Prognostic Relevance of Ploidy, Proliferation, and Resistance-predictive Tests in Ovarian Carcinoma

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ABSTRACT

In a cooperative study specimens of 37 patients with stage III and IV ovarian carcinomas who had been treated with chemotherapy were investigated utilizing flow cytometry and an *in vitro* short-term test for predicting resistance. Patients with aneuploid tumors had significantly shorter survival rates than did those with diploid tumors. Patients whose tumors showed a low Go/G1 cell proportion or a high proliferation pool (S- and G2/M cell-proportion) seemed to die earlier. There was also a tendency for patients with *in vitro* resistant tumors to die earlier under chemotherapy than with sensitive tumors.

INTRODUCTION

Flow cytometry provides a fast and precise method for determination of DNA ploidy and distribution of the cell cycle in tumors. The major question concerning the clinical applications of flow cytometry is their relation to prognosis, metastatic activity, and sensitivity to treatment (1). The relationship between DNA abnormality or proliferative activity in solid tumors and prognosis remains to be elucidated, in spite of some preliminary studies which demonstrate that high DNA index values and a high proportion of S-phase cells are prognostic factors for survival times (2–10).

The few studies of ovarian carcinomas using flow cytometry (6, 11–15) have also shown a possible relationship between ploidy and biological behavior in these tumors (6, 11). However, the DNA content was determined from archival paraffin-embedded tumor tissue from patients whose clinical course was known. Therefore we determined in a prospective study whether indeed DNA content and distribution of cell cycle phases in operative specimens have prognostic importance in patients with ovarian carcinomas. In addition the resistance of tumors under chemotherapy was determined using a short-term test (16–19) and the results were related to the survival time of the patients.

MATERIALS AND METHODS

Patients. Thirty-seven patients with previously untreated ovarian carcinomas were entered into the study (Table 1). These patients were chosen from one specific clinic involved in a multicenter study. The deadline for the compilation of data for this analysis was October 1, 1984. The median length of follow-up of patients was 2 years.

Three different treatment regimens were compared, the results of which will be published in a future paper: Regimen A, cyclophosphamide, 1000 mg/m² i.v. (n = 9), three cycles, with an interval of 4 weeks, and afterwards 80 mg/m² p.o. per day over 9 months ; Regimen B, cyclophosphamide, 600 mg/m² i.v. (day 1), and cis-platinum, 80 mg/m² i.v. (day 3) 7 cycles, with an interval of 4 weeks; Regimen C, cyclophosphamide, 500 mg/m² i.v., Adriamycin, 50 mg/m² i.v., and cis-platinum 50 mg/m² i.v. (all day 1), 10 cycles, with an interval of 4 weeks (n = 15).

In his prospective randomized study only those patients were included who had received more than 80% of the chemotherapy dosage, who had been treated within 4 weeks after surgery, who were alive more than 6 weeks after surgery, and the results of whose therapy could be evaluated (eight patients were excluded).

Staging was performed according to the Cancer Committee of the International Federation of Gynecology and Obstetrics. Histological classification was based on the standards of the federation and WHO. The tumors included 33 serous carcinomas (IA3a), 3 undifferentiated carcinomas (IG), and 1 endometrioid carcinoma (IC3a). Nine of the 37 tumors were highly differentiated, 8 were moderately differentiated, and 20 were poorly differentiated. Twenty of the patients had stage III disease and 17 had stage IV disease. The median age at the time of operation was 60 years (range, 29 to 82 years). The following surgical procedures were performed: 6 patients underwent an inspection-laparotomy and only biopsies of metastases were possible (residual tumor >5 cm rest); in 7 patients at least one ovarian tumor could be resected and designated as "adnex resec tion" (residual tumor >5 cm rest); in 24 patients debulking surgery could be performed with bilateral ovariectomy and salpingectomy, hysterectomy, and omentectomy, but in most of the cases residual tumor >2 cm in diameter had to be left (2 cases 1–2 cm, 22 cases 2–4 cm).

Flow Cytometry. The tissue pieces were collected in vials containing methanol. Before measurement tumor material was minced, suspended in Hanks' balanced salt solution, and pipeted several times using a sharp-edged glass pipet; the resultant cell suspension was then filtered through a 70-mm filter and centrifuged for 5 min at 200 x g, and washed with Hanks' balanced salt solution. The cell count was adjusted to 10⁶ cells/ml. Adjacent parts of the specimens were used for additional histological examination of tumors. The biological variability within the individual samples was minimized by using large segments of the tumors. Aliquots for the measurements were removed from cell suspensions made from the whole sample.

The nuclear fluorescence of the specifically stained DNA was measured with an ICP 22 pulse cytophotometer (Plywe AG, Göttingen, Federal Republic of Germany) using a mixture of propidium iodide (10 μg/ml) and 4',6'-diamidino-2-phenylindole (2 μg/ml). These DNA-specific dyes were applied simultaneously with RNase (1 mg/ml) after protease digestion (solution of 0.5% pepsin). The G0-G1 diploid peak was identified by adding peripheral blood leukocytes from healthy donors.

Predicting Drug Resistance. The short-term test for predicting resistance has been described previously (16–19), its basic feature is measurement of changes in incorporation of radioactive nucleic acid precursors into tumor cells after addition of cytostatic agents. Briefly the solid tumors are first mechanically disrupted and then filtered through gauze (pore size, 200 μm); the cells are then sedimented by centrifugation (200 x g, 5 min) and subsequently resuspended at a defined cell density (5 x 10⁶ cells/ml). The suspensions are then incubated with the particular cytostatic agent to be tested in different concentrations in a water bath for 3 h. The radioactive precursor is added during the last hour of incubation. Then aliquots of the cell suspensions are pipeted onto filter...
Paper discs and the acid-soluble radioactivity is extracted (5% ice-cold trichloroacetic acid, twice for 30 min; 100 filters to 1). The filters are then washed in ethanol:ether (1:1, 20 min) and ether (10 min) and air-dried. The incorporated activity is measured by scintillation counting. Uptake values for the individual concentrations are expressed as percentage of controls. The test threshold between sensitive and resistant tumors was taken from an earlier clinical study (17).

Analysis of Data. The method for analysis of survival is the statistical failure time model with censored data according to the method of Kaplan and Meier (20). For comparison of the functions of different populations the log-rank test and the generalized Wilcoxon test for univariate data analysis were used (21, 22). They are rank tests based on exponential or Wilcoxon scores. Both statistical methods were integrated in a program package by Edler ef al. (23).

The cutoff point for low or high fractions of S-G2-M-phase cells was determined by the CRITLEVEL procedure (24). This is a simple but efficient graphic tool for determining those levels of quantitative prognostic factors at which the most pronounced deterioration of prognosis takes place.

RESULTS

Tumor samples for flow cytometry analysis and predicting resistance were obtained from 37 patients with previously untreated ovarian carcinomas of stages III and IV (Table 1). Chart 1 shows representative DNA histograms. From tumors of 37 patients we classified seven cases as tumors with DNA diploidy (Chart 1A), 27 cases as tumors with DNA aneuploidy containing one abnormal DNA stem line (Chart 1B), and 3 cases as tumors with DNA aneuploidy containing more than one abnormal DNA stem line (Chart 1C). All but one tumor with DNA aneuploidy contained a population of cells with a normal DNA stem line (DNA diploid). Overlapping of subpopulations (near DNA diploid and DNA multiploid) was the reason for omitting tumor samples from cell cycle analysis (see Table 1).

In Chart 2 the observed DNA indices are demonstrated. Included are all DNA stem lines measured apart from DNA diploid lines within tumors with abnormal DNA stem lines. The DNA index values range from a value of 1.6 to a hyperploid value of 5.4.

A relationship between DNA content and distribution of the cell cycle phases was observed. Tumors with a normal DNA stem line had a higher proportion of Go-G1-phase cells and a lower proportion of S-phase or G2-M-phase cells than tumors with abnormal DNA stem lines (Table 2).

The prognosis of patients with ovarian carcinomas is largely determined by the extent of residual disease and tumor grade followed by stage, age, and histological type (25, 26). In our study the stage was an important prognostic factor (Chart 3; }
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Chart 1. Representative examples of the DNA histograms of ovarian carcinomas. A, normal DNA stem line (DNA diploid); B, abnormal DNA stem line (DNA aneuploid); C, more than one abnormal DNA stem line (DNA multiploid). Cell cycle analysis was performed in areas marked by darkening. CV, coefficient of variation; SYN, S-phase cells.

Chart 2. Distribution of DNA indices in ovarian carcinoma. The DNA index represents the ratio of relative DNA contents of tumor versus normal G0/G1 cells. Tumors were classified as DNA diploid when only a diploid cell population was present. Diploid DNA stem lines of heterogeneous tumors are omitted.

= 0.036). The different surgical procedures and cytostatic treatment, histological tumor type, and differentiation had no significant effects on the survival time of the patients of our study. Perhaps it is the result of the small sample size.

The results of DNA content analysis have prognostic importance with regard to the length of survival time. Patients with aneuploid tumors had significantly shorter survival times than did those with diploid or near diploid tumors (Chart 4; P = 0.029). The distribution of diploid and aneuploid tumors is similar in stage III and IV disease.

Cell cycle analysis was possible in 27 tumors. Patients whose tumors had a high G0/G1 cell proportion (>83%) seemed to survive longer than did patients with a low G0/G1 cell proportion. If the proliferative pool (S-G2-M phase cells, >17%) of tumors was high, it appeared that the patients died earlier (Chart 5; P = 0.094).

Samples of ovarian carcinomas were investigated by means of the short-term test for predicting resistance. There was a tendency for patients with in vitro resistant tumors to die earlier under chemotherapy than did those with sensitive tumors (Chart 6; P = 0.080).

DISCUSSION

In our study we investigated the specimens of 19 primary tumors and 18 metastases. The value of this investigation would be limited if primary tumors exhibited a variation of DNA ploidy and a different proportion of cell cycle phases when compared to metastases. Therefore we examined the heterogeneity of cellular DNA content and of the cell cycle distribution in primary ovarian carcinomas and their metastases in eight patients (Table 3). Primary tumors and metastasis material were available from these patients. We found a high stability in DNA content and of the proportion of cell cycle phases. Although the proportion of diploid and aneuploid cells is variable between primary carcinomas and metastases, the similarity of the DNA index and of the proportion of cell cycle phases shows that DNA content is a stable feature in most cases of ovarian carcinomas. These results justified our considering primary carcinomas and metastases together.

The few studies of ovarian cancer using flow cytometry have shown aneuploid DNA content between 55 and 76% (1, 6, 13). We found that 81% of the patients had tumors with abnormal DNA stem lines. The majority of the aneuploid tumors were in the triploid range. Seven of the tumors were near diploid and
PROGNOSTIC FACTORS IN OVARIAN CARCINOMA

Table 2

<table>
<thead>
<tr>
<th></th>
<th>G0-G1 Mean ± SD</th>
<th>Median</th>
<th>Range</th>
<th>S Mean ± SD</th>
<th>Median</th>
<th>Range</th>
<th>G0-M Mean ± SD</th>
<th>Median</th>
<th>Range</th>
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<tbody>
<tr>
<td>Total</td>
<td>79.5 ± 8.3</td>
<td>80.5</td>
<td>(65-92)</td>
<td>10.8 ± 5.8</td>
<td>11.0</td>
<td>(2-23)</td>
<td>9.8 ± 4.5</td>
<td>8.5</td>
<td>(4-18)</td>
</tr>
<tr>
<td>Diploid</td>
<td>89.2 ± 2.2</td>
<td>89.0</td>
<td>(86-92)</td>
<td>4.2 ± 1.8</td>
<td>4.5</td>
<td>(2-6)</td>
<td>6.6 ± 1.4</td>
<td>6.5</td>
<td>(5-9)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>76.3 ± 7.0</td>
<td>76.0</td>
<td>(65-88)</td>
<td>13.9 ± 4.9</td>
<td>12.0</td>
<td>(8-23)</td>
<td>10.9 ± 4.7</td>
<td>10.5</td>
<td>(4-18)</td>
</tr>
<tr>
<td>Diploid-aneuploid</td>
<td></td>
<td></td>
<td></td>
<td>*P = 0.005</td>
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* x² test (median).

In our study diploid tumors had an S-phase cell fraction of 4.2 ± 1.8 (SD) while aneuploid tumors had a significantly higher S-phase (6.6 ± 1.4). A possible reason that diploid tumors have a lower proportion of S-phase cells might be that admixed normal cells could lower the proportion of S-phase cells. Corresponding values reported by Friedlander et al. (13) were 9.8 and 19.6%. Labeling indices hitherto published using autoradiography show an average fraction of S phases which is lower than in studies using flow cytometry (27). The discrepancy between data obtained using autoradiography and flow cytometry disappeared largely after carrying out appropriate correction for systematic errors by subtraction of background debris and cell aggregates (28). The data that we acquired by flow cytometry corresponds better with data obtained by autoradiography.

A recent multivariate analysis of 430 patients with ovarian carcinomas indicated that extent of residual disease and histological grade were the most important prognostic factors for survival, followed by stage, age, and histological type (26). In our study the prognosis of patients with ovarian carcinomas is determined only by the stage. Different surgical procedures, chemotherapeutic regimens, and hormone therapy may also influence the prognosis.

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other therapy schedules, histological grade, and type of tumor had no significant effect on the survival time of the patients. This may be due to the small number of patients.

The present report shows that DNA content analysis has prognostic importance with regard to length of survival times of patients with ovarian carcinomas. Patients with tumors with diploid or near diploid DNA stem lines survived significantly longer than patients having tumors with abnormal DNA stem lines. The reason for these differences in biological behavior in diploid or near diploid and aneuploid tumors is unknown. The aneuploid tumors seem to be genetically more unstable which could give rise to new cell variants (6).

In our study patients with tumors having a high proliferative activity seemed to die earlier than did patients whose tumors only had a small fraction of S-phase and G2-M-phase cells. Corresponding results were obtained in studies of non-small cell lung carcinomas (27). The question arises whether groups of patients classified according to the newly observed prognostic factors experience an advantage or disadvantage from particular modalities of therapy. We consider it important to carry out such investigations to identify groups which are either sensitive or resistant to commonly used chemotherapy than those with sensitive tumors (17–19). Because the assessment of clinical response is often subjective, we restricted the investigation to survival of the patients.

In conclusion our experiments have shown that measurement of DNA ploidy and proliferative activity as well as short-term tests might be additional prognostic indicators for patients with ovarian carcinomas.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Berna McGuckin for reading the manuscript.

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*Cancer Res* 1985;45:5180-5185.

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