Clonal Heterogeneity of Small-Cell Anaplastic Carcinoma of the Lung Demonstrated by Flow-Cytometric DNA Analysis

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ABSTRACT

Flow-cytometric DNA analysis yields information on ploidy and proliferative characteristics of a cell population. The analysis was implemented on small-cell anaplastic carcinoma of the lung using a rapid detergent technique for the preparation of fine-needle aspirates for DNA determination and a newly developed procedure for storing aspirates at —80°C. Thirty-eight different metastases from 30 consecutive patients with small-cell anaplastic carcinoma of the lung were examined with a total of 273 fine-needle aspirations. The results on ploidy are reported in this paper. The degree of contamination of the aspirates with normal cells was determined by differential counts. The ratio of the peak channel numbers for the G1 phase of the tumor cells to that of the diploid standard (DNA index) was calculated and used for ploidy identification. Twenty-nine patients were evaluable with respect to DNA index determination. The coefficient of variation of the DNA index determination was estimated as 0.039. In 23 (79%) patients, only one cell line could be detected. Evidence of the presence of 2 tumor cell clones with different ploidy was obtained in the remaining 6 (21%) patients. Of the 35 malignant clones thus demonstrated, 26 (74%) were significantly different from diploid (p ≤ 0.01). Four (11%) were hypodiploid, 3 (9%) were hypotriploid, and 19 (54%) were hypo- or near-tetraploid. Clonal heterogeneity in the tumors of 21% of the patients is a conservative estimate. Assessment of the detection limit set by the methodology used and the restricted number of samples studied in each patient indicate that the true occurrence of clonal heterogeneity in small-cell carcinoma of the lung may be much higher.

INTRODUCTION

Small-cell anaplastic carcinoma of the lung is highly sensitive to multiple chemotherapeutic agents. The results of treatment indicate objective response in 80 to 90% of all patients receiving intensive combination chemotherapy, and about 40% of the patients obtain a complete remission (3, 15). However, the response to treatment is usually of short duration, and less than 10% of the patients attain a 2-year disease-free survival (12).

Studies of DNA content and of cell kinetics have shown considerable heterogeneity in small-cell carcinoma of the lung. In tumors from different patients, ploidies between near-diploid and near-pentaploid have been demonstrated (4) as well as a wide range of TD’s (5) and labeling indices (18). If this heterogeneity is correlated to the clinically obvious differences in the sensitivity of individual tumors to individual drugs, one would expect a subclassification based on ploidy and cell kinetics to result in subgroups with a more homogeneous sensitivity pattern. Subclassification would thus be a useful tool in improving the results of treatment.

Earlier studies of karyotype anomalies by single-cell DNA analysis and of cell kinetics are scarce, inasmuch as they were hampered by methodological problems (1). Flow-cytometric DNA analysis yields information on G1 cell modal DNA content (ploidy) and the percentage of cells in the cell cycle phases. By sequential analysis of treatment-perturbed populations, further cell kinetic information can be obtained. Flow cytometry combines precision with speed of measurement. Its main limitation is the requirement of a monodisperse cell suspension for analysis. This requirement was met by using a recently developed, simple method for the preparation of fine-needle aspirates for flow cytometry (19). The results of ploidy of the tumors of 30 patients are given in this paper.

MATERIALS AND METHODS

Thirty consecutive patients entered the study from August 1977 to September 1978 (Table 1). All patients had small-cell anaplastic carcinoma of the lung with metastases easily accessible to fine-needle aspiration. Eleven of the patients were females (mean age 59 years; range, 50 to 71 years) and 19 were males (mean age 60 years; range, 48 to 71 years). The histological diagnosis and subtyping were based on the WHO 1967 classification (11) and were performed by one pathologist (F. R. Hirsch) according to the criteria described elsewhere (10). Eight of the 30 patients had been treated previously with chemotherapy or chemotherapy and radiotherapy. Thirty-eight different metastases were examined. Twenty-eight metastases were located in lymph nodes, mainly in the supraclavicular region. Nine were skin nodules, and 1 was a metastasis in the liver aspirated during peritoneoscopy. The size of palpable tumors was measured in 2 dimensions with calipers before each aspiration. The tumor volume was calculated by use of the formula for an ellipsoid.

A total of 273 aspirations were performed with the technique described previously (19). The aspirate was transferred to 400 μl ice-cold buffer (5% dimethyl sulfoxide-250 mM sucrose-40 mM sodium citrate, pH 7.6). One half of this cell suspension was used for cytocentrifuge preparations, and the other half was used for DNA analysis. The cytocentrifuge slides were prepared in duplicate. The cell number was counted in a

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hemocytometer, and the cell concentration was adjusted to 600 cells/μl. Equal volumes of cell suspension and the above-mentioned buffer with 40 mM spermine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) added were mixed. Sixty μl of this suspension were used for each cytocentrifuge slide. The slides were stained by the May-Grünwald-Giemsa technique. A differential count of 500 cells was done for each aspirate. Samples that were not analyzed on the day of aspiration were stored by the following procedure. The cell suspension was transferred to polypropylene tubes, frozen rapidly in a mixture of dry ice and 99% ethanol, and stored in a freezer at −80°C. Before analysis, the samples were thawed rapidly in a water bath at 37°C. When treated in this way, aspirates can be kept for at least 1 year without any detectable change in the DNA distributions. Staining with propidium iodide was done with the low-salt procedure as described previously (19). The yield of stained nuclei was 10^4 to 5 × 10^6 per aspirate. The number of nuclei analyzed per sample was 10^4 to 10^5.

The flow cytometer used was a Model 4802 cytofluorograph (Bio/Physics Systems Inc., Mahopac, N. Y.). A diploid standard was analyzed before each sample. Whole blood from AKR mice, containing 80 to 90% lymphocytes, was used for this purpose. Control experiments showed no difference in fluorescence between human leukocytes and the diploid standard. For each aspirate, the DNA distribution was recorded, and photographs were taken of the diploid standard, the DNA distribution, and the DNA scatter dot plot. The fractions of cells in the cell cycle phases were calculated. The statistical method used was a modified version of the procedure described elsewhere (6). For diploid tumors, the fractions were corrected for the admixture of normal cells as estimated by the differential counts. The correction was made assuming that all normal cells were in G1. The DI defined as the ratio of the peak channel number for the G1 phase of the tumor cells to that of the diploid standard (1) was calculated. The zero-point location of the multichannel analyzer was not stable when tested on different days. A correction for this was made as follows: the means of the Gaussian curves fitting the G1 cells and the G2 cells were determined by statistical analysis. The corrected zero point was defined such that the G2 mean is twice that of the G1 mean.

### RESULTS

#### Differential Counts. Tumor cells obtained by in vivo aspiration.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histological classification (WHO 1967)</th>
<th>Previous treatment</th>
<th>No. of samples</th>
<th>Observation time (days)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>II, 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−</td>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>II, 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−</td>
<td>1</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>II, 2</td>
<td>+</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
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<td>II, 3</td>
<td>+</td>
<td>2</td>
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</tr>
<tr>
<td>5</td>
<td>II, 3</td>
<td>+</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>II, 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>1</td>
<td>13</td>
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<td>7</td>
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<td>+</td>
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</tr>
<tr>
<td>13</td>
<td>II, 3</td>
<td>−</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

* Days between first and last aspiration.

### Notes

<sup>a</sup> Polygonal subtype.

<sup>b</sup> Lymphocyte-like subtype.

<sup>c</sup> Lymphocytoid subtype.

<sup>d</sup> Number of samples from each tumor site.

<sup>e</sup> Fusiform subtype.

<sup>f</sup> Small cell anaplastic carcinoma of the lung; subclassification not possible.
tion of a metastasis are inevitably mixed with a small amount of blood. In addition, the metastasis may contain other diploid host cells. In the case of a diploid or near-diploid tumor, the DNA analysis cannot be fully interpreted unless the admixture of normal diploid cells is known. Cytocentrifuge slides were therefore prepared for each aspirate. In strongly aneuploid tumors, normal cells and tumor cells can be distinguished by their DNA content. To evaluate the precision of the differential counts, a plot was made (Chart 1) showing the correlation between the percentage of normal cells in the differential counts and the percentage of normal cells determined from the DNA distribution. The latter percentage was calculated as the area of the diploid peak in percentage of the area of the whole DNA distribution. A correction was made for necrotic cells by subtracting the area below an interpolation line as shown in Chart 6B. The percentage of diploid cells estimated by the 2 methods should be equal, provided that all nonneoplastic cells were in the G1 phase and that no diploid tumor cells were present. A clear correlation is seen in Chart 1. Deviations of the magnitude observed do not impair the conclusions of this paper (see "Discussion").

Ploidy. All patients with the exception of one (Patient 30), for whom no diploid standard was run, were evaluable with respect to ploidy (DI). The distribution of the DI determined in the 29 patients is shown in Chart 2. Bars indicate standard deviations based on 2 to 20 observations. The coefficient of variation of the DI determination based on all measurements was estimated as 0.039. In 23 (79%) patients, only one cell line could be detected. Evidence of the presence of 2 tumor cell clones with different ploidy was obtained in the remaining 6 (21%) patients. Of the 35 malignant clones thus demonstrated, 26 (74%) were significantly different from diploid (p ≤ 0.01). Four clones (11%) were hypodiploid, 3 (9%) were hypotetraploid, and 19 (54%) were hypo- or near-tetraploid. In 5 patients (Patients 4, 5, 23, 27, and 29), 2 metastases were analyzed with identical results.

The finding of more than one malignant clone in some pa-

![Chart 1. The percentage of diploid or near-diploid cells, determined as the area of the diploid peak in percentage of the area of the whole DNA distribution, is plotted against the percentage of normal cells found in the cytocentrifuge slides. △, 18 different aspirates from Patient 14; ○, 6 different aspirates from Patient 28; □, 10 aspirates from 10 different aneuploid metastases prior to chemotherapy; ×, the aspirates of Patient 1 and Metastasis 2 of Patient 3, where evidence of near-diploid tumor clones was found.](chart1.png)

![Chart 2. DI's of the 35 different clones demonstrated in 29 patients. The patients were numbered according to the lowest DI found in each patient. Two clones were found in one metastasis in Patients 1, 3, 16, 22, and 28, and in different metastases in Patient 13. Circles (C) close together indicate that 2 metastases were examined with results not significantly different. Bars, S.D. based on 2 to 20 observations.](chart2.png)

![Clonal Heterogeneity of Small-Cell Carcinoma of the Lung](image.png)

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Chart 3. DNA distribution from Patient 16 showing 2 different aneuploid clones in one metastasis. The G₁ peaks are located in Channels 67 and 81. The corresponding G₂ peaks are located in Channels 136 and 160.

Chart 4. DNA distributions of 3 different metastases from Patient 13. A and B, distributions of Skin Nodules 1 (A) and 2 (B) prior to therapy. A: G₁ = 62%, S = 34%, G₂ + M = 4%, coefficient of variation = 3.6%, and DI = 1.65. B: G₁ = 64%, S = 32%, G₂ + M = 4%, coefficient of variation = 4.0%, and DI = 1.66. C and D, DNA distributions of Skin Nodules 1 (C) and 2 (D) 2 days after initiation of treatment with CCNU, cyclophosphamide, and vincristine. C: G₁ = 31%, S = 60%, G₂ + M = 9%, and coefficient of variation = 4.6%. D: G₁ = 37%, S = 56%, G₂ + M = 7%, and coefficient of variation = 4.1%. E, DNA distribution of a surgically removed lymph node metastasis; differential count showed 69% tumor cells. The percentages of cells in the cycle phases were corrected accordingly: G₁ = 73%, S = 13%, G₂ + M = 14%, coefficient of variation = 5.6%, and DI = 1.05. Part of this lymph node was transplanted to nude mice. F, DNA distribution of the resulting transplantable tumor: G₁ = 74%, S = 11%, G₂ + M = 15%, coefficient of variation = 5.7%, and DI = 0.98. A differential count was not done.

Chart 5. DNA distributions of one metastasis in Patient 22. A, the distributions prior to therapy. The shoulder on the left side of the G₁ peak was ignored in the statistical analysis. G₁ = 46%, S = 48%, G₂ + M = 6%, coefficient of variation = 3.3%, and DI = 1.85. B and C, DNA distribution on Days 2 (B) and 6 (C) after start of treatment with cyclophosphamide, CCNU, and vincristine. B: G₁ = 26%, S = 66%, G₂ + M = 8%, and coefficient of variation = 3.9%. C: G₁ = 6%, S = 90%, G₂ + M = 4%, and coefficient of variation = 3.6%. D, DNA distribution on Day 13 after start of chemotherapy. The shoulders on the right side of both the G₁ and the G₂ peaks were ignored in the statistical analysis. G₁ = 68%, S = 24%, and G₂ + M = 8%. All signs of a second cell clone had disappeared on Day 30 after start of the treatment (E). G₁ = 71%, S = 25%, G₂ + M = 4%, coefficient of variation = 4.0%, and DI = 1.67. The tumor relapsed locally after 14 months in complete remission (F). G₁ = 74%, S = 12%, G₂ + M = 14%, coefficient of variation = 4.4%, and DI = 1.79.

had a lower DI (1.67). Radiotherapy was started on Day 40 and included the monitored supraclavicular metastasis. A complete remission was obtained, and the patient was in remission for 14 months on treatment with cyclophosphamide, CCNU, vincristine, and methotrexate. The tumor relapsed at the same location (Chart 5F) with an S phase further reduced to 12% and a DI of 1.79. DI's of 1.67 and 1.79 in different samples are not significantly different (p = 0.17). The main source of variation in the DI determination is instability of the flow cytometer. Reproducible double peaks or shoulders in one distribution are on the other hand firm evidence of the presence of 2 clones. In this way, DI differences so small that they are overlooked if different samples are compared can be detected.

In Patient 28 results similar to those just described were found. A DNA distribution with double G₁ and G₂ peaks was found (Chart 6A). Treatment resulted in a partial remission of the metastasis and disappearance of the clone with the highest
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There was a marked reduction in the size of the S phase as seen in Chart 6B (Day 9).

In Patient 3, 2 lymph node metastases were aspirated. Prior to treatment, very similar DNA distributions were obtained from the 2 lesions (Chart 7, A and B). The admixture of normal cells in Metastasis 1 was estimated as 41% by the differential count versus 34% by integration. The corresponding values for Metastasis 2 were 1% versus 17% (see Chart 1). The DI's of the near-diploid peaks were 0.98 and 0.90, respectively. These findings suggest the presence of a second hypodiploid tumor cell clone in Metastasis 2.

When treatment with cyclophosphamide, CCNU, and vincristine was started, the DNA distributions showed on Day 3 the often seen accumulation of cells in mid-S phase of both hypotetraploid tumors [peaks at Channels 140 to 150 (Chart 7, C and D)]. In addition, Metastasis 2 showed an accumulation of cells between the hypodiploid and the hypotetraploid G1 peaks (peak at Channel 70 in Chart 7D). This finding strongly supports the presence of a second hypodiploid tumor cell clone in Metastasis 2.

In Patient 1, only one aspiration was obtained (not shown). The majority of cells belonged to a tumor cell clone with a DI of 1.80. There was a near-diploid peak accounting for 25% of the distribution area and with a DI of 0.88. This low DI in combination with a differential count showing only 4% admixture of normal cells (see Chart 1) makes it probable that 2 different tumor cell clones were present.

**DISCUSSION**

In this study on small-cell carcinoma of the lung, fine-needle aspirates were examined by differential counts and flow-cytometric DNA analysis. The results were used to estimate ploidy and identify clonal heterogeneity.

The differential counts were found to be relatively inaccurate. The impact of this can be illustrated by calculating the effect of an admixture of, e.g., 10% normal diploid G1 cells, to a typical diploid tumor with 70.0% G1 cells, 20.0% S cells, and 10.0% G2 + M cells. This will change the ratios of cells in G1:S:G2 + M from 70:20:10 to 80:20:10 which corresponds to 72.7% in G1; 18.2% in S, and 9.1% in G2 + M. Deviations of this magnitude are insignificant when compared with the substantial differences and changes found in this study.

Small-cell anaplastic carcinoma of the lung is a heterogeneous group of tumors with respect to DI. The ploidy is not scattered at random but is grouped into near-diploid, -triploid, and -tetraploid values. Earlier results obtained by absorption cytophotometric DNA analysis on Feulgen-stained slides have been reviewed by Böhm and Sandritter (4). The results from a total of 17 patients, mainly autopsy cases, showed in 7 patients (41%) near-diploid DI's, in 7 patients (41%), near-triploid values, and in 3 patients (18%), DI's from 2.5 to 3.75. In contrast to our results, no near-tetraploid tumors were found.

In our study, aneuploidy was clearly present in 26 (74%) of total (of the tumor cell clones of Patients 1 to 4 and 13 to 29 (Chart 2). In 9 of the tumor cell clones of Patients 5 to 13, the DI's ranged from 0.96 to 1.05. The resolution of the DI determination does not allow detection of such small deviations from normality as would result from the loss or gain of 1 to 4 average-sized chromosomes (coefficient of variation, 0.039).

In 6 (21%) of the 29 patients evaluable for ploidy, 2 different tumor cell clones were demonstrated either in the same metastasis or in different metastases. Five of the 6 patients were previously untreated. This finding clearly demonstrates that some small-cell anaplastic carcinomas are not monoclonal at the time of diagnosis. The true occurrence of di- or possibly polyclonality is likely to be higher than the 21% found in our study. A closer examination of each patient, including multiple biopsies of several metastases, would probably have shown more heterogeneity. However, it should be realized that quite narrow limits for detection are set by the relative representation of each clone in a metastasis. If the difference in DI's is small (see Charts 5A and 6A), clones representing less than 30 to

![Chart 6](image-url)

**Chart 6.** DNA distributions of one metastasis in Patient 28. A, the distribution prior to therapy interpreted as 2 overlapping clones with DI's estimated as 1.92 and 2.07; B, the DNA distributions on Day 9 after start of chemotherapy. G1 = 80%, S = 7%, G2 + M = 13%, coefficient of variation = 4.8%, and DI = 1.89. DI of the near-diploid peak = 0.98. C and D, interpolation line (broken line separating diploid peak and debris) was used as a correction for necrotic cells in the estimate of the percentage of normal cells (see Chart 1).

![Chart 7](image-url)

**Chart 7.** DNA distributions of 2 different metastases in Patient 3. A and B, distributions prior to chemotherapy. A (Metastasis 1): G1 = 64%, S = 25%, G2 + M = 11%, and coefficient of variation = 4.8%. DI of the near-diploid peak = 0.98. DI of the hypotetraploid peak = 1.79. B (Metastasis 2): G1 = 64%, S = 26%, G2 + M = 10%, and coefficient of variation = 4.7%. DI of the near-diploid peak = 0.90. DI of the hypotetraploid peak = 1.81. C and D, DNA distribution on Day 2 after start of therapy with cyclophosphamide, CCNU, and vincristine. C (Metastasis 1): G1 = 30%, S = 55%, G2 + M = 15%, and coefficient of variation = 3.8%.

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40% of all cells may easily be overlooked, and clones representing less than 5 to 10% cannot be identified with certainty, even when the G1 peaks are favorably separated.

Based on cytogenetic studies, the concept of "clonal evolution" (13) has been developed. It encompasses the principle that tumors progress on the basis of genetic instability within the neoplastic population. This leads to the sequential emergence of mutant subpopulations with increasingly "malignant" properties (14). It is a consequence of this theory that each neoplasm has its own individual characteristics, which is in excellent agreement with our DI determinations (see Chart 2).

Two clones were demonstrated in some metastases. With the above-mentioned detection limits in mind, this points to either a high mutation rate or a substantial difference in TD's of the clones. To reach 10⁶ to 10¹² cells (1 g to 1 kg of tumor), 30 to 40 tumor volume doublings have occurred. If the 2 clones have identical TD's, they must both have been present within the first 4 TD's of the tumor. Otherwise, one of them would constitute less than 6.3% (one-sixteenth) of the total mass and would therefore escape detection. Similar doubling times therefore imply a high mutation rate. Conversely, the emergence of a second clone at a later point in growth (low mutation rate) implies a substantial increase in TD to allow the second clone to catch up with the first.

Heterogeneity of tumor cells was recently demonstrated by Dexter et al. (7) in a mouse mammary tumor. Three sublines of this tumor differed significantly when tested in vivo for drug sensitivity (9). Similar observations have been made by Barranco et al. (2, 3) and Håkansson and Tropé (8). Taking these studies into account, our results suggest that drug resistance of a substantial fraction of a small-cell anaplastic carcinoma of the lung may exist prior to therapy, a notion fully compatible with clinical observations of partial remissions of short duration, or the emergence of one metastasis with the simultaneous progression of another. In contrast to this are the results of extensive trials in transplantable animal tumors where the ratio of drug-resistant cells to drug-sensitive cells has been estimated as 1:10⁶ to 1:10⁷ cells (16).

The emerging concept of a fully developed small-cell carcinoma as different tumor cell clones in a mixture, unique for each patient, is somewhat discouraging from a clinical point of view. It suggests that if curative therapy is to be designed, it may have to be individualized for each patient. Methods for guidance of such therapy are at present scarce. Sequential flow-cytometric DNA analysis may prove useful in selected patients, as will be shown in a forthcoming paper.

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