demonstrated in 2 of 27 cases using MNF and in 6 of 14 cases using pNF160 (Table 4).

Double immunofluorescence studies using antisera to cytokeratins and neurofilaments on frozen sections demonstrated that coexpression of cytokeratins and neurofilaments occurred in some cells within poorly differentiated SQCs, while other tumor cells expressed only cytokeratins. No cells expressing only neurofilaments and no cytokeratins were seen (compare Fig. 1, g and h).

Adenosquamous Cell Carcinomas

Lung tumors, histologically showing adenomatous and squamous cell differentiation also exhibited this bipartite nature immunohistochemically. Most of these tumors are diffusely positive with pKer [6 of 8 (Table 3)] and RCK102 [5 of 5 (Table 3)] and have both keratinizing areas detectable with RK560 [4 of 8] and areas reacting diffusely with RGE53 [4 of 8]. In one case also the presence of the MOC-1 antigen could be demonstrated, albeit in a small part of the tumor (see Table 3).

Adenocarcinomas

Most adenocarcinomas show a diffusely positive staining reaction with the antibodies pKer and RCK102 (Fig. 2a), both in frozen and in paraffin sections (Tables 3 and 4). Also a diffuse staining reaction is seen in most frozen sections with the antibody RGE53 [39 of 45 (Table 3; Fig. 2b)], although in some poorly differentiated adenocarcinomas [4 of 45 cases], only a focal positive reaction with RGE53 was found, while in two cases no reaction with RGE53 was observed. Also no reaction was seen with RK560 [6 of 45 (Table 3)], indicating the absence of keratinization in these tumors. The adenocarcinomas did not react with the antibody to MOC-1, except for one case, in which some scattered tumor cells were positive with this antibody or with the neurofilament antibodies in frozen sections. In paraffin sections a focal staining reaction for neurofilaments was observed in 3 of 9 cases of poorly differentiated adenocarcinomas (Table 4). These selected cases were initially diagnosed as SCLC but upon revision appeared to be poorly differentiated adenocarcinomas.

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Small Cell Lung Cancer

Nearly all cases of SCLC examined showed the expression of cytokeratins. The polyclonal antibody pKer reacted in 15 of 20 cases on frozen sections while RCK102 reacted in all cases of frozen sections examined [16 of 16 (Table 3; Fig. 3a)]. In paraffin sections the presence of cytokeratins could be detected in 78 of 91 cases using pKer (Table 4; Fig. 3e), in 17 of 20 cases using RCK102 (Table 4), and in 47 of 51 cases using CAM5.2 (Table 4). RGE53 reacted in nearly all cases available as frozen sections [19 of 20 (Table 3; Fig. 3b)], while no reaction was seen with RKSE60 [0 of 20 (Table 3)]. In all cases of frozen sections [19 of 19] a staining reaction was observed with the MOC-1 antibody [Fig. 3c], although in 3 cases a reaction was seen in only part of the cells. In some cases of SCLC reacting only focally or not at all with cytokeratin antibodies a focal

Fig. 2. Immunoperoxidase staining on frozen sections of the lung. Note the strong staining reaction of both the broadly cross-reacting monoclonal cytokeratin antibody RCK102 (a, × 275) and the monoclonal antibody RGE53 (b, × 275).

Fig. 3. Immunoperoxidase and immunofluorescence staining patterns in frozen sections (a–d, g, h) and peroxidase of the lung. Note the typical granular staining reaction in all tumor cells with the broadly cross-reacting monoclonal cytokeratin antibody RCK102 (a, × 310) and with most tumor cells using the monoclonal antibody to cytokeratin 18, RGE53 (b, × 275). In all cases the presence of the MOC-1 antigen could be demonstrated (c, × 235), while in some cases neurofilament proteins were detected in part of the tumor cells, using the monoclonal antibody MNF (d, × 335). The presence of cytokeratins could also be demonstrated in paraffin sections of SCLC using the polyclonal cytokeratin antibody pKer (e, × 390), while additionally in some tumors a reaction with the neurofilament antibody pH160 was observed (f, × 390). Double-label immunofluorescence studies using the monoclonal cytokeratin antibody RCK102 (g, × 235) in combination with the polyclonal neurofilament antibody pH160 (h, × 235) show a coexpression of cytokeratins and neurofilaments in some cells (arrows in g and h), while most neurofilament-positive cells are negative for cytokeratins.
staining reaction with the neurofilament antibody MNF was seen [4 of 20 cases (Table 3; Fig. 3d)]. Also in paraffin sections neurofilaments could be demonstrated, reacting in 1 of 20 cases with MNF and in 3 of 72 cases with pNF160 (Table 4; Fig. 3f).

Using the double immunofluorescence technique in frozen sections of SCLC containing neurofilaments it could be demonstrated that coexpression of cytokeratins and neurofilaments seems to occur in only a few tumor cells (compare Fig. 3g with Fig. 3h).

Combined Small Cell Carcinomas

Mixed Small Cell-Squamous Cell Carcinomas. This tumor type with admixtures of SCLC- and non-SCLC (squamous cell) differentiation could be demonstrated histologically in four cases. Immunohistochemically their bipartite nature was characterized by a diffuse staining reaction with RGE53 in all tumor cells in one part of the tumor, similar to the staining reaction found in poorly differentiated SQC (Fig. 4c), while in other parts a speckled reaction pattern is seen, similar to the staining reaction of SCLC (Fig. 4b). In all cases a staining reaction with the MOC-1 antiserum was observed. As far as we can conclude from the 4 cases examined at least the histologically evident SCLC parts of the tumors showed a MOC-1 staining reaction (Fig. 4c), while in the poorly differentiated SQC areas only scattered MOC-1-positive cells were seen. The presence of neurofilaments could not be demonstrated.

Mixed Small Cell-Adenocarcinomas. In one case histologically this tumor type could be distinguished, showing a staining reaction with the cytokeratin antibodies pKer and RGE53. In part of this tumor a reaction with MOC-1 occurred, while neurofilaments could also be demonstrated in some tumor cells in frozen sections and in paraffin sections (results not shown).

Large Cell Undifferentiated Carcinomas

This group comprises all lung carcinomas in which at the light microscopic level no signs of differentiation could be observed. In frozen sections these types of tumors reacted diffusely with the cytokeratin antibodies pKer, RCK102, and RGE53, while in one case a reaction with MNF was also observed. In paraffin sections most cases reacted with pKer [13 of 16 (Table 4)] and all cases reacted with CAM5.2 [16 of 16]. In addition, one case in paraffin sections showed neurofilament-positive cells.

**Lung Carcinoids**

All lung carcinoids examined contained cytokeratins, as concluded from a positive reaction with pKer (Fig. 5a, RCK102, and RGE53 (Fig. 5h) in frozen sections. Also, in all cases of frozen sections (9 of 9) the MOC-1 antigen was present (Fig. 5e), while in 4 of 9 cases neurofilaments could be detected (Fig. 5d). Also in paraffin sections the three cases of lung carcinoids examined were positive for cytokeratins (Table 4; Fig. 5e), and two cases also reacted with the pNF160 neurofilament antibody (Fig. 5f). In contrast to SCLC, a clear coexpression of cytokeratins and neurofilaments was detected in virtually all cells of lung carcinoids using the double immunofluorescence technique (compare Fig. 5g and 5h). Both cytokeratin and neurofilament fluorescence patterns showed a dot-like concentration of these intermediate filament proteins in close proximity to the nucleus.

**Comparison of Primary Lung Cancers with Their Metastases**

In order to obtain an impression of the role of lung tumor heterogeneity in the metastatic process and to see if metastases showed a similar or different immunohistochemical reactivity pattern, we have examined 61 tumor specimens from 6 patients known with a primary SCLC. At all primary sites of the tumors as well as in all metastases the presence of cytokeratins could be demonstrated, although in variable amounts. In one case the presence of neurofilaments next to cytokeratins could be demonstrated at the metastatic site in the adrenal gland, while no neurofilaments were observed at other metastatic sites or at the primary tumor site of the same patient.

**DISCUSSION**

It has been suggested that in the future biomarkers will permit differentiation between different subtypes of major tumor groups (37).

The use of such markers on lung tumors, however, has occasionally led to confusing or contradictory results. In our studies we have used well-characterized biomarkers with known specificity for certain subtypes of lung tumors. As a general marker for epithelial differentiation broadly cross-reacting cytokeratin antibodies, which react with both simple (columnar) and squamous epithelia, were used. Keratinizing areas in SQCs can be detected by our monoclonal antibody to cytokeratin 10, RKE60. Adenocarcinomas on the other hand contain merely simple epithelium-type cytokeratins such as cytokeratin 18,
Fig. 5. Immunoperoxidase and immunofluorescence staining reactions in frozen sections (a-d, g, h) and peroxidase anti-peroxidase staining reactions in paraffin sections (e, f) of lung carcinoids. Note the typical dot-like immunostaining patterns with the monoclonal cytokeratin antibodies RCK102 (e, × 335) and RGE53 (h, × 170). The membrane surface antigen MOC-1 reacts with all carcinoids in a characteristic manner (c, × 220). In some of these tumors also the monoclonal neurofilament antibody MNF reacts in a typical dot-like fashion (d, × 220). In paraffin sections the presence of cytokeratins can be demonstrated using the polyclonal antibody pKer (e, × 390), while in some tumors coexpression of neurofilaments also can be detected with the polyclonal pNF160 antibody (f, × 390). Double-label immunofluorescence studies clearly demonstrate this coexpression of cytokeratins and neurofilaments using RCK102 (g, × 225) and pNF100 (h, × 225) in the same tumor cells.

generally not occurring in squamous epithelia or in SQCs (27–29). Cytokeratin 18 is also found in neuroendocrine tumors such as SCLC and lung carcinoids (38).

Recently, several monoclonal antibodies have been developed against SCLC cell surface antigens such as SM-1 (39), TF1-4 (40), LAM8 (41), and MOC-1 (10), each of them suggested to be more or less specific for SCLC. One of these, the monoclonal MOC-1 antibody, used in this study, appears to be a marker for neuroendocrine differentiation and reacts with normal neuronal and neuroendocrine tissues, as well as with neuroendocrine lung tumors (10). In SCLC and SCLC cell lines also neurofilaments have been detected (42, 43). Cell culture studies suggest that these intermediate filament proteins occur in the variant type of SCLC (20, 21). Furthermore, in some lung carcinoids neurofilaments have been described to be coexpressed next to cytokeratins (11). Therefore neurofilament antibodies were also added to the panel of biomarkers used in this study. The fact that these latter antibodies as well as some of the cytokeratin antibodies are reactive in paraffin sections allowed us to perform a retrospective study using a large series of paraffin blocks. In this way we could obtain an impression about the frequency of occurrence of cytokeratins and neurofilaments in lung cancer.

Our studies with these antibodies elucidate some of the confusion concerning intermediate filament protein expression in SCLC. Some authors demonstrated only neurofilaments in SCLC and in SCLC cell lines (42, 43), while others (38, 44, 45) have demonstrated only cytokeratin intermediate filaments and neurofilaments in SCLC. Also coexpression of both cytokeratins and neurofilaments in SCLC cell lines has been suggested (22, 23). These discrepancies have been partly explained by our recent findings (20, 21) that classic SCLC cell lines contain cytokeratins, while variant SCLC cell lines may contain neurofilaments and are negative for cytokeratins. Our results with tissue sections of SCLC, showing that about 5–10% of all SCLC examined contain focal areas positive for neurofilaments, seem to point to the variant-type differentiation of SCLC in these tumors. The frequency of the presence of neurofilaments matches quite well with the frequency of the variant type of SCLC as observed at first diagnosis by Radice et al. (17) and
Hirsch et al. (18). One should keep in mind, however, that a
cytokeratin-negative reaction may be a fixation artifact (24),
which may explain possible false negative findings (42, 43, 46,
47).
Our results in paraffin sections show that, when using the
proper antibodies, in most tumors cytokeratins can be demon-
strated. Some of the lung tumors investigated had been stored
for up to 23 years in formalin, and yet in most cases cytokeratins
and in some cases neurofilaments were demonstrable. The
pNF160 antibody reacts with more cells and lung tumors than
the MNF monoclonal antibody, probably due to the fact that
both antibodies recognize different neurofilament proteins or
that the polyclonal antibody pNF160 recognizes more antigenic
determinants than the monoclonal antibody.
Also with respect to the intermediate filament protein content
of lung carcinoids discrepancies in the literature have arisen.
Some authors demonstrated neurofilaments in lung carcinoids
(48), others only cytokeratins (38, 44, 49), while Lehto et al.
(11) reported the coexpression of neurofilaments and cyto-
keratins in three cases of such neoplasms. Our results clearly
demonstrate that indeed coexpression of cytokeratins and neu-
rofilaments can occur, however, only in some of the cases.
Coexpression of cytokeratins and neurofilament proteins
seems to be restricted to (neuro)endocrine tumors and has been
shown to occur in neuroendocrine skin carcinomas [Merkel cell
tumors (12-15)], in some pancreatic islet tumors [insulinomas
(16)], and in parathyroid adenomas (50). The possible cells or
origin for these tumors, i.e., Merkel cells in skin (51), pancreatic
islet cells, and parathyroid gland cells (50), respectively, express
only cytokeratins and do not contain neurofilaments. These
findings are in parallel with the detection of only cytokeratins
in the so-called Kulchitsky cells of the lung (36, 52), a possible
candidate for the cell type from which SCLC and lung carci-
noids arise. Apparently (neuro)endocrine cells have the poten-
tial to induce neurofilament expression as a result of malignant
growth.
Although not immediately obvious from histopathological
examinations, lung cancers may show a profound heterogeneity
when examined at the ultrastructural or immunocytochemical
level. Heterogeneity of lung cancer has been reported previ-
ously. In fact, histochemical examination of different
dises of entire lung tumors by Roggli et al. (53), who showed
that only 34% of all lung carcinomas examined were homoge-
neous. This large heterogeneity was also observed in cytological
specimens (3) and after electron microscopic examinations (4).
In some cases within the same tumor cell squamous cell differen-
tiation, adenomatous and small cell differentiation can be
observed at the ultrastructural level (54).
In our study, histological examination of lung cancers re-
vealed a combined phenotype only in about 6% of the cases
from which frozen sections were available. After the immuno-
histochemical procedures, in about 66% (144 of 218) of all
tumors, tested as frozen sections, a more or less heterogeneous
staining reaction was seen. In 29% of histologically homoge-
neous SQc a nonsquamous differentiation (adenocarcinoma or
SCLC) is observed in virtually all tumor cells using the RGE53
antibody, while nearly all cases of SQc showed at least a partial
staining reaction with this antibody. A neuroendocrine or neu-
ronal differentiation was detected by MOC-1 and the neurofil-
ament antibodies in 12 and 5% of the SQc cases, respectively.
The degree of heterogeneity seen with these immunohistoche-
tical techniques in SQcS increased with decreasing degree of
differentiation (see Table 3). The finding that in 28% of the
cases of poorly differentiated squamous cell carcinoma and in
one case of poorly differentiated adenocarcinoma cells express-
ing MOC-1 are found suggests that these latter tumors contain
cells with neuroendocrine differentiation. The presence of neu-
rofilaments in the moderately to poorly differentiated SQcS
and in some poorly differentiated adenocarcinomas might point
to the occurrence of the variant type of SCLC within poorly
differentiated non-SCLC.
Also in histologically homogeneous adenocarcinomas heter-
goeneity could be detected, as concluded from focal staining
reactions with RGE53 in some tumors, indicating a less pro-
nounced adenocarcinomatous differentiation in large parts of
these tumors.
Lung tumors, histologically recognizable as combined SCLC-
non-SCLC, also showed a combined nature immunohisto-
chemically. Since normally combined small cell carcinomas are
treated as SCLC (8) it is our belief that poorly differentiated
carcinomas containing MOC-1, and therefore having features
in common with the "large cell neuroendocrine tumors" as
described before (55), should be treated as SCLC using com-
bined chemotherapy and radiotherapy. This opinion is sup-
ported by the findings by Hammond and Sause (55) who could
demonstrate that histologically undifferentiated large cell car-
cinomas containing neuroendocrine elements (neurosecretory
granules) at the electron microscopic level behaved clinically
like SCLC. Interestingly, coexpression of neurofilaments and
MOC-1 in poorly differentiated lung carcinomas was quite rare.
This is in parallel with our findings in SCLC cell lines that the
variant type of cell lines expressed only low amounts of MOC-
1 as compared to the classic SCLC cell lines (21). Therefore it
might well be that variant SCLC cells gradually lose their
expression of MOC-1 with ongoing (de)differentiation.
We have examined autopsy material of patients with SCLC
to detect a possible differential expression of cytokeratins and/
or neurofilaments in metastases as compared to the primary
tumor site. It is well known that in up to 30% of all cases which
were first diagnosed as SCLC, non-SCLC components can be
observed at autopsy, especially after chemotherapy or radio-
therapy (19). These mixed tumors respond less well to combined
therapy and have a worse prognosis than pure SCLC (56).
The absence of neurofilaments in all but one case of meta-
stases indicates that transition from classic SCLC (which are
positive for cytokeratin) to the variant SCLC (positive for
neurofilaments) is not a frequent event in the course of the
disease, at least as far as we can conclude from our limited
study.
In summary, we can state that the main lung tumor types can
be distinguished from each other on the basis of the panel of
intermediate filament and surface protein antibodies used in
this study. A profound heterogeneity can be detected with these
antibodies in lung tumors, especially in poorly differentiated
(squamous cell) carcinomas. Within SCLC the variant subtype
may possibly be distinguished by the absence of cytokeratins in
large parts of the tumor and the presence of neurofilaments in
some cases. The immunocytochemical detection of neuroen-
docrine components in histologically nonneuroendocrine tu-
mors as well as subtyping within SCLC may become of clini-
copathological significance.

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