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Mitotic index in the subrenal capsule assay as an indicator of the chemosensitivity of ovarian cancer

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Abstract The subrenal capsule assay (SRCA) is used in clinical oncology to assess the sensitivity of individual malignant tumors to various anticancer agents and their combinations. Mitotic indices reflect cancer cell proliferation and have prognostic value in epithelial neoplasms, including ovarian carcinoma. We combined the two tests (SRCA, mitotic index) by evaluating the numbers of mitotic figures per square millimeter of neoplastic epithelium (M/V) in paraffin-embedded tumor samples after SRCA. The M/V index was compared with the tumor size measurement (dTS), which is used in conventional SRCA to predict the drug response. Histology examination showed insignificant changes in the size of tumor transplants due to host reaction but disclosed a number of potential errors in the use of dTS to evaluate transplant growth and drug effects. In our series of 62 patients with advanced ovarian carcinoma the M/V value was superior to the dTS in explaining the clinical response after 6 months as assessed at second-look laparotomy. Patients showing no response had significantly higher M/V values than did those displaying complete or partial responses ($P < 0.033$). The use of 6 mitotic figures/mm^2 as a limit differentiating responders from nonresponders resulted in an overall predictive accuracy of 79% in the logistic regression analysis. In comparison to the FIGO stage, residual tumor size, and the dTS, the M/V value obtained for the cytostatic combination given to the patient was the single most significant factor predicting the 6-month clinical response. The results indicate that the combined use of the M/V index and SRCA is a promising new approach to prediction of the drug response in ovarian adenocarcinoma.

Key words Mitotic index · Chemotherapy · Sensitivity · SRCA · Morphometry

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Introduction

The use of cytotoxic agents is an essential part of the treatment of ovarian epithelial malignancies [19]. To improve the survival rate of ovarian cancer patients, several methods of chemosensitivity testing have been developed. Currently, most chemosensitivity tests are performed in vitro on cultured cells. The subrenal capsule assay (SRCA) is an in vivo test whereby small tumor samples are transplanted under the renal capsule of mice. Originally, nude mice were used as tumor recipients [4], but subsequently, normal immunocompetent mice were found to be equally suitable, provided that the grafts were allowed to grow for only 6 days [5]. Our previous studies have shown that in addition to single agents, the effects of combination chemotherapy can be tested in the SRCA [29–31]. In conventional SRCA the drug effect is measured as the difference between the final and the initial transplant diameter (dTS). However, tumor tissue may also grow in terms of depth, and the 6-day period is a relatively short time for any significant difference in tumor diameter to develop.

The view was developed that methods directly assessing the proliferative activity of cancer cells might give more accurate results in the SRCA. Immunohistochemical staining of cancer cells at the DNA-synthesizing stage

[39] and biochemical determination of chemosensitivity by measurement of the specific activity of succinate dehydrogenase in the tumor implants [38] have been combined with the SRCA, but data on their predictive clinical value are not presently available. The mitotic index is a simple and rapid method for determination of the proliferative activity of cancer cells, and it provides significant prognostic information in a wide variety of different malignancies [1, 17, 27]. In this study the use of dTS in a standard SRCA [7] was compared with the use of the mitotic index [16] in evaluation of the chemosensitivity of ovarian cancers in a series of 62 patients.

Patients and methods

Patients

The tumor transplants were obtained from 62 ovarian cancer patients who had been randomized to the SRCA arm of a study in which the efficacy of SRCA-guided chemotherapy was compared with that of a standard regimen, cyclophosphamide-doxorubicin-cisplatin (CAP) [34]. We excluded 5 patients from this analysis due to poor tumor growth in the control group as assessed by the numbers of mitotic figures per square millimeter of neoplastic epithelium (M/V), reducing the number of evaluable assays to 57. The mean age of the patients was 58 (SD 8) years (range 39–72 years). In all, 14 of the tumors were of FIGO stage IIb, 28 were of stage III, and 15 were of stage IV. Altogether, 45 of the samples were derived from primary tumors and 12, from metastases.

All the patients received six courses of a drug combination selected individually on the basis of the SRCA as assessed by the dTS. The drug combinations given to the patients are shown in Table 1. The clinical responses were confirmed at relaparotomy and classified by standard criteria as a complete (CR) or a partial (PR) response, no change (NC), or progressive disease (PD) [36].

Tumor samples

The presence of tumor tissue in the biopsy sample was confirmed perioperatively by a board-certified pathologist. Ten tumor pieces measuring 3 mm × 3 mm in diameter were immersed in a test tube containing 10 ml Medium 199 supplemented with Earle's salt, L-glutamine, and $NaHCO_3$ at 2.2 g/l (Gibco, UK). To prevent bacterial contamination of the medium, 160 μg gentamycin sulfate (Sigma Chemical Company, UK) was added to each test tube. The test tubes were protected against heating and freezing, and the time elapsing from the tumor excision to implantation never exceeded 24 h. Before implantation the tumor samples were cut into 1-mm^3 pieces under a stereomicroscope.

Table 1 The drug combinations given to the patients

Group	Combination	Patients
1	Carboplatin + cyclophosphamide + doxorubicin (CARAP)	11
2	Cisplatin + cyclophosphamide + doxorubicin (CAP)	19
3	Carboquone + methotrexate + tegafur (MTQ)	9
4	Cisplatin + hexamethylmelamine +etoposide (PAE)	12
5	Bleomycin + epirubicin + cisplatin (BEP)	6

Subrenal capsule assay

The basic SRCA technique [5, 6] was used, with slight modifications. Normal immunocompetent female CD2F1 mice (barrier maintained at the breeder) aged 8 weeks were purchased from the National Laboratory Animal Center (Kuopio, Finland). Initial body weights were 17–30 g. The animals were anesthetized with 0.22 *M* chloral hydrate given i.p. at 0.1 ml/10 g body weight. The left kidney was exteriorized and a small incision was made through the renal capsule. Two tumor pieces of approximately 1 mm^3 were inserted under the translucent renal capsule with the aid of a trocar and a stereomicroscope fitted with an ocular micrometer. The exact sizes of the fragments [$(length + width)/2$] were measured with the micrometer and were expressed in terms of ocular micrometer units (OMU), where 10 OMUs are equal to 1 mm. The kidney was then repositioned into the body cavity and the wound was closed (day 0). On days 1–5 the drug treatment was carried out once daily. The chemotherapeutic agents, their doses, and the routes of administration are summarized in Table 2. Each treatment group consisted of three mice. The control group (four mice) received physiological saline. On day 6 the animals were killed by cervical dislocation and the final body weights and tumor sizes were recorded. The difference between the final and the initial tumor size was calculated ($dTS = final\ TS - initial\ TS$). An assay was considered evaluable if the mean dTS in the control group was positive. The drug combination that caused the dTS most divergent from that of the control group was given to the patient, even if it did not invariably cause a real implant regression.

From the implantation until day 6 the mice were kept in stainless-steel cages with solid floors at one group per cage, with standard R3 pellets (EWOS AB, Södertälje, Sweden) and tap water being available ad libitum. The duration of light/dark periods was 12/12 h. On the completion of each test the kidneys with the tumor transplants were removed, fixed in 10% formalin, and embedded in paraffin. Toxicity was evaluated by the mouse weight ratio (W_{d6}/W_{d0}). A weight loss of more than 20% was considered to be the result of excessive toxicity due to the cytostatic combination. No test had to be eliminated because of such toxicity.

Histology methods

From the paraffin blocks, 5-μm-thick sections were cut at 200-μm intervals and stained with hematoxylin and eosin. Usually, 10–20 sections were studied to assess tumor histology and mitotic activity. Counting of the mitotic figures in the transplants was done in a blind manner using an objective magnification of ×40 (field diameter 490 μm). The criteria used in identifying the mitotic figures have been described elsewhere [16]. The number of mitotic figures identified to ten consecutive microscopic fields (corresponding to 1.94 mm^2 in a section) of the most cellular areas in the sample was corrected for the fraction of neoplastic epithelium [16]. This volume-corrected M/V index expresses the mitotic activity per square

Table 2 The drugs, their doses, and the routes of administration to mice

Preparation (trademark)	Generic name	Dose mg/kg	Route of administration
Bleomycin	Bleomycin	8.00	s.c.
Carboplatin	Carboplatin	3.00	s.c.
Carboquone	Carboquone	0.25	s.c.
Doxorubicin	Doxorubicin	3.00	s.c.
Farmorubicin	Epirubicin	4.00	s.c.
Ftorafur	Tegafur	160.00	p.o.
Hexastat	Hexamethylmelamine	100.00	p.o.
Platinol	Cisplatin	1.00	s.c.
Syklofosfamid	Cyclophosphamide	30.00	i.p.
Trexan	Methotrexate	3.00	s.c.
Vepesid	Etoposide	10.00	s.c.

millimeter of neoplastic tissue in the section. The histological type of all tumors collected in this multicenter study was reascertained by a board-certified pathologist.

Statistical analysis

For the basic statistical calculations the SPSS/PC+ program package was used in a Comper computer. The statistical tests used are indicated in Results when appropriate.

Results

In all cases the transplantation was adequately performed and tumor biopsy was detectable under the renal capsule. In most control transplants, tumor tissue was vital and rapidly proliferating (Fig. 1a). In 3 of the 62 assays the M/V value of the control group was 0, and in an additional 2 assays it was 1 and 2, respectively. These 5 assays were considered unevaluable by the M/V index and were excluded from the analysis; thus, 57 assays (92%) were evaluable.

If the transplanted tumor tissue was sensitive to a given combination of chemotherapeutic agents, only scar tissue was usually present under the renal capsule (Fig. 1b). If the cancer transplant was insensitive or only partially sensitive to a given drug, proliferating cancer cells (Fig. 1c) were present in the transplant, which had also increased in size. In some sections, large cystic cavities filled with inflammatory exudate and inflammatory cells were present (Fig. 1d).

M/V determination before the SRCA

Altogether, 48 of the 57 tumor samples were available for determination of the M/V index on day 0 of the SRCA. In two tests, no mitotic figure could be detected in the pretest sample, although there were numerous mitoses in the corresponding control groups. These zero values were not considered representative and were excluded from the subsequent tests. In the remaining 46 tumor samples the M/V value was 37 ± 21 (mean ± SD). In the control groups of the corresponding 46 tests (and also in the control groups of all 57 tests) the M/V index was 22 ± 12, which is significantly lower than the pretest values ($P < 0.0001$, t-test).

M/V index in control tumors

The tumor growth (dTS) in the control animals in all 57 tests was 3.1 ± 2.4 OMU (mean ± SD; 1 OMU = 0.1 mm). The histological type of the tumor was determined in 45 cases. In 36 of these the pretest samples were available. There was no statistically significant difference between the mitotic indices of different histological types in the pretest and control groups (Table 3).

The mitotic index seemed to be higher in metastatic (Table 4, Source), more advanced (Table 4, FIGO stage), and less differentiated (Table 4, Grade) pretest tumors, but the differences were not statistically significant (one-way analysis of variance, Student-Newman-Keuls procedure). The M/V value was equally high in the group of patients with no residual tumor/small residual tumor and the group with large residual tumor (Table 5).

On average the control M/V values were 85 ± 76% of the corresponding pretest values. When the clinical responses were compared with the assay results the M/V index was superior to the dTS (Table 6). The M/V (as a percentage of the control M/V) was statistically significantly higher ($P < 0.007$) in the tumors with PD (104 ± 125) than in those showing a complete (CR) or partial response (PR; 17 ± 37).

M/V index in tumors treated with cytostatics

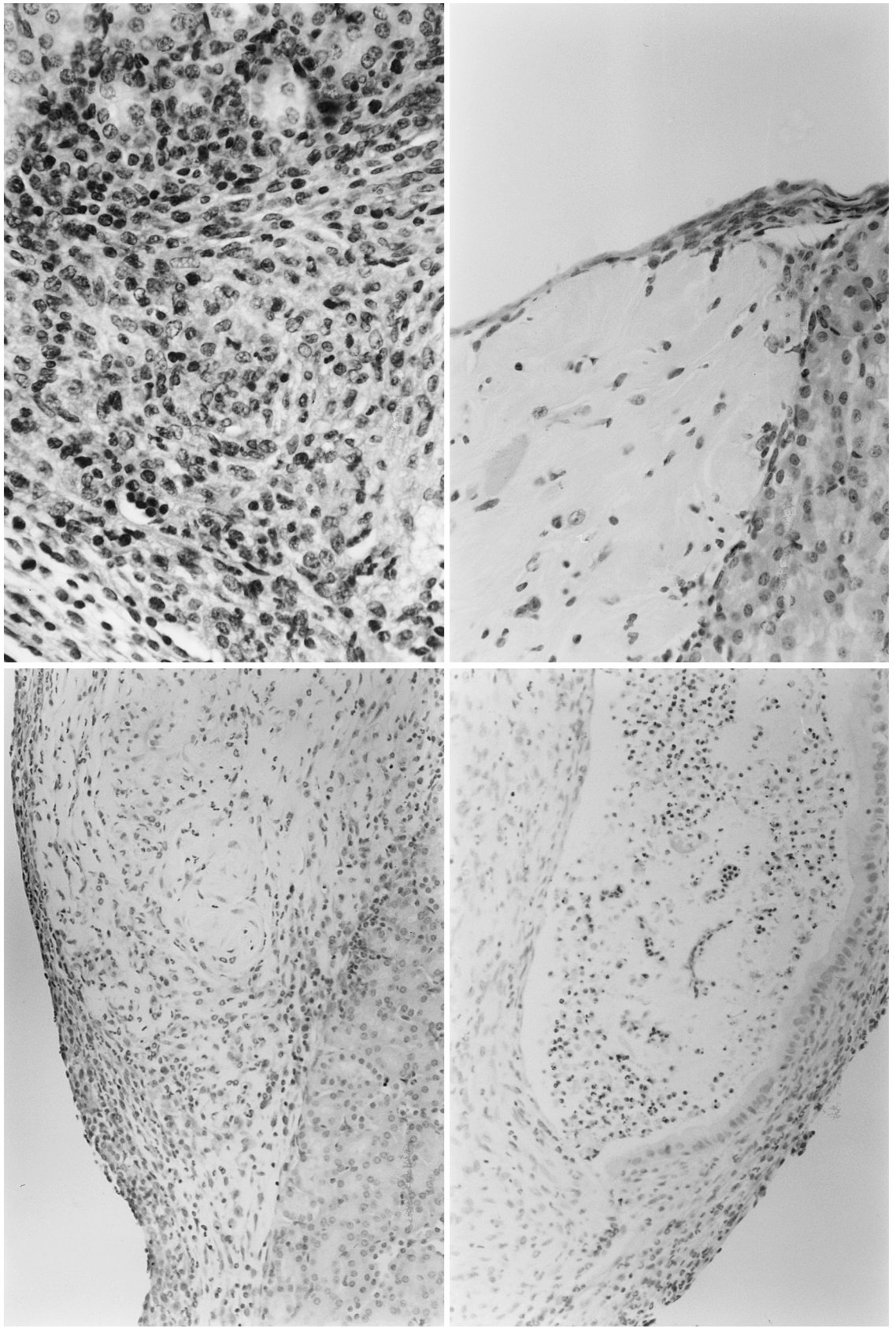
In assessment of the drug response by the M/V, the combination with the smallest M/V index was considered the most effective. To take the intrinsic tumor growth potential into account, the drug effect was measured as a percentage of the control growth. In three patients the M/V value of the control group was 0; they were excluded from further M/V analyses.

Stepwise logistic regression analysis showed the M/V index to be the most powerful predictor of the clinical response (Table 6). The use of 6 mitotic figures/mm^2 as the cutoff to divide the patients into responders and nonresponders resulted in an overall predictive accuracy of 79%. Multivariate regression analysis showed that the drug response was better explained by the M/V index than by the conventional dTS. The patient's age and the origin of the test specimen (primary tumor or metastasis) were not significant individual explanatory factors for the 6-month clinical response, whereas the FIGO stage and the residual tumor size (diameter < 2 or > 2 cm) were.

Discussion

The SRCA was originally developed to identify the best chemotherapeutic agent/combination for the treatment of malignant solid tumors [5]. The interpretation of SRCA results is based on the assumption that measurable changes in tumor size are the net effect of cell division and cell death. The tumor size increases when cell division exceeds cell death and decreases when cell death outnumbers cell division. Since the introduction of the SRCA using immunocompetent mice, there has been some doubt and criticism as to the reliability of direct tumor diameter measurement [11, 12].

The most critical commentaries have been focused on problems with the inflammatory responses in immunocompetent mice [13, 14]. T-cell activity is a prominent early component of the immunological rejection of grafted tumors [15]. Lymphocyte infiltrates exerting



a b c d

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Fig. 1a Tumor growth in a control transplant. ×400. **b** Only fibrous tissue and some nonproliferating cancer cells are visible in the SRCA when the transplant is sensitive to a given chemotherapeutic agent. ×250. **c** A transplant showing proliferating cancer cells in the margin of the transplant and fibrous tissue in the center of the transplant. ×250. **d** The center of the transplant contains fluid-filled cavities with inflammatory cells and necrotic cell debris. At the margins, proliferating cancer cells are also present. The increase in the transplant size is mainly due to the presence of noncancer cells and fluid-filled cavities. ×100

Table 3 Tumor pretest and control M/V and dTS values by histological type[a]

Histological type	*n*	M/V pretest	M/V control	dTS control
Serous	11	40 ± 26	23 ± 11	3.87 ± 3.12
Mucinous	1	29	18	1.00
Endometrioid	7	36 ± 19	15 ± 10	2.81 ± 2.50
Mesonephroid	5	27 ± 8	15 ± 12	4.96 ± 2.63
Undifferentiated	10	34 ± 19	23 ± 9	3.14 ± 2.28
Brenner	1	55	43	4.29
Other	1	41	30	1.80
Total	36			

[a] Data represent mean values ± SD

Table 4 Pretest and control M/V and control dTS values by origin of the tumor sample, FIGO stage, and grade of differentiation[a]

	n	Pretest M/V	Control M/V	Control dTS
Source:				
Primary tumor	27	33 ± 23	21 ± 11	3.60 ± 2.72
Metastasis	9	44 ± 19	21 ± 10	3.17 ± 2.28
FIGO stage:				
IIb	11	25 ± 16	18 ± 11	2.87 ± 1.88
III	16	40 ± 22	21 ± 11	4.09 ± 3.07
IV	9	41 ± 17	24 ± 11	3.17 ± 2.43
Grade[b]				
1	4	23 ± 15	26 ± 5	3.23 ± 2.15
2	10	32 ± 17	19 ± 12	3.52 ± 2.61
3	22	40 ± 21	21 ± 11	3.52 ± 2.76

[a] Data represent mean values ± SD
[b] 1 = Well differentiated, 2 = Moderately differentiated, 3 = Poorly differentiated

Table 5 Tumor pretest M/V, control M/V, and control dTS values by amount of residual tumor[a]

Residual	*n*	Pretest M/V	Control M/V	Control dTS
<2 cm	17	35 ± 19	21 ± 12	4.00 ± 3.01
>2 cm	19	36 ± 21	21 ± 10	3.03 ± 2.13
Total	36			

[a] Data represent mean values ± SD

Table 6 Variables predicting a good[a] versus poor[b] clinical response at the 6-month second-look laparotomy: a logistic regression analysis

Variable	Score	*df*	*P* value	*R*
M/V	11.7952	1	0.0006	0.3739
dTS	1.1016	1	0.2939	0.0000
Primary/metastasis	2.2689	1	0.1320	0.0620
FIGO stage	4.8236	1	0.0281	0.2008
Residual tumor size	9.7460	1	0.0018	0.3325
M/V best drug	2.9333	1	0.0868	0.2691

[a] Complete responses + partial responses
[b] Progressive disease

various adverse effects on the measurement of tumor size have been reported on histological examination [24, 35]. To prevent the infiltration of tumor implants by host immunocompetent cells, some authors have employed immunosuppressive pretreatment [3, 15, 41] or sequential immunosuppressive treatment of the mice [42], whereas others have shortened the total assay time to 4 days [24].

Many problems with poor tumor growth in control animals can be avoided by removal of the tissue for assay from a point adjacent to the sample selected for histopathology. We have previously concluded that the SRCA can be used to assess the response of ovarian cancer to chemotherapy without routine histological control [29]. Even without the previously described changes in the assay protocol, we have succeeded in establishing a predictive clinical accuracy of 77% [32, 33]. Thus, the changes in the size of the transplanted tumor may reflect the increase in the tumor cell mass, but the measurement is subject to several sources of error.

The microscopic analysis of more than 500 sections from the present series of tumor transplants revealed that in addition to real proliferation of tumor cells, the increase in the size of a transplant may be due to various confounding factors such as scar tissue, edema, inflammatory cells, or fluid-filled cystic cavities. The viable tumor cells were usually situated at the margins of the transplant, since the compromised nutrition in the central areas of the transplant prevents tumor cell proliferation [37]. Accordingly, in many cases the increase in the size of the tumor transplants as measured by dTS in the original SRCA may be due to the confounding factors rather than to actual tumor mass growth. The present histopathology findings explain many of the difficulties encountered in application of the standard SRCA in clinical oncology [2, 10, 23].

The direct measurement of cell proliferation in tumor biopsies by different methods [17, 26, 37] has led to accurate prognostic estimates in a variety of human cancers. The volume-corrected mitotic index (M/V index) is a simple, economical, reproducible, and rapid method for the assessment of cell proliferation in neoplastic tissue [1, 16–18, 27]. Accordingly, it was considered relevant to apply the M/V index in the measurement of cell proliferation in the SRCA as well to improve its accuracy.

The present analysis clearly showed that the mitotic frequency per square millimeter of neoplastic epithelium was a more accurate method than the conventional SRCA for prediction of the response of ovarian carcinoma to chemotherapy. This type of quantitative microscopic analysis avoids several of the confounding factors related to the SRCA as discussed above. The presence of cystic cavities and scar tissue can easily be detected. The proliferating and potentially proliferating cancer cells can be identified at the margins of the transplant. Frequently, the proliferating cancer cells spread as a thin (macroscopically invisible) layer beneath the renal capsule into a large area. In these cases the conventional SRCA (i.e., dTS) will give a false impression of a favorable response to chemotherapy.

However, neither the dTS nor the M/V index accurately predicted the drug response in all cases. It should be emphasized that in this study the dTS and the M/V cannot be compared as equal predictive factors, since the choice of the drug combination was always based on the dTS, not on the M/V value. Malignant tumors show intratumoral heterogeneity in their proliferation rate [8, 26], expression of oncoproteins [22], and other features related to malignancy. In the SRCA the heterogeneity of the tumors was (taken into account by transplantation of at least six randomly selected tumor pieces per drug combination and eight tumor-piece transplants per control group in each test. The mean changes in the size of the originally 1-mm^3 pieces over the 6-day assay period were considered to represent the growth capacity and drug response of the whole tumor. Despite these precautions, the tumor pieces used in the SRCA may not necessarily represent the malignant potential and sensitivity to the tested drugs of the entire tumor cell population. For example, differences in the sensitivity to cytostatics of primary tumors and metastatic lesions have been reported [21, 28].

In the present report a new method of assessment is suggested for the SRCA. Previously the test was considered evaluable if the tumor implants did not decrease in size (dTS $>$ 0). It now seems reasonable that the test result be assessed by M/V and that a certain level above zero be required in the control group. How high this level should be set can be proposed only after analysis of a larger series. In addition, different evaluability criteria would be needed for tumors representing different histological types.

Despite the limited number of cases included in the present series, the methods used to assess the chemotherapy response were reliable. All the patients had a second-look laparotomy, and the chemotherapy response was accurately defined [34]. Both the conventional SRCA [7] and mitotic frequency analysis are well-established methods [18]. By combining these two techniques we have established a novel and simple method for assessment of the individual chemotherapy response of advanced ovarian cancer. This test system is of potential usefulness in clinical oncology. The results of this analysis are fully comparable with more complicated methods described recently [9, 25].

The results of this analysis must be confirmed in a larger cohort of patients and in different tumor types before this modified test can be adopted into clinical use. This group of tumors was also too small for the establishment of relevant group limits for responders and nonresponders in assays using the M/V index, although the limit of 6 mitotic figures/mm^2 gave the highest rate of correct predictions.

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