Phenotypic Heterogeneity Studied by Immunohistochemistry and Aneuploidy in Non-Small Cell Lung Cancers

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ABSTRACT

Non-small cell lung cancers (non-SCLC) differ from small cell lung cancers (SCLC) by many clinical features and prognosis. However, recent studies suggest that lung cancer heterogeneity frequently leads to the association of SCLC and non-SCLC in the same tumor. This phenotypic heterogeneity can be analyzed by immunohistochemistry using monoclonal antibodies (Mab) raised against differentiation related antigens. It may have clinical relevance inasmuch as the diversification of malignant cells is a well-known factor of tumor progression and may be due to chromosomal instability because inappropriate gene expression leads to the formation of antigens unrelated to cell lineage. Chromosomal instability in cancer leads to aneuploidy detectable by cell DNA content analysis. In a prospective study, we analyzed, in parallel, the expression of neuroendocrine related antigens by immunohistochemistry and the cell DNA content in frozen specimens from 40 patients who underwent complete surgical resection of primary non-SCLC in an attempt (a) to characterize the phenotypic heterogeneity and (b) to determine whether this heterogeneity is correlated with aneuploidy and clinical staging. Three Mabs were used in association as a marker of neuroendocrine antigen expression (S-L 11.14, MOC-1, and NE-25); reactivity of these Mabs in 9 SCLC and 3 lung carcinoid tissue sections was used as positive control. All SCLC and 2 of 3 lung carcinoids tested were homogeneously positive with Mabs S-L 11.14, MOC-1, and NE-25; 13 of 40 non-SCLC were homogeneously positive and 11 additional specimens focally positive with Mabs S-L 11.14, MOC-1, and NE-25. The percentage of cells in the modal DNA of Go-G1 phase was significantly lower in non-SCLC homogeneously positive with Mabs S-L 11.14, MOC-1, and NE-25 (x2 = 5.93; P < 0.02), and in tumors involving mediastinal lymph nodes (x2 = 10.08; P < 0.005). The percentage of cells in the modal DNA of G0-G1 phase was significantly lower in non-SCLC homogeneously positive with Mabs S-L 11.14, MOC-1, and NE-25 (x2 = 5.93; P < 0.02), and in tumors involving mediastinal lymph nodes (x2 = 10.08; P < 0.005). The percentage of cells in the modal DNA of G0-G1 phase was significantly lower in non-SCLC homogeneously positive with Mabs S-L 11.14, MOC-1, and NE-25 (x2 = 5.93; P < 0.02), and in tumors involving mediastinal lymph nodes (x2 = 10.08; P < 0.005).

INTRODUCTION

Non-SCLC differ from SCLC by many clinical features including curability by surgical resection and poor sensitivity to chemotherapy (1). However, light (2, 3) or electron (4, 5) microscopic studies suggest that lung cancer heterogeneity is frequent and can lead to the association of SCLC and non-SCLC in the same tumor. More recently immunohistochemistry, using Mabs raised against differentiation related antigens, has been used to analyze lung tumor phenotype (6, 7). These studies have shown that all lung tumors arise from endodermal lineage (6); moreover, using Mab MOC-1 raised against a specific neural and neuroendocrine antigen, it has been demonstrated that some poorly differentiated SQCs express neuroendocrine antigens (7). This finding suggests that heterogeneity is likely to be underestimated by conventional techniques. A new Mab (S-L 11.14) was raised against a neuronal neuroendocrine antigen present on neuroblastoma cells (8) and found to be in competitive inhibition with Mab MOC-1 (9). Mab NE-25 is also a Mab raised against a neuroendocrine membrane antigen expressed by SCLC (10). Using these three Mabs in the same study, it is possible to characterize the expression of neuroendocrine antigens in non-SCLC. This heterotopic expression of antigens unrelated to cell lineage may be the consequence of chromosomal instability leading to abnormal regulation of gene expression and aneuploidy (11). Since phenotypic diversification partially explains tumor progression it may be important to find out if there is a link between the expression of an unrelated antigen, the degree of histological differentiation and aneuploidy and to see whether there is a correlation between lung tumor heterogeneity and clinical stage in non-SCLC.

Forty patients underwent a surgical resection for primary non-SCLC following a complete clinical staging. Tumor specimens were analyzed by immunohistochemistry using Mabs S-L 11.14, MOC-1, and NE-25 and by Feulgen DNA staining for quantitative analysis of cell DNA content. In addition, 9 SCLC and 3 lung carcinoid specimens were analyzed under the same conditions as a positive control of the reactivity of Mabs against neuroendocrine related antigens. This study was undertaken (a) to find out if phenotypic heterogeneity of non-SCLC is related to aneuploidy and (b) to determine whether this heterogeneity has some significance in the staging of the tumor.

MATERIALS AND METHODS

Patients

Forty patients (36 men, 4 women, ages 41–76 years; mean ± SD, 59.6 ± 8.8 years) underwent a surgical resection for primary non-SCLC. Staging of lung carcinoma was performed according to the American Joint Committee (12) and included clinical examination, performance index assessment, chest tomodensitometry, fiberoptic bronchoscopy, liver and adrenal gland tomodensitometry, and bone scanning. It was completed by surgical observations. Modalities of resection was lobectomy (26 of 40) or pneumonectomy (14 of 40) and all patients underwent lymphadenectomy. In addition 12 patients underwent a surgical resection for primary SCLC (9 cases) or lung carcinoids (3 cases) following the same procedure.

Methods

Histology of Lung Carcinomas

All lung tumors were classified according to the latest WHO classification (13) by light microscopy following hematoxylin-eosin staining.
Keratinization was used as definitive criteria for well-differentiated SQCs. Other SQCs were considered poorly differentiated.

Lung Specimens

During the surgical resection, a specimen of the tumor from a nonnecrotizing area was deep frozen in liquid nitrogen until histochemical study and DNA content analysis were performed.

Immunohistochemical Study

Antibodies Used

Monoclonal Antibodies against Neuroendocrine Antigens. Mabs MOC-1 and NE-25 have been described extensively before. Mab MOC-1, an IgG1 cluster 1 antibody, was kindly given by L. de Leij (6) (State University, Groningen, The Netherlands), Mab NE-25 (10) was obtained from the First International Workshop on Lung Cancer Antigens, and Mab S-L 11.14 was obtained from Sanofi Research Center, Montpellier, France. This IgG2a Mab was clustered in group 1 (which included Mabs MOC-1 and NE-25) at the First International Workshop on Lung Cancer Antigens (9). The reactivity of Mab S-L 11.14 is similar to the cluster 1 Mab reactivity. In normal tissues, Mab S-L 11.14 reacts with brain and nerve, thyroid, striated muscle, Leydig cells, and erythrocytes; it is unreactive with normal lung. In malignant tissues, it reacts with SCLC, carcinoids, neuroblastoma (8), melanoma, and renal carcinoma; it is unreactive with most epithelial tumors. The antigen is lost by a fixation procedure using paraffin. Immunoprecipitation performed with either Mab S-L 11.14 or other cluster 1 Mabs characterizes the S-L 11.14-related antigen as a Mr 145,000 nonglycosylated membrane protein, identified as the neural cellular adhesion molecule.3

Monoclonal Antibodies against Epithelial Lineage Antigens. Since it has been demonstrated previously that both non-SCLC and SCLC express epithelial lineage antigens, an IgG Mab raised against cytokeratin (14) and an IgG2a Mab raised against EMA (15) (Dakopatts, Glostrup, Denmark) were used as control of the immunohistochemical reaction. Anti-EMA Mab has been shown previously to react with some normal and neoplastic lymphoid cells (16); consequently anti-cytokeratin Mab was used in conjunction with anti-EMA Mab as epithelial markers.

Immunohistochemical Reactions

Frozen tissue sections were fixed with cold acetone. Indirect immunoperoxidase tests were carried out using the biotin-streptavidin-peroxidase system (Amersham, Les Ulis, France) following the three stage procedure (17): stage 1, briefly, after rehydration in PBS and inhibition of endogenous peroxidase, sections were incubated with 10 μg/ml of purified Mab for 40 min at room temperature; in stage 2, sections were then washed in PBS and incubated with sheep biotinylated anti-mouse IgG diluted 1:50 in PBS for 30 min at room temperature; in stage 3, sections were finally incubated with streptavidin-biotinylated horseradish peroxidase at 1:200 for 30 min at room temperature. Immunohistochemical reaction was then revealed in the dark with 3-aminop-ethylcarbazole (Merck, Nogent/ Marne, France) using hydrogen peroxide as a substrate and counterstained with hematoxylin. Positive and negative controls were performed for each reaction. The immunohistochemical reaction was analyzed without any knowledge of clinical and light microscopy data. Reactions were classified into homogeneous reaction (Fig. 1), focal reaction (less than 20% cells reacting with the Mab, scattered cells, or islets of cells) (Fig. 2), or no reaction.

Cell DNA Content Analysis

The cell DNA content analysis was done after staining by the pararosaline Feulgen technique (18) on cytological prints of the specimens. The stoichiometric reaction was analyzed using computerized interactive morphometry (19, 20) (SAMBA 2002; TITN, Grenoble, France). For each specimen, cell DNA content analysis was performed in 300 randomized malignant cells. The nuclear DNA values were computerized in order to produce histograms (Fig. 3). The percentage of malignant cells in the modal DNA of G0-G1 phase was taken as index of ploidy using normal hepatic tissue as a control.

Study Design

All patients had a complete staging. Surgical resection of the tumor was deep frozen until it was analyzed by conventional histology, immunohistochemistry, and cell DNA measurement. Neither chemotherapy nor radiotherapy were performed prior to surgical resection.

Statistical analysis was performed by nonparametric tests (χ2 square test and Mann-Whitney U test).

RESULTS

Histological Hematoxylin-eosin Staining

According to the latest WHO classification we studied 40 non-SCLC; among them were 34 SQCs (17 well-differentiated, 17 poorly differentiated), 5 adenocarcinomas and 1 adenosquamous cell carcinoma. In addition, 9 SCLC (among them 2 mixed SCLC-non-SCLC) and 3 lung carcinoids were analyzed.

Clinical Staging of Non-SCLC

At the time of surgical resection 20 patients were in stage I, 6 patients in stage II, and 14 patients in stage III according to the American Joint Committee Criteria. None of the patients had a performance index more than 2 or a weight loss more than 5%.

Immunohistochemical Study

Reactivity of Mabs against Neuroendocrine Related Antigens. The reactivity of Mabs S-L 11.14, MOC-1, and NE-25 demonstrated a concordance of 97% of the 52 specimens tested. Homogeneous reactivity of Mabs S-L 11.14, MOC-1, and NE-25 was seen in 7 of 7 SCLC, 2 of 2 mixed SCLC-non-SCLC, and 2 of 3 lung carcinoids. Reactivity on non-SCLC tissue sections is described in Table 1. A homogeneous reaction was seen in 11 of 34 SQCs, in 1 of 5 adenocarcinomas, and in the adenosquamous cell carcinoma. No reaction was observed for 13 of 34 SQCs and for 3 of 5 adenocarcinomas. A focal reactivity was seen in 10 of 34 SQCs mainly in malignant cells from islets of poorly differentiated areas of the preparation. In SQCs the reactivity differed significantly (χ2 10.08; P < 0.005) according to their degree of differentiation: 6 of 17 well-differentiated versus 15 of 17 poorly differentiated SQCs showed a positive reaction (Table 2).

Reactivity of Epithelial Lineage Antigens. There were no significant differences in staining between the tumors when using either anti-cytokeratin or anti-epithelial membrane antigen Mabs. All specimens, including SCLC, showed a homogeneous reactivity mainly on membranes of the malignant cells for anti-EMA Mab and in the cytoplasm for the anti-cytokeratin Mab.

Cell DNA Content Analysis

Cell DNA content analysis was performed in 39 of 40 non-SCLC tumors (34 SQC, 4 adenocarcinomas, and 1 adenosquamous cell carcinoma). The mean percentage of cells in the modal DNA of G0-G1 phase was 46.0 ± 22.3% (SD) for all the non-SCLC tumors and 45.6 ± 22.3% for SQCs. For 8 of 9 SCLC and mixed SCLC-non-SCLC the percentage of malignant cells in the modal DNA of G0-G1 phase was 15.5 ± 7.2%. All carcinoid tumors were in the near diploid range.
Correlations

Staging and Immunohistochemistry of the Non-SCLC. The reactivity of Mabs S-L 11.14, MOC-1, and NE-25 was significantly higher ($\chi^2 5.93; P < 0.02$; Table 3) in stage III non-SCLC (12 of 14 positive specimens) by comparison with stage I and II non-SCLC (12 of 26 specimens). Moreover, reactivity of Mab S-L 11.14, MOC-1, and NE-25 differed significantly according to mediastinal nodal status: 9 of 10 positive specimens from N2 tumors; 15 of 30 positive specimens from NO-1 tumors ($\chi^2 5.07; P < 0.03$; Table 4).

Staging and Cell DNA Content of Non-SCLC. There was no difference in the percentage of malignant cells in the modal DNA of G$_0$-G$_1$ phase between stage I non-SCLC (46.0 ± 24.0%), stage II non-SCLC (43.8 ± 19.3%), and stage III non-SCLC (47.0 ± 20.9%).

Cell DNA Content and Immunohistochemistry of Non-SCLC. The percentage of malignant cells in the modal DNA of G$_0$-G$_1$ phase was significantly lower in tumors reacting homogeneously with Mab S-L 11.14 (27.4 ± 10.3%) when compared with tumors with a focal reaction (53.2 ± 19.9%; $P < 0.01$, Mann-Whitney U test) or without reaction (56.8 ± 21.3%, $P < 0.01$; Fig. 4).

Phenotypic Heterogeneity of Lung Cancer. A mixed SCLC-non-SCLC differentiation was seen in 3 of 52 tumors using light microscopy following hematoxylin-eosin staining. By
PHENOTYPIC HETEROGENEITY OF NON-SMALL CELL LUNG CANCERS

Fig. 3. Computer drawn histograms of DNA distribution. A, histogram of DNA distribution from a SQC in the near diploid range. B1, modal DNA of cells in G0-G1 (2N) which contains 80% of malignant cells; B2, modal DNA of cells in the G2-M phase (4N). B, histogram of DNA distribution from an aneuploid SQC. B1, modal DNA of cells in G0-G1 (2N) contains 18% of malignant cells; M1, modal DNA of aneuploid cells (3N).

Table 1 Indirect immunoperoxidase reactivity of Mabs S-L 11.14, MOC-1, and NE-25 on lung tumor tissue sections

<table>
<thead>
<tr>
<th>Reaction</th>
<th>SQC</th>
<th>Ad*</th>
<th>AdSQC</th>
<th>SCLC</th>
<th>SCLC-non-SCLC</th>
<th>Carcinoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Focal</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Ad, adenocarcinoma.

Table 2 Indirect immunoperoxidase reactivity of Mabs S-L 11.14, MOC-1, and NE-25 in SQC tissue sections (n = 34) according to the degree of tumor differentiation

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Well differentiated (n = 17)</th>
<th>Poorly differentiated (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Focal</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

χ² 10.08; P < 0.005

Table 3 Indirect immunoperoxidase reactivity of Mabs S-L 11.14, MOC-1, and NE-25 in non-SCLC tissue sections (n = 40) according to clinical staging

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Stage I and II</th>
<th>Stage III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Focal</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

χ² 5.93; P < 0.02

Table 4 Indirect immunoperoxidase reactivity of Mabs S-L 11.14, MOC-1, and NE-25 in non-SCLC tissue sections (n = 40) according to nodal status

<table>
<thead>
<tr>
<th>Reaction</th>
<th>N0</th>
<th>N1</th>
<th>N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Focal</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>3</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

χ² 5.07; P < 0.03

![Figure 4](image)

DISCUSSION

The first results of this study confirm previous data suggesting that a neuroendocrine antigen can be expressed by some non-SCLC, in particular by some poorly differentiated SQCs. Thus, immunohistochemical analysis disclosed a greater phenotypic heterogeneity than did conventional hematoxylin-eosin staining. Moreover, we found that this heterotypic phenotype is mainly seen in advanced clinical stage. An important heterogeneity of cell DNA content was also found in our homogeneous group of non-SCLC by means of computerized image analysis. Finally, we demonstrated that the heterotypic expression of a neuroendocrine antigen in non-SCLC is related to aneuploidy.

The neuroectodermal origin of SCLC was commonly accepted because they have histological features (21) and biological properties (22, 23) of the amine precursor uptake and decarboxylation system. However, recent studies based on immunohistochemistry disclosed that SCLC express cytokeratin (24, 25) which is a cytoskeletal antigen from endodermal lineage (26, 27) and suggested that SCLC arise from epithelial origin. Thus, the occurrence of the association of SCLC and non-SCLC in the same tumor may be related to this common epithelial origin.

Previous light and electron microscopic studies showed lung tumor heterogeneity (2-4). More recently, investigations were made by immunohistochemistry using Mab to see whether the tumor phenotypes of SCLC and non-SCLC were different or not (28-33). It appeared that some antigenic determinants are expressed by either SCLC or non-SCLC tumors (34). This fact might explain the difficulty in establishing a clearcut difference between phenotypes of tumor subsets. Mab MOC-1 is a highly specific antibody directed against a neuroendocrine membrane antigen (6). Reactivity with Mab MOC-1 was found in some poorly differentiated non-SCLC (7). Recently Mab S-L 11.14 was also reported to be specific against a neuroectodermal membrane antigen and found to be in competitive inhibition with Mab MOC-1 (9). Mab NE-25, MOC-1, and S-L 11.14 react with a M, 145,000 nonglycosylated protein expressed on SCLC membrane. Using three different Mabs we found a homogeneous immunohistochemical reaction in all SCLC, in mixed SCLC-non-SCLC, and in lung carcinoids confirming means of immunohistochemistry using Mab S-L 11.14, MOC-1, and NE-25 we found 13 additional non-SCLC in which a homogeneous positive reaction clearly demonstrated a neuroendocrine differentiation. Thus, the occurrence of a mixed SCLC-non-SCLC differentiation was higher when it was studied by immunohistochemistry (χ² 9.67; P < 0.01).
their neuroendocrine selectivity. Interestingly, reaction of Mabs S-L 11.14, MOC-1 and NE-25 was also seen in some tumors classified as pure non-SCLC by light microscopy. This reaction was mainly disclosed in poorly differentiated SQC s. Moreover, in some individual cases, a focal reactivity with Mabs S-L 11.14, MOC-1, and NE-25 was seen only in very poorly differentiated areas of the specimen. This finding suggests that some poorly differentiated tumors may express antigens unrelated to the differentiation observed by conventional light microscopy. This is in accordance with the hypothesis suggesting that all lung cancers are part of a spectrum of differentiation and that adjacent cell types frequently overlap (3). It also demonstrates that the phenotypic heterogeneity of lung carcinoma is underestimated by conventional light microscopy and that immunohistochemistry is useful for the evaluation of tumor phenotypes. This phenotypic heterogeneity occurs mainly in tumors at an advanced stage involving mediastinal lymph nodes or distant metastases. Thus, heterogeneity of non-SCLC and tumor growth seem to be linked.

Cell DNA content analysis disclosed aneuploid malignant cell populations in 90% of lung tumors independently of pathological subsets (35–38). Moreover, there is increasing evidence that aneuploid lung cancers have a poor prognosis in comparison to near diploid ones (38–40) as previously reported for breast cancer (41). The image analysis, by means of interactive computerized morphometry, is a useful tool for pathological diagnosis (42) and makes it possible to analyze cell DNA content in the tumor itself (18). Using this technique we found that aneuploidy and stage are not correlated. Staging of lung cancer is a well-known prognostic factor and it may appear paradoxical that aneuploid and stage are not linked. This apparent discrepancy may be explained by heterogeneity of clinical behavior of stage III lung cancers suggesting that tumor growth and progression are not necessarily correlated (43).

Because aneuploidy is the consequence of chromosomal instability, known as a critical factor for diversification (11), we made this study to see whether there is a relationship between aneuploidy and the heterotopic expression of neuroendocrine antigens in non-SCLC. We found that the mean percentage of malignant cells in the modal DNA of the G0-G1 phase was significantly lower in tumors strongly reacting with Mabs NE-25 and S-L 11.14 monoclonal antibody recognizing neuroectodermal tumors with epithelial-specific components of the human milk fat globulin membrane: production and reaction with cells in culture. Int. J. Cancer, 28: 17–21, 1981.


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