The prognostic value of NCAM, p53 and cyclin D1 in resected non-small cell lung cancer

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Abstract

Expression of the neural cell adhesion molecule, NCAM, in frozen sections has been associated with decreased postoperative survival in non-small cell lung carcinoma. Of the various isoforms of NCAM described, the highly sialylated isoform plays a role in the migration of embryonal cells from the neural crest and is expressed by highly malignant tumours such as small cell lung carcinomas. We investigated the clinical significance of expression of this NCAM isoform as a prognostic factor in a series of 96 non-small cell lung carcinomas resected with curative intent. We also evaluated the effect of microwave pretreatment of formalin-fixed, paraffin-embedded sections on the NCAM immunostaining and related the outcome to the postoperative clinical course of disease. In addition, in an attempt to extend our search for possible molecular markers of unfavourable prognosis in lung cancer, we evaluated increased immunostaining for p53 and cyclin D1 in the same series. We did not find a significant relation between expression of NCAM or its highly sialylated isoform and the length of postoperative survival. The numbers of positive cases (9 and 14, respectively) were relatively low. Increased p53 and cyclin D1 immunostaining (50 and 55 of
Keywords: Non-small cell lung carcinoma; Prognostic factors; NCAM; P53 protein; Cyclin D1; Immunohistochemistry

1. Introduction

Lung carcinomas are often subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC shows neuroendocrine differentiation and very aggressive clinical behaviour, whereas NSCLC represents a histologically heterogeneous group of tumours [1] with a significant variability in clinical behaviour. One of the factors associated with a poor prognosis of NSCLC is neuroendocrine differentiation [2–4], which is most consistently associated with the expression of the neural cell adhesion molecule (NCAM) [5,6]. NCAM is expressed by SCLC and carcinoids and by 15–20% of NSCLC [7,8]. In NSCLC its expression has been associated with a higher tumour stage and a worse prognosis [7–9]. The monoclonal antibody (mAb) 123C3, raised in our institute, binds to the polypeptide backbone of NCAM [9,10]. In a previous study, positive immunohistochemical reaction on frozen sections of NSCLC with this antibody was associated with a shorter survival [8]. With routine techniques, this mAb does not reliably recognise NCAM in paraffin-embedded tissue, which detracts from its potential diagnostic usefulness [8]. However, pre-heating of paraffin-embedded sections in the microwave oven can improve the results of immunohistochemistry in formalin-fixed, paraffin-embedded tissues considerably [11,12]. Using this method, we investigated the binding of this antibody in paraffin-embedded tissue.

In contrast to the adult isoform, NCAM-L, the embryonal isoform, NCAM-H, has long side-chains of polysialic acid with an unusual χ(2,8)-linkage. NCAM-H is expressed by all SCLC tumours and some atypical carcinoids [10,13,14]. A decrease in adhesive properties of cells expressing the NCAM-H isoform was found and is thought to be due to the large negative charge of the long side-chains. This may be biologically significant in view of the highly aggressive behaviour of SCLC tumours [15–17]. MAb 735, raised against live meningococci, binds to the polysialic side-chains of NCAM-H [13] and can be used to detect NCAM-H in formalin-fixed, paraffin-embedded material [8]. We investigated the binding of this mAb with resected NSCLC on paraffin-embedded sections and its possible usefulness as a prognostic factor.

Mutations of the p53 gene, located on chromosome 17p, are among the most frequent genetic abnormalities in lung cancer [18,19]. The majority of mutations result in missense codons and accumulation of a non-functional gene product [19,20], which can be detected by immunohistochemical methods [21]. However, p53 protein overexpression is not always correlated with a p53 gene mutation [22]. Conflicting data are available on the significance of p53 protein overexpression as
a prognostic factor in lung cancer [23–29]. In this study, we investigated the prognostic value of immunostaining with mAb DO-7 [28] on resected NSCLC.

The p53 gene product exerts its regulatory function on the cell cycle through p21/WAF 1 protein, which has an inhibitory effect on the cyclin D1/cyclin dependent kinase 4 (cdk 4) complex [30,31]. This complex is responsible for the G1/S transition by phosphorylation of the retinoblastoma gene product (Rb protein), thereby releasing transcription factor E2F. High levels of cyclin D1 result in a shorter G1 phase and may contribute to tumorigenesis [32,33]. Amplification and overexpression of cyclin D1 are found in some breast cancers and head and neck carcinomas and are associated with a poor prognosis in these tumours [34–36]. In this study, polyclonal rabbit anti-cyclin D1 antibodies [36] were used to investigate the prognostic value of overexpression of this protein in resected non-small cell lung carcinomas.

2. Material and methods

2.1. Clinical evaluation

Ninety-nine NSCLC, resected at the University Hospital in Leiden between January 1986 and December 1991, were available for study. Clinical and pathological staging was done according to the New International Staging System [37]. In three patients, no curative resection of the tumour was possible and these patients were excluded from further analysis. Of the remaining 96 patients, follow up data were available in all instances. The tumours were classified according to the WHO criteria [1].

2.2. Antibodies

MAb 123C3 is an IgG1 murine antibody, raised at the Netherlands Cancer Institute against a membranec fraction of a fresh SCLC specimen. It belongs to cluster 1 according to the International Workshop on Small Cell Lung Cancer Antibodies [38] and recognises an epitope on NCAM [10]. For immunohistochemistry it was used in a dilution of 1:200. MAb 735 is a murine IgG2a, raised against live group B meningococci, and was kindly provided by Dr D. Bitter-Suermann [8,10]. It reacts with α(2,8)-polysialic residues present on embryonal NCAM-H isoform [10,13,14] and was used in a dilution of 1:8000. For the detection of p53, murine anti human p53 mAb DO-7 (Dako Corporation, Denmark) was used in a dilution of 1:200 [28]. For immunohistochemical detection of cyclin D1 overexpression, a polyclonal rabbit serum, developed in our institute against the carboxyl-terminal part of this protein, B31S, was used in a dilution of 1:80 [36]. No cross-reactions with cyclins D2 and D3 were found. The highest dilution of an antibody solution showing positive staining of a positive control tissue or tumour was considered the optimal dilution for a specific antibody.
2.3. Immunohistochemistry

For this study, sections were cut from formalin-fixed, paraffin-embedded tumour samples. Without pre-treatment, no immunohistochemical staining of these sections can be observed with mAb 123C3. Successful antigen retrieval was obtained by pre-treatment with microwaves for 3 × 5 min at 100°C in sodium citrate buffer (pH 6.0). The duration of optimal microwave pre-treatment and mAb 123C3 concentration were established on the basis of a series of control experiments, using paraffin-embedded nerves and an intestinal carcinoid, which are known to express NCAM. At the optimal concentration and duration of the microwave pre-treatment, nerves and carcinoid tumour cells were distinctly positive whereas surrounding tissues were entirely negative or showed a very faint, diffuse staining which could be easily distinguished from the positive staining of nerves and carcinoid cells. The intensity of staining of these nerves and carcinoid cells was somewhat weaker than the staining seen when frozen sections were used. However, the morphology was better preserved, so that false positive interpretations were less likely to occur. For immunohistochemistry, the ABC technique was used as previously described [39]. As positive control for mAb 123C3, a sample from a typical bronchial carcinoid was used. For mAb 123C3 as well as mAb 735, small nerves present within each test sample served as internal positive controls. Immunohistochemical evaluation was done on at least one entire section, which was randomly taken from the tumour sample. For both mAbs 123C3 and 735, only distinct cell membrane-pattern staining of tumour cells was scored as positive, even when the number of positive tumour cells was small (in the order of a few percent). P53 staining was considered positive when 10% or more of the tumour cells exhibited distinct nuclear positivity. For cyclin D1 there is evidence that the number of tumour cells showing nuclear staining is correlated with the prognosis in carcinomas of the head and neck [36]. Therefore, we have scored the tumour cells for cyclin D1 nuclear staining on a semi-quantitative basis. All negative controls consisted of omission of the antiserum from the primary incubation.

2.4. Statistical analysis

For correlations between the results of immunostaining with individual antibodies, the Spearman rank test was used. Fisher’s exact test was used to correlate immunohistochemical data with clinical parameters. The frequency of positive staining according to histology and pathologic TNM staging was analyzed using the $\chi^2$ test. This test is not reliable when one of the groups consisted of 0 or 1 tumour. Therefore, the $\lambda$-value is calculated to evaluate the correlation between histology and the results of immunohistochemical staining. Analysis of survival and disease free interval was performed with the Kaplan–Meier method [40] and the differences between the groups were calculated with the log-rank test. Multivariate analysis was done on all prognostic factors. Results with $P$-values under 0.05 were considered significant. For the statistics, SPSS for Windows™ software was used.
3. Results

The population characteristics are shown in Table 1. All patients underwent a radical resection for Stage I, II or IIIa disease. The histological classification of the tumours showed a preponderance of squamous cell carcinomas.

The results of the immunohistochemical evaluation of the tumours are shown in Table 2 and Table 3. Staining with mAb 123C3 after treatment with microwave irradiation yielded nine positive tumours, eight of which were also positive for mAb 735. Six other tumours were positive for mAb 735 only. This suggests that mAb 735 is more sensitive in detecting NCAM than mAb 123C3, despite antigen retrieval by microwave incubation, but probably not all NCAM positive tumours can be detected by either of these mAbs. No significant correlation between NCAM expression, detected by these mAbs, with histology and differentiation grade was found. Most of the tumours showed only focal staining (Fig. 1a). Many adenocarcinomas showed extensive granular staining of the cytoplasm without any membrane staining and were scored as negative (Fig. 1b). The same pattern of cytoplasmic staining was also found in some cells of the normal bronchial surface epithelium (not shown). Probably, this staining represents a cross-reaction with a yet unidentified intracellular epitope of these cells. No correlation between mAbs 123C3 or 735 staining and tumour stage was observed. Survival analysis did not reveal any association of the immunohistochemical staining with any of these mAbs and a shorter survival, but the numbers of positive tumours were small (Table 3).

P53 protein accumulation, as detected with mAb DO-7, was found in 50 tumours, and cyclin D1 overexpression was detected in 55 tumours. P53 protein accumulation was not correlated with cyclin D1 overexpression (r = 0.08) and no association was found between overexpression of either p53 or cyclin D1 with any histological type. Specification of the percentage of stained cells for cyclin D1

Table 1
Population characteristics

<table>
<thead>
<tr>
<th>Population</th>
<th>Criteria</th>
<th>n</th>
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<tbody>
<tr>
<td>Total number</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Age (years)</td>
<td>&lt; 60</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>≥ 60</td>
<td>68</td>
</tr>
<tr>
<td>Median (years)</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13</td>
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<tr>
<td>Histology</td>
<td>Squamous cell carcinoma</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Mixed histology</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Large cell carcinoma</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Carcinoid tumour</td>
<td>3</td>
</tr>
<tr>
<td>Postoperative Stage</td>
<td>I</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>IIIa</td>
<td>17</td>
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Table 2
Immunohistochemical staining related to histology and postoperative stage

<table>
<thead>
<tr>
<th>Positive staining</th>
<th>NCAM</th>
<th>NCAM-H</th>
<th>Cyclin D1</th>
<th>p53</th>
</tr>
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<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>5</td>
<td>8</td>
<td>39</td>
<td>33</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Mixed carcinoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Carcinoid tumour</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Postoperative stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>8</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>4</td>
<td>15</td>
<td>9</td>
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<tr>
<td>III</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>11</td>
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</table>

*χ²-test results

<p>| | | | | |</p>
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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Histology*</td>
<td>3.28 (0.00)</td>
<td>8.98 (0.02)</td>
<td>5.48 (0.07)</td>
<td>3.82 (0.05)</td>
</tr>
<tr>
<td>Postoperative T-stage</td>
<td>0.05</td>
<td>0.06</td>
<td>1.65</td>
<td>7.59*</td>
</tr>
<tr>
<td>Postoperative N-stage</td>
<td>0.15</td>
<td>0.22</td>
<td>3.66</td>
<td>0.24</td>
</tr>
<tr>
<td>Postoperative stage</td>
<td>0.71</td>
<td>0.57</td>
<td>3.51</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Very few adenocarcinomas stain with mAb 123C3 or 735.

The only significant correlation is between postoperative T-stage and p53 staining.

*χ²-test not reliable when there are values of 0 or 1. The χ²-values (in parentheses) are very low indicating that there are no correlations between histology and immunohistochemical staining.

overexpression did not provide additional information (Table 3). Therefore, we considered staining of ≥ 10% of the tumour cells as cyclin D1 overexpression. P53 protein overexpression was associated with a higher postoperative tumour stage (χ² = 7.59, P = 0.022) but not with a shorter survival. No correlations were found between cyclin D1 overexpression with tumour stage or prognosis even when a semi-quantitative analysis is done (Table 3). The analysis for the disease free interval for all immunohistochemical parameters showed the same pattern as the survival analysis. The only significant prognostic factors found in this study were the postoperative N classification and the tumour stage (Table 3, P < 0.05). The postoperative T stage did not reach significance as a predictor of survival (P = 0.09).

4. Discussion

NCAM plays a role in the intercellular adhesion by homotypic binding and the adhesion of cells to the extracellular matrix [15]. Polysialylation of NCAM results in a decrease in its adhesive properties; in neuroendocrine lung cancers, its expression has been associated with a more aggressive tumour phenotype [17]. MAb 735 binds to epitopes on the polysialic acid side-chains and detects only the embryonal isoform [10,14]. The anti-NCAM mAb used in this study, mAb 123C3, binds to an epitope on the protein backbone of NCAM [41] and recognises all major isoforms of this molecule [10]. Before the advent of antigen retrieval
techniques [11,12], immunohistochemical studies with this mAb were possible only on frozen sections [8]. We found that the pre-treatment of formalin-fixed, paraffin-embedded, sections with microwaves enables detection of NCAM with mAb 123C3. However, the lower percentage of positive tumours suggests that this method is less sensitive than when frozen sections are used [8]. More tumours were stained with mAb 735, indicating a higher sensitivity of this mAb in detecting NCAM, in the subset of tumours expressing NCAM-H. In contrast to an earlier report [4], we could find no association of NCAM expression with histology, especially with adenocarcinoma. Not infrequently, an adenocarcinoma showed cytoplasmic staining with mAbs 123C3 and 735, often with a granular appearance. In the absence of membrane staining, we considered such tumours negative. However, it seems possible that some similar tumours, when evaluated with immunostained frozen sections, with generally a far less optimal morphology, were previously considered positive. Thus, the superior morphology of paraffin-embedded tissue may have resulted in fewer false positives (superior specificity) but also in the lower number of true positive tumours (inferior sensitivity). In contrast to a previous study [8], no correlation was found between mAb 123C3 positivity and a decrease in survival. Other investigators using other anti-NCAM mAbs did find a correlation between NCAM expression and survival [7]. These investigators also found a correlation of NCAM expression with a higher tumour stage and especially N-stage. This finding suggested that analysis of material from more advanced stage tumours may provide more information.

Mutations of p53 are the most frequent genetic abnormality in lung cancer, with a prevalence of 77% of SCLC tumours and a slightly lower frequency in NSCLC.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Disease free interval P-value</th>
<th>Survival P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.76</td>
<td>0.70</td>
</tr>
<tr>
<td>Histology</td>
<td>0.47</td>
<td>0.52</td>
</tr>
<tr>
<td>ABO blood group</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Rh blood group</td>
<td>0.94</td>
<td>0.82</td>
</tr>
<tr>
<td>Postoperative stage</td>
<td>0.008*</td>
<td>0.02*</td>
</tr>
<tr>
<td>Postoperative T stage</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Postoperative N stage</td>
<td>0.002*</td>
<td>0.01*</td>
</tr>
<tr>
<td>NCAM (mAb 123C3)</td>
<td>0.65</td>
<td>0.55</td>
</tr>
<tr>
<td>NCAM-H (mAb 735)</td>
<td>0.38</td>
<td>0.43</td>
</tr>
<tr>
<td>P53 (mAb DO-7)</td>
<td>0.27</td>
<td>0.22</td>
</tr>
<tr>
<td>Cyclin D1 staining (B31S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10% positive</td>
<td>0.80</td>
<td>0.93</td>
</tr>
<tr>
<td>≥20% positive</td>
<td>0.82</td>
<td>0.93</td>
</tr>
<tr>
<td>≥30% positive</td>
<td>0.66</td>
<td>0.54</td>
</tr>
<tr>
<td>≥40% positive</td>
<td>0.70</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Cyclin D1 staining is indicated by the fraction of tumour cells that stained with the polyclonal antibodies B31S.

*P < 0.05.
The majority of p53 mutations results in an altered protein with an increased stability [42]. As a result, the concentration in cancer cells with such a p53 mutation is higher than normal, so that it can be detected by immunohistochemical methods [24,28,29]. However, not all cases of p53 protein overexpression are associated with a p53 gene mutation, especially after DNA damage due to ionising radiation [22]. We found accumulation of the p53 protein in 50 of the 96 tumours, mainly in the higher tumour stage (T stage). In contrast to some investigators [28,29] we could not find an association with a shorter survival, possibly because the study popula-
tions differed with respect to histology and stage and due to the use of a different antibody. However, our finding is in accordance with other investigators, some of whom have used the same mAb [24,25]. Other factors, such as K-ras, may play a more important role in determining the clinical outcome [24].

Overexpression of cyclin D1, generally the result of gene amplification, is found in some breast carcinomas and squamous cell carcinomas of the head and neck (HNSCC) and has been associated with a poor prognosis [34–36]. Semi-quantitative analysis showed a correlation of the percentage of stained tumour cells and a shorter survival in HNSCC [36]. In this study, we found that 55 of the 96 resected NSCLC showed staining for cyclin D1 in $\geq 10\%$ of the cells. Even when the percentage of positive cells is considered, no correlation with tumour stage, histological type or differentiation grade was observed, and there was no association with a poor prognosis. Although theoretically, a negative correlation between accumulation of p53 and cyclin D1 could be expected, this was not found in this study. Furthermore, there was no correlation between NCAM expression and p53 or cyclin D1 overexpression.

In conclusion, NCAM expression was not a significant prognostic factor in the present series of resected NSCLC. The superior quality of formalin-fixed, paraffin-embedded material enabled a better morphological evaluation of the tumours at the cost of a lower sensitivity in detecting NCAM expression. Immunostaining for p53 and cyclin D1 did not provide prognostic information in NSCLC and semi-quantitative analysis of cyclin D1 staining did not provide additional information. Cyclin D1 overexpression was the most frequent finding. The only prognostic factors identified were the tumour and nodal stage. In these series no immunohistochemical marker was found to have prognostic value in resected NSCLC. Our findings are in contrast to some other studies. However, the study populations described in the different reports vary considerably regarding histology and tumour stage. In addition, many different antibodies are used in different studies and comparisons between the results are difficult to make. The use of more homogenous and comparable study populations may facilitate the detection of prognostic factors in NSCLC.

Acknowledgements

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References


