Five-Year Follow-up Study of Independent Clinical and Flow Cytometric Prognostic Factors for the Survival of Patients with Non-Small Cell Lung Carcinoma

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ABSTRACT

Fresh surgical specimens of tumors of 187 patients with previously untreated non-small cell lung carcinomas were investigated by means of flow cytometry. The aim of the study was to look for cellular prognostic indicators for survival times of these patients in addition to the well-known clinical prognostic factors. All patients had a minimum of 5 years follow-up. Patients with aneuploid tumors had significantly shorter survival times than did those with diploid tumors ($P < 0.001$). Identical results are obtained when the analysis is restricted to just those patients with $T_3$ tumors or to patients with metastatic tumors at time of surgery or who were classified as Stage III ($P < 0.01$). These data indicate that DNA ploidy is a strong and independent prognostic factor in patients with non-small cell lung carcinoma. Patients having tumors with a high proliferative activity died significantly ($P < 0.05$) earlier than patients having tumors with lower proliferative activity. As with tumor ploidy, survival time in patients with high or low proliferative tumor activities was independent of whether the patients had $T_3$-tumors, metastases, or were in Stage III. Univariate and multivariate analyses of the data in this study demonstrate two groups of independent prognostic factors for the survival of patients with non-small cell lung carcinoma: a group of clinical factors and a group of flow cytometric factors.

INTRODUCTION

Although many histological types of lung cancer exist, the vast majority of cases fall into one of the two groups that are treated very differently: small and non-small cell lung carcinomas. Histological features, clinical course, and response to treatment indicate that small cell lung carcinomas are a separate entity indeed, whereas NSCLC$^2$ represent a mixed group of tumors with overlapping histologies, clinical courses, and responses to treatment. Thus, an accurate prediction of tumor progression and patient survival remains a major problem for these tumors. The aim of this study, therefore, was to look for cellular prognostic indicators in NSCLC in addition to the well known clinical prognostic factors.

There is increasing evidence that in a variety of tumors DNA content may be of considerable prognostic value with respect to survival (1-14). Some studies on lung carcinomas have also shown a possible relationship between DNA ploidy and biological behavior of these tumors (15-18), while others provide no such evidence (19).

In a preliminary investigation we found that patients with NSCLC whose tumors were aneuploid had a shorter survival time than those with diploid tumors (18). Subsequently, we were able to demonstrate that patients whose tumors had a high proliferative activity also had a worse prognosis (20). In this early study the follow-up was only 1.5 years and even while there may be a difference in the survival 2 years after surgery, by 3 years the differences can disappear (21). In this investiga-

MATERIALS AND METHODS

Patients. Patients with previously untreated NSCLC were entered into this investigation. All patients ($n = 187$) were surgically treated and fresh specimens of the tumors were investigated by means of flow cytometry. The morphological classification of the bronchogenic carcinomas was based on the WHO Study (22) and comprised 105 epidermoid carcinomas, 55 adenocarcinomas, and 27 large cell carcinomas and the histological examination and classification of the tumors were carried out by two pathologists. The stage distribution of the patients (166 men, 21 women) included 5 patients younger than 40 years, 21 between 40 and 49, 93 between 50 and 59, 50 between 60 and 69, and 18 older than 70 years. All patients were staged at time of surgery (lobectomy: 115; pneumonectomy, thoracotomy, resection: 72). The classification of the stage (pTNM) was made according to the guidelines of the American Joint Committee for Cancer Staging and End Results Reporting (23). Of the 187 patients 34 had Stage I, 16 Stage II, and 137 had Stage III tumors. Seventy-four patients did not have metastases whereas 111 patients had metastases (2 patients could not be defined). One hundred and thirty patients were treated only by surgical procedures, 23 patients were additionally treated with cytotoxic drugs, and 32 patients (mainly epidermoid carcinomas) were treated with irradiation (2 cases could not be defined precisely). The majority of the patients were treated with 5-fluouracil (40 mg/m$^2$, days 1-5) and 1,3-bis(2-chloroethyl)-1-nitrosourea (400 mg/m$^2$, days 1-5) every 6 weeks for 2-6 cycles. Radiation therapy was given by $^{60}$Co; the total radiation dose was 50-60 Gy in 5-6 weeks.

Follow-up data were obtained through hospital charts and correspondence with the referring physicians. The survival times were determined from the day of surgery. Only patients who were alive more than 4 weeks after surgery ($n = 175$) were included in the survival analysis. DNA index analysis was carried out in all 187 tumors and cell cycle analyses in 122 cases. Sixty-five patients could not be included in cell cycle analysis because of overlapping of subpopulation in the tumors (near-diploid or multiploid).

Flow Cytometry. Flow cytometry analysis was carried out using an ICP-22 (PHYWE AG; Goettingen, Federal Republic of Germany). Preparation of the cell suspensions and measurements of nuclear fluorescence were performed according to the method described earlier (20, 23, 24). Only fresh tumor specimens were processed. The biological variability within the individual samples was minimized by using large segments of the tumors. Briefly, the tumor material was freed from fat and necrotic parts and then mechanically minced with scissors. The tissue pieces were suspended in Hank's balanced salt solution, passed 10-20 times through a sharp edged glass pipet, and the resultant cell suspension filtered through gauze. Adjacent parts of the specimens were used for additional histological examination.

For measurements of DNA content a mixture of propidium iodide (10 $\mu$g/ml) and 4',6-diamidino-2-phenylindole (2 $\mu$g/ml) was added simultaneously with RNase (1 mg/ml) after methanol fixation and protease digestion (0.5% pepsin). Peripheral blood leukocytes of healthy donors were applied as a calibration standard for DNA diploidy.

Fig. 1 shows representative DNA histograms demonstrating tumors with DNA diploidy (left), tumors with DNA aneuploidy containing one abnormal DNA stemline (middle), and tumors with DNA aneuploidy containing more than one abnormal DNA stemline (right). Cell cycle analyses (areas marked by darkening) were performed using integrated
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classification according to the method of Fleischer and Pelka (25). A computerized subtraction of exponentially decreasing corrections beginning with the peak of cellular debris was included in the evaluation program (26). Cell cycle analyses with interspersed cell populations (e.g., Fig. 1, right) were deleted from the study.

Documentation and Analysis of Data. The data were stored by the central data processing system of the German Cancer Research Center (VW/CMS operating system, program package SURVIVAL). The method for analysis of survival was the statistical failure time model with censored data according to the method of Kaplan and Meier (27). The multivariate relationship was analyzed according to Cox regression (28, 29). Both statistical methods were integrated in a program package by Edler et al. (30). The cutoff point for low or high fraction of cell cycle phases was determined by the CRITLEVEL procedure (31). This is an efficient graphic tool for determining those levels of quantitative prognostic factors at which the most pronounced deterioration of prognosis takes place.

RESULTS

The prognosis of patients with NSCLC is largely determined by the stage of disease and this is confirmed by the present study (Fig. 2). Extent and location of primary tumor (T), nodal involvement (N), and distant metastases (M) are well-known prognostic factors and their significance is also clearly established in our study. Histology, on the other hand, was not of significant prognostic value.

The results of DNA content analysis and length of survival are summarized in Fig. 3. Analysis of all patients show that those with aneuploid tumors had significantly shorter survival times ($P < 0.001$) than did those with diploid tumors (Fig. 3, top left). While the overall number of patients with diploid tumors is only 27, representing about 15% of the total number of patients studied, it is noteworthy that 16 or almost 60% of these patients survived 5 years or more. Although we have not been able to determine whether all of these 16 patients had no evidence of disease at 5 years the finding that no deaths occurred after the 120th week would tend to substantiate this.

To exclude the influence or possible introduction of a bias into the analysis we further analyzed these data according to patients with T3 tumors, NM+, and in Stage III. The analysis of these homogeneous groups of patients may be found in Fig. 3, top right and bottom. Patients classified as Stage III or with T3 tumors or metastasis at time of surgery and having diploid tumors lived longer ($P < 0.05$) than did those with aneuploid tumors. This finding can also be extended to other homogeneous groups of patients who were treated for instance with surgery alone. Similarly, patients with diploid tumors receiving surgery alone survived significantly longer ($P < 0.002$) than did those with aneuploid tumors. This means that DNA ploidy is a significant prognostic factor for the survival of patients with NSCLC.

The results of the analyses in 122 cases of the proportion of tumor cells in different stages of the cell cycle are demonstrated in Fig. 4 and Table 1. Patients who had tumors with a low proliferative activity as demonstrated by more than 78% of the cells in G0-G1, or less than 22% of the cells in S-G2-M lived longer ($P < 0.05$) than patients with more active tumors (Fig. 4). The same trends can be demonstrated when the S-phase and G2-M cells are equal to or less than 8 and 14%, respectively. The same results are obtained when the analysis is restricted to just those patients with T3 tumors, or with metastases at the time of surgery, or who were classified Stage III (Table 1). This finding means that proliferation is a strong prognostic factor for the survival of patients with NSCLC.

The correlation coefficient according to Pearson was tested.

Fig. 1. Representative examples of the DNA histograms of NSCLC. Left, diploid; middle, aneuploid; right, multiploid (more than one abnormal DNA stemline). Cell cycle analysis was performed in the areas indicated by darkening. Abscissa, relative DNA content; ordinate, number of cells.
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Fig. 2. Survival curves of patients with NSCLC (Kaplan-Meier-estimates) subdivided according to histology (top left) T (top right), lymph node involvement and metastases (NM, bottom left), and stage (bottom right). NM−, without lymph node involvement and without metastases; NM+, with lymph node involvement or with metastases.

DISCUSSION

With most diseases and in particular with cancer it is important to have a complete work-up before treatment. This affords the physician some evaluation of the prognosis and the treatment to use. In lung cancer correct staging is important because the prognosis for survival depends on size and location of the neoplasm and whether it has metastasized.

In addition to histology which in our study has no prognostic value flow cytometry of the primary tumors allows further characterization of the tumors. Flow cytometry can provide two pieces of information about the tumor: assessment of ploidy which seems to be correlated with malignant aggressiveness and measurement of the fraction of the different phases of the cell cycle, which is an index of proliferative activity.

The present report shows that cytometric analysis clearly has prognostic importance in patients with NSCLC. Regardless of stage, or whether the patients had a T3 tumor or were metastatic, patients with DNA diploid tumors lived longer than patients having DNA aneuploid tumors. This difference in survival was still significant in patients who had a minimum of 5 years' follow-up. Therefore, we conclude that DNA ploidy is an independent prognostic factor from clinical characteristics in patients with NSCLC. In addition, we found that the survival curves of patients with diploid tumors plateaued at 2 years, whereas patients with aneuploid tumors continued to die. Hedley et al. (10) working with breast cancer patients published similar results.

Several authors (10, 32, 33) measured the DNA content in nuclear suspensions prepared from archival paraffin-embedded tumor size. The advantage of their procedure is that survival and disease progression data are already available. A disadvantage of this technique is that the overall quality of DNA histograms obtained from paraffin-embedded tissue is generally inferior to that obtained with fresh tissue (32). Using this method Zimmerman et al. (33) substantiated our results by recently publishing a report that patients with aneuploid lung tumors had significantly shorter survival than those with diploid tumors. In our study only fresh tumor specimens were processed and we could therefore also examine cell cycle parameters.

A disadvantage with flow cytometry is that cell cycle analysis cannot be done in all cases. Whereas in this study we were able to obtain DNA indices of all 187 tumors, cell cycle analyses...
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Fig. 3. Survival curves of patients with NSCLC (Kaplan-Meier-estimates) subdivided according to ploidy in all patients (top left) and according to T3 (top right), NM+ (bottom left), and Stage III (bottom right).

Fig. 4. Survival curves of all patients with NSCLC (Kaplan-Meier-estimates) subdivided according to cell cycle phases (G0-G1; S phase; G2-M; S-G2-M).
were possible in only 122 cases (65%). One-third of the patients (n = 65) could not be included in cell cycle analysis because of the overlapping of DNA stemlines. The results of the analyses show that patients whose tumors had a high proliferative activity, (G2–G1 proportion <78%; S-phase proportion >8%; G2–M proportion >14%; S–G2–M proportion >22%) died significantly earlier than patients with lower proliferative activity. As with tumor ploidy, survival time in patients with high or low proliferative tumor activities was independent of whether the patients had T3 tumors, metastases (NM+), or were in different stages.

Each method of cell cycle analysis has its own specific limitations with both cell preparation and the computer evaluation. In our analysis the original distribution of S-phase cells is separated from background debris by computerized subtraction. The overlapping of DNA stemlines. The results of the analyses (n = 65) could not be included in cell cycle analysis because of

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Total no. of patients (dead)</th>
<th>MST* (wk)</th>
<th>Log rank test (P)</th>
<th>Total no. of patients (dead)</th>
<th>MST* (wk)</th>
<th>Log rank test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>45 (27)</td>
<td>117</td>
<td>0.041</td>
<td>60 (37)</td>
<td>119</td>
<td>0.027</td>
</tr>
<tr>
<td>T1</td>
<td>30 (20)</td>
<td>84</td>
<td>0.103</td>
<td>41 (29)</td>
<td>79</td>
<td>0.065</td>
</tr>
<tr>
<td>NM+</td>
<td>24 (16)</td>
<td>88</td>
<td>0.025</td>
<td>37 (24)</td>
<td>83 (26)</td>
<td>0.019</td>
</tr>
<tr>
<td>Stage III</td>
<td>32 (21)</td>
<td>84</td>
<td>0.074</td>
<td>41 (21)</td>
<td>144</td>
<td>0.041</td>
</tr>
<tr>
<td>Surgery alone</td>
<td>33 (17)</td>
<td>144</td>
<td>0.074</td>
<td>41 (21)</td>
<td>144</td>
<td>0.041</td>
</tr>
</tbody>
</table>

* Median survival time.

**Table 1** Median survival times of patients with NSCLC subdivided according to cell cycle distribution


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