Value of quantitative pathological variables as prognostic factors in advanced ovarian carcinoma

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Abstract

Aims—To evaluate correlations among clinical, pathological, morphometric, stereological, and DNA flow cytometric variables and their prognostic value in advanced ovarian cancer.

Methods—Tissue was collected from 180 patients with advanced ovarian cancer. All 180 had undergone debulking surgery and were being treated with cisplatin. Long term follow up was available for all patients. The mitotic activity index (MAI), volume % of epithelium (VPE), mean nuclear area (MNA), standard deviation of the nuclear area (SDNA), estimates of volume weighted mean nuclear volume ($\bar{v}_v$), and variables obtained from minimum spanning tree (MST) analysis were assessed in the least differentiated tumour section in each case. DNA flow cytometry was also performed.

Results—Quantitative pathological features differed significantly with respect to histological grade. The MAI, MNA, SDNA, and the number of points connected to three neighbours differed significantly among the different DNA ploidy groups. The VPE and number of points connected to two or three neighbours differed significantly between FIGO stages III and IV. Fifty two (29%) patients survived. FIGO stage, residual disease and SDNA had prognostic significance on both univariate and multivariate survival analysis. In patients with FIGO III stage disease and residual tumour nodes ≤ 2 cm in diameter (67 patients, 29 (43%) survivors) a prognostic index was established based on SDNA and of the line length of the MST. The median survival time was not reached in a subgroup of patients with favourable prognosis (overall survival 57%). Median survival was 32 months for patients with an unfavourable index score (overall survival 28%).

Conclusion—Morphometric variables have important additional value in predicting prognosis in patients with advanced ovarian cancer.

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Ovarian cancer is the fifth most common type of cancer diagnosed in women in the Netherlands and Denmark, with age standardised incidence rates of 14.6 and 15.2, respectively.^{1,2} The initial symptoms are usually non-specific and therefore

most patients present with advanced disease.³ Despite recent improvements in treatment strategies for advanced ovarian malignancies, including optimal debulking surgery and cisplatin combination chemotherapy,⁴⁻⁶ clinical outcome is still fairly poor. Cisplatin has considerable side effects and a significant proportion of patients ultimately does not respond adequately to therapy. About 75% of these women will die of their disease; the median survival time is only two years.⁷ Objective variables that can accurately predict prognosis may be helpful for selecting patients who will benefit most from cisplatin therapy. Alternative treatment may be considered for other patients.

FIGO stage and performance status based on the Karnofsky index (KI) at initial diagnosis predict clinical outcome.⁸⁻¹⁰ The KI, however, is based on subjective criteria and its prognostic relevance may therefore vary between different populations and evaluators.¹⁰ Optimally debulked patients with residual tumour nodes of less than 2 cm in diameter do better,⁵⁻⁸ possibly as the tumour is more accessible to cytotoxic drugs because of better vascularisation and oxygenation, and because the number of cycling cells is relatively larger than in bulky tumour masses.^{11,12} However, some patients with relatively favourable indicators of prognosis will die of recurrent disease. Histological grade is also of prognostic importance,^{7,9,13} but cannot always be reproduced reliably.^{14,15}

Several studies have shown that quantitative pathological features, such as morphometric and stereological variables¹⁶⁻²⁰ and DNA flow and image cytometric features,^{13,21,22} are predictive of survival in patients with ovarian cancer undergoing treatment with cisplatin. An important advantage of these techniques is that objective and reproducible values are obtained. Moreover, these features are correlated with biological processes taking place in the tumour, such as genetic instability (DNA ploidy), proliferation (mitotic indices), nuclear differentiation (nuclear area, nuclear volume), and architectural differentiation (volume % epithelium, minimum spanning tree analysis). Most of these studies have evaluated relatively small patient groups and the patients have not always been treated uniformly with cisplatin based chemotherapy and optimal surgery. Therefore, firm conclusions could not be drawn from these studies. The aim of the present study was to evaluate the prognostic value of clinical, pathological, morphometric, stereological, and DNA flow cytometric variables in 180 patients with advanced ovarian cancer.

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Methods

The patients and tissue included in the present study have also been included in previous studies.^{16 20 23} The patients were between 26 and 70 years of age (median 54 years) at diagnosis. Of these patients, 140 (78%) had FIGO stage III and 36 (20%) had FIGO stage IV disease. The FIGO stage was unknown in four patients with advanced ovarian cancer. Disease was bulky (the diameter of the largest tumour node was greater than 5 cm) at diagnosis in 105 (81%) of 130 patients.

All of the patients underwent cytoreductive surgery and treatment with cisplatin combination chemotherapy. In 77 (45%) of 172 patients the size of the largest tumour node was surgically reduced to ≤ 2 cm; residual disease was > 2 cm in 95 patients. The post-operative residual disease status was unknown in eight patients. Seventy three patients received CAP-1 (cisplatin, doxorubicin and cyclophosphamide), 36 received CHAP-5 (cisplatin, doxorubicin, cyclophosphamide, and hexamethyl-melamine), 22 received CP (cisplatin and cyclophosphamide), and 49 were randomised for treatment with cisplatin and cyclophosphamide with or without adriamycin. No significant differences in survival were noted between the patients treated with the different chemotherapeutic regimens ($p = 0.15$).

Follow up information was available in all cases. Fifty two (29%) patients survived. Median follow up in non-survivors was 21 months (range two to 82 months) and was 72 months (range 19 to 114 months) in survivors.

TISSUE PROCESSING, QUALITATIVE AND QUANTITATIVE METHODS

Tumour tissue was cut into slices 0.5 cm thick and fixed in 4% buffered formaldehyde. Haematoxylin and eosin stained sections, 4 μ m thick, were cut for diagnosis, morphometry and stereology. In each case a poorly differentiated, epithelium rich primary tumour section was selected for further quantitative analysis. An area measuring 0.5 $\times$ 0.5 cm was chosen for analysis. This area was rich in epithelium and had the highest number of mitotic figures. Areas with necrosis, inflammation or calcification were avoided.

For DNA flow cytometry, sections 50 μ m thick were cut from the paraffin wax embedded tumour specimens; haematoxylin and eosin stained sandwich sections 4 μ m thick were checked for tumour content. The sections were processed routinely to obtain single cell suspensions according to standard procedures in the Netherlands^{16 20} and Denmark.^{24 25} Paraffin wax embedded material was not available for 47 patients, precluding DNA ploidy analysis.

All primary tumour sections were graded as well, moderately or poorly differentiated.²⁶ Morphometric and stereological assessments were carried out as described previously.^{16 23}

The total number of mitotic figures was counted in 25 contiguous fields of vision with a conventional light microscope (final magnification $\times 400$, numerical aperture 0.75, field

diameter 450 μ m). Once focused, no further adjustment was permitted. The total number of clearly identifiable mitotic figures, fulfilling the criteria described previously,²⁷ was counted and taken as the mitotic activity index (MAI).

The volume percentage of epithelium (VPE) was estimated (final magnification $\times 200$, objective $\times 20$) using a 42 point parallel Weibel grid, assuming that the tissue was isotropic, uniform and randomly oriented. At least 300 points covering epithelial or stromal cells were counted in each tumour section. The percentage of points overlying epithelial cells was taken as the VPE.

Nuclear morphometric analysis was carried out using the PRODIT interactive digitising video overlay system (software version 5.1) (BMA, de Meern, The Netherlands). This system is composed of a video camera mounted on a microscope and linked to a video overlay board in a MS-DOS based computer. A final magnification on screen of approximately $\times 3000$ (objective $\times 100$) was used for assessing the mean nuclear area (MNA) and the volume weighted mean nuclear volume ($\bar{v}_v$). An automatic scanning stage is linked to the system, which permits electronic demarcation of the measurement area and the random systematic selection of fields of vision.²⁸ To assess the MNA, the contours of 100 nuclei were traced using the mouse in 100 randomly selected fields of vision in each case. Thus, the mean (MNA) and standard deviation of the nuclear area (SDNA) were calculated in each case. The estimation of the $\bar{v}_v$ was carried out as described elsewhere.²⁹⁻³³ Briefly, the intercepts of at least 90 nuclei in 14 fields of vision were measured. Thus, the intercepts were point sampled with a chance proportional to their volume. The unbiased estimate of $\bar{v}_v$ was then obtained by raising the measured mean intercept lengths to the third power and multiplying by $\pi/3$.

Minimum spanning tree (MST) analysis was also performed using the PRODIT system. The final magnification displayed on the monitor was $\times 1900$ (objective $\times 63$). MST analysis was performed in 12 randomly selected fields of vision (each of which was 570 $\times$ 570 μ m). In each field of vision the centres of gravity of all tumour cell nuclei present within the same epithelial layer were marked. These points were then automatically connected by the minimal total line length without forming loops, thus forming the MST. The variables obtained from the MST were the number of points, total line length, average line length, minimum line length, maximum line length, standard deviation of the line length, and percentage of points connected to one neighbouring nucleus, two neighbouring nuclei and three neighbouring nuclei.³⁴

The DNA index (DI) was calculated by dividing the channel numbers of the second and first G_0/G_1 peak, being 1.0 in case of a single peak. Based on their DI, tumours were classified as diploid ($DI = 1.0$), hyperdiploid ($1.0 < DI < 1.9$), tetraploid ($1.9 \leq DI \leq 2.1$), and hypertetraploid ($DI > 2.1$).

Table 1 Correlations among quantitative pathological features and histologic grade, FIGO stage and DNA ploidy

Variable	Grade (n = 180)				FIGO stage (n = 176)	DNA ploidy (n = 133)
	Kruskal p value		Mann-Whitney p value		Mann-Whitney p value	Kruskal p value
	I v II v III*	I v II	I v III	II v III	III v IV	D v HD v T v HT
Mitotic activity index	0.00001	NS	<0.05	<0.05	0.09	0.01
Volume percentage epithelium	0.00001	<0.05	<0.05	<0.05	0.01	0.45
Mean nuclear area	0.0003	NS	<0.05	<0.05	0.79	0.05
Standard deviation of nuclear area	0.0005	NS	<0.05	NS	0.77	0.02
Volume weighted mean nuclear volume	0.00001	NS	<0.05	<0.05	0.57	0.30
DNA index	0.12	NS	NS	NS	0.22	NP
MST variables						
No. of points	0.00001	NS	<0.05	<0.05	0.53	0.24
Total line length (µm)	0.00001	<0.05	<0.05	<0.05	0.93	0.49
Average line length (µm)	0.00001	NS	<0.05	<0.05	0.37	0.44
Minimum line length (µm)	0.0001	NS	<0.05	<0.05	0.36	0.20
Maximum line length (µm)	0.0001	NS	<0.05	NS	0.20	0.70
SD of line length	0.13	NS	NS	NS	0.36	0.62
Connected to one nucleus (%)	0.06	NS	NS	NS	0.005	0.75
Connected to two nuclei (%)	0.00001	NS	<0.05	<0.05	0.006	0.06
Connected to three nuclei (%)	0.00001	NS	<0.05	<0.05	0.08	0.02

* Grade I = well differentiated, II = moderately differentiated, III = poorly differentiated; DNA ploidy: D = diploid; HD = hyperdiploid; T = tetraploid; HT = hypertetraploid; SD = standard deviation; NS = not significant; NP = not performed.

DATA EVALUATION

The differences in quantitative features between the three histological grades, DNA ploidy and FIGO stage were assessed using the Kruskal–Wallis and Mann–Whitney tests. The level of significance was set at $p < 0.05$.

For survival analysis, the time from diagnosis to death or last date of follow up was used as the overall survival time. Cut off points for the quantitative variables were chosen (1) to form three groups of equal size, (2) to form four groups of equal size and according to (3) the median value, (4) the percentage of surviving patients, and (5) previously established prognostically important thresholds (the thresholds resulting in the strongest prognostic value are shown). Kaplan–Meier curves were plotted and differences between the curves were tested for significance using the Mantel–Cox statistic. The additional prognostic value of features was assessed by the multivariate Cox regression model (enter and exit limits 0.1). Multivariate analysis was performed initially on the whole group, including FIGO stages III and IV. Thereafter, multivariate analysis was performed

separately on the subgroups of patients with FIGO stage III disease, with residual tumour ≤ 2 cm in diameter, residual tumour > 2 cm in diameter, and with FIGO stage IV disease. Variables that reached significance in univariate analysis were used as categorical variables as determined by the established cut off point.

Results

Quantitative pathological features (especially the VPE and the total line length of the MST) were significantly different with respect to histological grades (table 1). The MAI, MNA, SDNA, and the number of points connected to three neighbouring nuclei differed significantly with respect to the different DNA ploidy groups. The VPE and number of points connected to two or three neighbouring nuclei differed significantly with respect to FIGO stage.

Univariate survival analysis demonstrated that FIGO stage was the best prognostic variable, followed by residual disease after surgery (table 2). Combining these two features pro-

Table 2 Results of survival analysis for clinicopathological variables in 180 patients with advanced ovarian cancer

Variable	n	Survival			
		%	Median time (months)	Mantel–Cox statistic	p value
FIGO stage					
III	140 (80%)	32	35	23.4	0.00001
IV	36 (20%)	11	17		
Unknown	4				
Residual disease					
≤ 2 cm	77 (45%)	40	48	14.2	0.0002
> 2 cm	95 (55%)	19	23		
Unknown	8				
FIGO and residual disease status (RD)					
FIGO III, RD ≤ 2 cm	67 (39%)	43	55	30	0.00001
FIGO III, RD > 2 cm	69 (40%)	23	29		
FIGO IV	36 (21%)	11	17		
Grade					
I	21 (12%)	52	50	5.46	0.07
II	40 (22%)	18	32		
III	119 (66%)	29	29		
Bulky disease status					
≤ 5 cm	25	48	51	3.8	0.05
> 5 cm	105	27	24		
Unknown	50				

* Grade I v grades II and III.

Figure 1 Survival curves for 180 patients with advanced ovarian cancer, combining FIGO stage and residual disease status (FIGO III, residual disease (RD) <2 cm; FIGO III, residual disease >2 cm; FIGO stage IV).

vided even more significant prognostic information on survival (fig 1). Bulky disease prior to surgery was less significant than the residual disease status after the debulking operation. Patients with well differentiated tumours survived significantly longer compared

with patients with moderately and poorly differentiated tumours.

The results of survival analysis for the quantitative pathological and MST variables which reached significance overall and in the relevant subgroups are presented in tables 3 and 4. The standard deviations of the nuclear area and of line length of the MST had important prognostic power overall in the subgroups of patients with FIGO stage III disease and residual tumour <2 cm in diameter. In FIGO stage IV disease only the MST variables total line length and the number of points connected to one neighbouring nucleus had prognostic power.

Table 5 shows the results of the multivariate analysis. When entering the clinical, pathological and quantitative variables, the FIGO stage was selected first, followed by residual disease and SDNA. This resulted in the FRS index as follows: $(0.8856 \times \text{FIGO}) + (0.6035 \times \text{residual disease}) + (0.5993 \times \text{SDNA})$ (FIGO = 3 or 4; residual disease = 1 (<2 cm) or 2 (>2 cm), SDNA $\leq 19.6 = 1$ or $>19.6 = 2$). Using this multivariate index three highly significant prognostic groups could be distinguished (Mantel-Cox = 35, $p < 0.00001$).

In patients with FIGO stage III disease and with residual disease <2 cm in diameter ($n = 67$), the SDNA was selected first, followed by SD of line length (SDLL) of the MST. The

Table 3 Results of survival analysis of quantitative pathological variables in each of the relevant substages (FIGO stage III and residual disease (RD) less than 2 cm, FIGO stage III and RD greater than 2 cm, FIGO stage IV) (only variables that reached significance are listed)

Variable	Cut off point	n	Survival			
			%	Median time	Mantel-Cox statistic	p value
FIGO stage III and IV						
Mean nuclear area (μm)	≤ 75.8	144	31	32	8.3	0.004
	> 75.8	36	19	20		
SD of nuclear area	≤ 19.6	118	35	43	15.8	0.0001
	> 19.6	62	18	21		
FIGO stage III, RD ≤ 2 cm						
SD of nuclear area	≤ 20.8	50	48	80	5.8	0.02
	> 20.8	17	29	29		
FIGO stage III, RD > 2 cm						
Mitotic activity index	≤ 60	36	28	31	5.0	0.03
	> 60	33	18	20		
SD of nuclear area	≤ 21.0	51	28	31	4.4	0.04
	> 21.0	18	11	20		

Table 4 Results of survival analysis of minimum spanning tree analysis in each of the relevant subgroups (FIGO stage III and residual disease (RD) less than 2 cm, FIGO stage III and RD greater than 2 cm, FIGO stage IV) (only variables that reached significance are listed)

Variable	Cut off point	n	Survival			
			%	Median time	Mantel-Cox statistic	p value
FIGO stage III and IV						
SD of line length	≤ 2.35	10	33	36	6.1	0.01
	> 2.35	75	23	26		
Connected to three nuclei (%)	≤ 18.9	95	34	40	4.4	0.04
	> 18.9	85	24	23		
FIGO stage III, RD ≤ 2 cm						
SD of line length	≤ 2.35	42	50	80	5.3	0.02
	> 2.35	25	32	35		
FIGO stage III, RD > 2 cm						
Connected to three nuclei (%)	≤ 19.4	35	34	36	7.2	0.007
	> 19.4	34	12	20		
FIGO IV						
Total line length (μm)	≤ 278	9	44	30	6.3	0.01
	> 278	27	0	16		
Connected to one nucleus (%)	≤ 28	17	0	14	5.8	0.02
	> 28	19	21	24		

Table 5 Results of multivariate survival analysis of MST and other quantitative pathological variables in each of the relevant subgroups (FIGO stage III and residual disease (RD) less than 2 cm, FIGO stage III and RD greater than 2 cm, FIGO stage IV)

Variable	Cut off point	n	Survival			
			%	Median time	Mantel-Cox statistic	p value
FIGO stage III and IV						
FIGO, residual disease, SD of nuclear area ^a	≤ 3.87	46	50	80	35	0.00001
	3.87–4.76	74	27	29		
	> 4.76	52	12	17		
FIGO stage III, RD < 2 cm						
SD of nuclear area, SD of line length ^b	≤ 1.35	35	57	NR	9.1	0.003
	> 1.35	32	28	32		
FIGO stage III, RD > 2 cm						
SD of nuclear area, Connected to three nuclei (%) ^c	≤ 1.24	26	42	50	12	0.0006
	> 1.24	43	12	21		

^a (0.8856 × FIGO) + (0.6035 × residual disease) + (0.5993 × SD of nuclear area), FIGO = 3 or 4, residual disease = 1 or 2, SD of nuclear area ≤ 19.6 = 1 or > 19.6 = 2.

^b (0.6970 × SD of nuclear area) + (0.6394 × SD of line length), SD of nuclear area ≤ 20.8 = 1 or > 20.8 = 2, SD of line length ≤ 2.35 = 1 or > 2.35 = 2.

^c (0.5414 × SD of nuclear area) + (0.6861 × connected to three nuclei), SD of nuclear area ≤ 21.0 = 1 or > 21.0 = 2, connected to three nuclei ≤ 19.4% = 1 or > 19.4% = 2.

NR = not reached the median survival time.

resulting formula was: (0.6970 × SDNA) + (0.6394 × SDLL), (SDNA ≤ 20.8 = 1 or > 20.8 = 2, SDLL ≤ 2.35 = 1 or > 2.35 = 2). The cut off point used (1.35) revealed that in the 35 patients with a low score long term survival was achieved in 57%, while in the group with a high score only 28% achieved long term survival (Mantel-Cox = 9.1, p = 0.003) (fig 2).

In patients with FIGO stage III disease and with residual disease > 2 cm in diameter (n = 67), the SDNA was selected first, followed by the percentage of points connected to three neighbouring nuclei (PCON3). The resulting formula was: (0.5414 × SDNA) + (0.6861 × PCON3), (SDNA ≤ 21.0 = 1 or > 21.0 = 2, PCON3 ≤ 19.4% = 1 or > 19.4% = 2). The cut off point used (1.24) revealed that of 26 patients with a low score, 42% survived with a median survival time of 50 months, while in the group with a high score, only 12% survived with a median survival time of 21 months (Mantel-Cox = 12, p = 0.0006).

In patients with FIGO stage IV disease the total line length (TLL) of the MST and the

percentage of points connected to three neighbouring nuclei (PCON3) were selected in the Cox regression model. The resulting formula was: (1.4746 × TLL) + (0.2272 × PCON3), (TLL ≤ 278 μm = 1 or > 278 μm = 2; PCON3 selected as continuous variable). Using this score function the four surviving patients belonged to the group of patients (n = 5) with the lowest score when all values obtained were sorted. However, the small number of patients in this group prevented further statistical evaluation.

Discussion

In this study, a new prognostic index (FRS index), comprising the FIGO stage, the size of residual tumour after debulking surgery and the standard deviation of the nuclear area, provided highly significant prognostic information in patients with advanced ovarian carcinoma. The major advantage of this prognostic index is that it is based on objective, reproducible variables. The size of residual tumour after debulking surgery provided better prognostic information than the preoperative status, which is biologically understandable and in agreement with previous reports.^{10,13} Indeed, large tumour nodules are less susceptible to cisplatin combination chemotherapy, possibly because of alterations in vascularisation, oxygenation and proliferation.^{11,12} Tumour nodules over 2 cm in diameter contain at least 10⁹ tumour cells and approximately 32 doublings will have taken place, inducing genetic liability which leads to the diversification of the dominant clone.^{35,36} Accordingly, more DNA aneuploid cases occur and there is a greater likelihood that more aggressive, cisplatin resistant cell types will develop.

In this study the standard deviation of the nuclear area and line length of the MST showed important prognostic power. Therefore, it is likely that intratumour heterogeneity in nuclear size, as reflected by the standard deviation of the nuclear area, and a low grade of architectural differentiation in the tumour tissue, as reflected by the standard deviation of the line length of

Figure 2 Survival curves for subgroups with FIGO stage III and residual disease < 2 cm, as determined by the multivariate prognostic index (cut off point 1.35), comprising the standard deviation of the nuclear area and line length of the MST.

the MST, are important determinants of disease progression. The larger the variability in the size of nuclei and the more their arrangement in relation to one another is disturbed, the worse the prognosis will be. Therefore, we speculate that either different clones of tumour cells exist in advanced ovarian cancer or that they represent different steps in the process of malignant nuclear differentiation, which is more prominent in the tumours of patients who are likely to die of their disease. Indeed, the absence of any organisation in the arrangement of tumour nuclei may hamper vascularisation and thereby reduce the effectiveness of chemotherapy. As expected, patients with FIGO stage III disease and residual tumour nodes <2 cm in diameter had a fairly good prognosis; however, clinical outcome in these groups was significantly less favourable when the standard deviations of nuclear area and line length of the MST were also high. Therefore, this prognostic index may be useful for predicting which patients are likely to benefit most from cisplatin combination chemotherapy and which are more likely to survive. Further prospective investigations are necessary to increase the sensitivity of this prognostic index.

The predictive value of quantitative pathological features (MNA, SDNA, MAI, VPE, and DNA ploidy) in the present study was not as good as in previous investigations.^{13-16,22} This may be because a relatively large number of patients with FIGO stage IV disease (20%), large tumours at diagnosis (81%) and/or post-operative tumour nodules over 2 cm in diameter (55%) were included in this study. The overall prognosis for these patients is very poor with current treatment regimens and therefore morphometric tumour characteristics may fail to predict a favourable outcome in these patients. The lack of prognostic significance of DNA ploidy assessed by flow cytometry may also be because of intratumour DNA heterogeneity in the large tumour masses, as has been found by other investigators.³⁷⁻⁴⁰

In this study no additional prognostic information was gained by estimating the $\bar{v}_n$, which is in agreement with the study performed by Mogensen *et al.*²³ The correction for sampling problems as performed in the current investigation, using a systematic random sampling method within the least differentiated area, did not improve the prognostic power of this variable.

Clinical outcome in patients with advanced ovarian cancer is mainly determined by the FIGO stage and the outcome of debulking surgery. However, a subgroup of patients with a favourable prognosis can be identified among those with FIGO stage III disease and residual tumour <2 cm in diameter using a new prognostic index based on the standard deviation of the nuclear area and line length of the MST. We conclude that morphometric variables have important additional value in predicting prognosis in patients with advanced ovarian cancer.

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