Multivariate analyses of DNA index, p62$^{c\text{-}myc}$, and clinicopathological status of patients with ovarian cancer

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Abstract

***Aim*—To determine if either DNA index or p62$^{c\text{-}myc}$ is an independent prognostic variable in ovarian cancer.**

***Methods*—Multivariate and univariate analyses of the relation between DNA index, p62$^{c\text{-}myc}$, FIGO stage, histological type, tumour grade, completeness of surgery, and patient survival in ovarian cancer were examined.**

***Results*—Multivariate analysis showed significant association of survival only with stage and grade. There was no relation between survival and DNA index.**

***Conclusions*—DNA index is not an independent prognostic variable in ovarian cancer.**

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Keywords: ovarian cancer; c-myc; DNA ploidy

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An increase in nuclear DNA content, aneuploidy, or DNA index[1] has long been known to be associated with ovarian malignancy.[2] It has also been shown to have prognostic value in various tumours[3–11] and in ovarian cancer.[11–34] It has been stated that the DNA index is an independent prognostic marker for ovarian cancer and either a very powerful or even the best indicator of survival.

However, the value of the DNA index as a truly independent prognostic variable must remain in doubt because some of the results are conflicting,[9 11 33] and very few studies have been designed to test for independence of the DNA index as a prognostic variable by using multivariate analysis techniques.[17 26 29 31 34]

Previously we have assayed the c-myc protein, p62$^{c\text{-}myc}$, simultaneously with DNA in archival serous papillary ovarian carcinoma, which expressed significantly higher p62$^{c\text{-}myc}$ than normal ovary.[35] The study reported here was undertaken in a large group of patients (233) to ascertain if either DNA index or p62$^{c\text{-}myc}$ is an independent prognostic variable in ovarian cancer. Both univariate and multivariate techniques were used to analyse the relation among DNA index, p62$^{c\text{-}myc}$, FIGO stage, histological type, tumour grade, completeness of surgery, and patient survival.

Methods

HISTOLOGICAL SPECIMENS

Paraffin wax embedded, formalin fixed biopsy material was obtained from patients entered in a prospective clinicopathological study of ovarian cancer in the North East Metropolitan (Thames) Region of London and the Home Counties between 1975 and 1980. Overall, 223 tumours were studied and the only criterion for entry to the present study was that sufficient archival material was available. All specimens were reviewed independently. Details of the tumours are given in table 1.

CLINICAL DATA

Full clinical details including FIGO stage, the extent of surgery performed, and the extent of residual disease after surgery were available from contemporary data.[36] The surgery performed was assessed as:

- complete macroscopic removal
- partial removal
- biopsy only.

Complete removal was subclassified as (1) clean removal; (2) removal with gross anatomical disturbance. The latter included those patients where major resections of adjacent tissues had to be carried out, for example of the sigmoid colon, in order to effect the removal.

Partial resection, where removal of the main tumour mass was carried out, was subclassified into two categories: (1) where widespread disease remained (heavy tumour burden); (2) where only minor scattered or local growth remained (optimal cytoreduction).

One hundred and fourteen patients underwent complete removal, 94 had partial resections, and 14 had biopsies only. Partial or incomplete resection naturally applied only to higher FIGO stage tumours (2B–4).

Data on cancer stage and histological grade are given in figs 1 and 2.

ANTIBODY PRODUCTION AND SPECIFICITY CONTROLS

Full details of the production and characterisation of the antibodies raised to p62$^{c\text{-}myc}$ have been reported elsewhere.[37–40]

NUCLEAR EXTRACTION AND STAINING

Sections of 40 μm thickness were cut from the paraffin embedded biopsies, and nuclei were

Table 1 Histological characteristics of tumours

(A) Classification (WHO)	
Serous papillary	95
Mucinous	46
Endometrioid	50
Mixed serous papillary plus endometrioid	16
Undifferentiated tumour	16
Total patients	223
(B) Architectural grade	
Well differentiated	64
Moderately differentiated	71
Poorly differentiated	63
Borderline — low potential malignancy	25

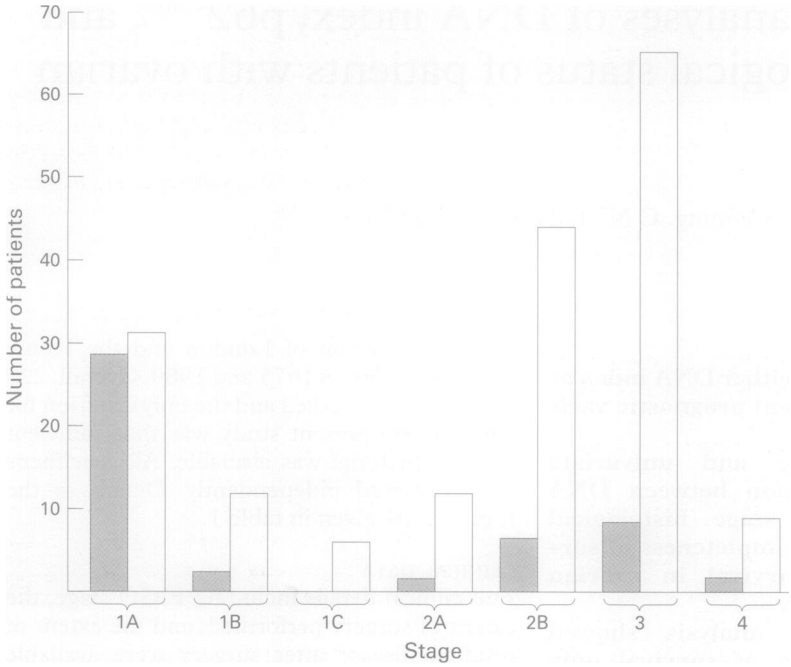

Figure 1 Number of patients (ordinate) versus stage (abscissa) for the mucinous tumours (solid columns) compared with the endometrioid, serous, mixed, and undifferentiated tumours combined (open columns); χ^2 was 30.84 with 6 degrees of freedom, $p<0.0003$.

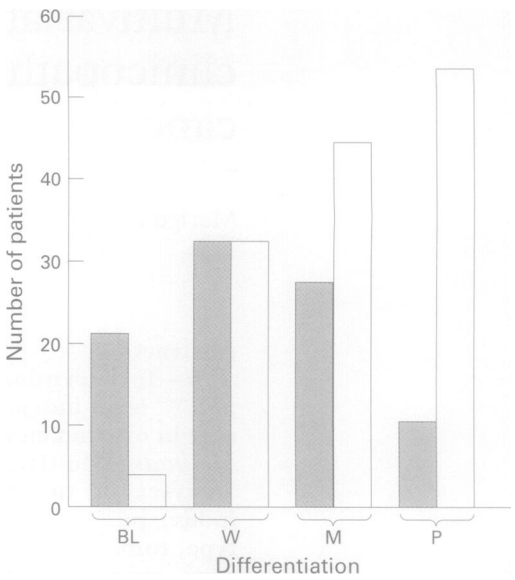

Figure 2 Number of patients (ordinate) versus histological grade (abscissa) for "early" tumours (stage 1+2a, solid columns) compared with "late" tumours (stages 2b+3+4, open columns); χ^2 was 34.8 with 3 degrees of freedom, $p<0.000001$.

released by enzymatic digestion as originally described by Hedley *et al.*[41] Samples were then stained for DNA with propidium iodide (PI; Calbiochem, Beeston, UK) 0.05 mg/ml and the nuclear associated protein $p62^{c\text{-}myc}$, as described in detail previously.[35 40]

FLOW CYTOMETRY

The nuclei were analysed blind and simultaneously for DNA and $p62^{c\text{-}myc}$ in the Cambridge MRC custom built dual laser flow cytometer,[43] which incorporates a modified flow chamber to increase light collection efficiency.[44] The instrument was aligned with microbeads and set up on the red fluorescence detector (DNA) with the same known diploid standard for each run, the peak being placed at channel 200. The high tension voltage of the green fluorescence detector ($p62^{c\text{-}myc}$) was then increased to record the short wavelength tail from the red (DNA-P1) fluorescence breaking through the filters at about channel 50.[35 42]

Table 2 DNA index versus tumour type

DNA index	*Muc*	*End*	*Ser*	*Mix*	*Und*	*Total*
DI < 1.1 (Dip)	37	21	46	4	3	111
DI > 1.1 (Anu)	9	29	49	12	13	112
Total	46	50	95	16	16	223

Anu, aneuploid; DI, DNA index; Dip, diploid; End, endometrioid; Mix, mixed serous papillary plus endometrial; Muc, mucinoid; Ser, serous papillary; Und, undifferentiated.

Table 3 χ^2 paired comparisons of DNA index within each tumour category; χ^2 is above and to the right of the diagonal with its associated p value in the complementary position below and to the left

	Muc	*End*	*Ser*	*Mix*	*Und*
Muc	–	13.24	11.38	13.91	17.13
End	<0.0003	–	0.32	0.85	1.923
Ser	<0.0059	0.5742	–	2.16	3.756
Mix	<0.0002	0.3555	0.1415	–	0.00
Und	<0.0001	0.1663	0.0525	1.0000	–

End, endometrioid; Mix, mixed serous papillary plus endometrial; Muc, mucinoid; Ser, serous papillary; Und, undifferentiated.

DATA COLLECTION AND PROCESSING

The data were collected in list mode on a 640 megabyte disc using dedicated LSI 11/23 and time sharing PDP 11/40 computers (Digital Equipment Corporation, Maynard, Massachusetts, USA) after digitisation of each signal into peak height, area, and width (time of flight through the laser beam) within the analogue to digital conversion range of 1–1024. However, the electronics were modified to incorporate variable sensitivity in the preamplifier circuits which increased the range to 6192. Processing was carried out in a VAX cluster of computers (Digital Equipment Corporation) which extracted seven variables from the list mode dataset. A five dimensional procedure was carried out which used a combination of forward and 90° scatter together with pulse analysis on the master channel, red fluorescence, to exclude debris and clumps.[35 42] Five variables—namely DNA, oncoprotein fluorescence, forward scatter pulse width, and 90° scatter width and area—were then written to disc as a fully cross correlated data file.[43]

FLOW CYTOMETRY DATA ANALYSIS

Tumour DNA indices were obtained as follows. The majority (> 94%) of aneuploid tumour specimens contained a definable diploid component with a DNA index within +7.5% of the known diploid standard (see above). Any tumour specimen in which there was only a single peak was regarded as aneuploid if its DNA index was ≥ 1.10, all of which were "large" cells (see below); otherwise it was scored as diploid. The $p62^{c\text{-}myc}$ fluorescence signals associated with the G1 component in the aneuploid tumours were calculated. Similarly, the $p62^{c\text{-}myc}$ signals associated with the larger G1 cells in the 90° scatter versus DNA data space of the diploid tumours were also calculated.

Table 4 DNA index (DI) of mucinous (Muc) tumours versus all the other varieties (ESMU), a combined group of endometrioid, serous, mixed, and undifferentiated tumours

DNA index	*Muc*	*ESMU*	*Total*
DI ≤ 1.1 (Dip)	37	74	111
DI > 1.1 (Anu)	9	103	112
Total	46	177	223

χ^2 = 20.27 with 1 degree of freedom, giving p = 0.000007. Anu, aneuploid; Dip, diploid.

STATISTICAL ANALYSES

Both univariate and multivariate analyses were conducted using algorithms in the BMDP computer statistical package (BMDP, Cork, Republic of Ireland). In the univariate analyses, patient survival according to FIGO stage, completeness of surgery, histological grade, DNA index, and tumour p62 $^{c\text{-}myc}$ levels was compared using the logrank test. The multivariate analysis used the Cox regression model[45] using a forward stepwise variable selection procedure with a significance level set at 0.05 for inclusion.

Variable reduction

A frequency distribution analysis strongly suggests that the histological categorisation of the tumours presented in this paper can be reduced from five to three, namely mucinous, endometrioid/serous, and undifferentiated. As the "watershed" of operability was in general between stages 2A and 2B, the stage categories were reduced from seven to two, namely early (1A–2A) and late (2B–4). Finally, the grade categories were reduced from four to three by amalgamating "borderline" and "well differentiated." Hence, the total number of possible categories based on tumour type, stage, and grade is reduced to 18.

Table 5 Relations between stage (<2a v >2b), tumour grade, and DNA index

	DNA index ≤1.1			*DNA index ≥1.1*			
Stage	*BL+W*	*M*	*P*	*BL+W*	*M*	*P*	*Total*
≤2A	41	13	3	12	14	7	90
≥2B	20	15	19	16	29	34	133
Total	61	28	22	28	43	41	223

BL, borderline; M, moderately differentiated; P, poorly differentiated; W, well differentiated.

Table 6 Summary of survival data according to stage, completeness of surgery, histological type, tumour grade, DNA index, and p62$^{c\text{-}myc}$

		Number of patients	*% 5 year survival*	*Log rank statistics* χ^2	*p Value*
FIGO stage	IA	59	76		
	IB	14	64		
	IC	6	50		
	IIA	11	55		
	IIB	50	38		
	III	73	4		
	IV	10	0	125.5	<0.00001 on 6 df
Completeness of surgery					
Complete removal		114	64		
Limited residual		50	20		
Major residual		44	0		
Biopsy only		15	13	103.8	<0.00001 on 1 df
Histological type					
Mucinous		46	57		
Endometrioid		50	40		
Serous papillary		95	35		
Mixed		16	31		
Undifferentiated		16	6	16.2	0.003 on 4 df
Tumour grade					
Well differentiated + borderline		89	64		
Moderately + poorly differentiated		134	21	16.2	<0.00006 on 1 df
DNA index	≤1.1	111	53		
	>1.1	112	23	18.7	<0.00002 on 1 df
p62$^{c\text{-}myc}$	≤500	100	31		
	>500	123	44	2.8	0.09 on 1 df

df, degrees of freedom.

Results

RELATION AMONG DNA INDEX, HISTOLOGICAL STATUS, AND STAGE

DNA index and histological type

The relation between DNA index and tumour type is given in table 2, showing a highly significant (p < 0.00005) statistical differences between tumour types.

These differences were then investigated with paired χ^2 comparisons as previously carried out. The results are given in table 3, which shows that as far as their DNA index was concerned the mucinous tumours were a distinct group and that the remaining tumour types—namely endometrioid, serous, mixed, and undifferentiated categories—could be combined for this purpose.

Table 4 shows the frequencies of DNA index in the mucinous tumours (Muc), demonstrating a statistically significant greater proportion of diploid tumours (DI < 1.1) in the mucinous category compared with the remaining tumour types.

DNA index, stage, and histological grade

The relation between aggregated stage, histological grades (with borderline plus well differentiated tumours as a combined group), and DNA index is given in table 5. Comparisons of the frequencies in this table gave a χ^2 value of 31.94 with 5 degrees of freedom (p = 0.000006). This shows a significant difference in the distribution of diploid and aneuploid tumours in early stage (< 2A) and late stage (> 2B) disease, with a greater relative proportion of aneuploid tumours (DI > 1.1) being observed in the latter.

CORRELATIONS WITH SURVIVAL

The univariate correlations of survival with FIGO stage, completeness of surgery, histological type, tumour grade, DNA index, and p62$^{c\text{-}myc}$ are summarised in table 6. As expected, the extent of disease at presentation showed a highly significant inverse correlation with survival.

The extent of surgical removal was also associated with survival (p < 0.00001). As expected there was no significant difference between the biopsy only, limited residual, and major residual disease categories. Complete removal is feasible in lower stage disease and may be curative on its own.

Histological type was significantly associated with survival (p < 0.003), with mucinous tumours showing the best five year survival (57%), and undifferentiated tumours the worst (6%). There was no significant difference between survival in the endometrioid, serous, and mixed tumours (fig 3).

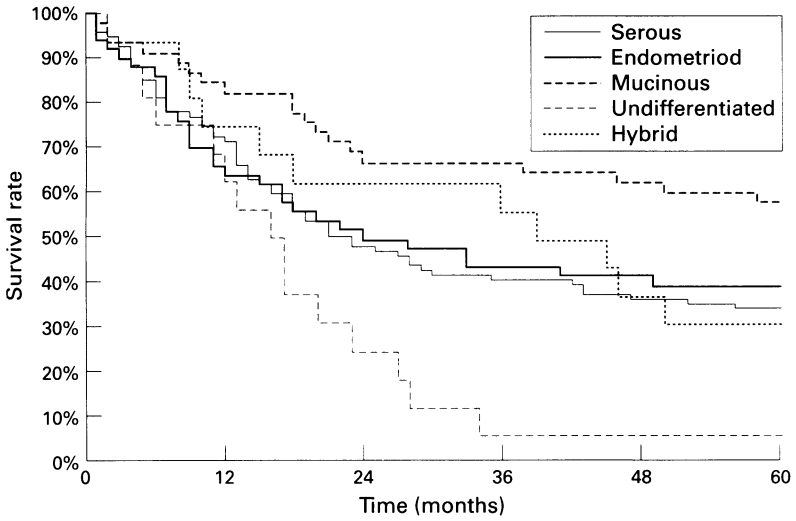

Figure 3 Dependence on survival on tumour type, namely serous papillary, endometrioid, mucinous, mixed (hybrid), and undifferentiated tumours.

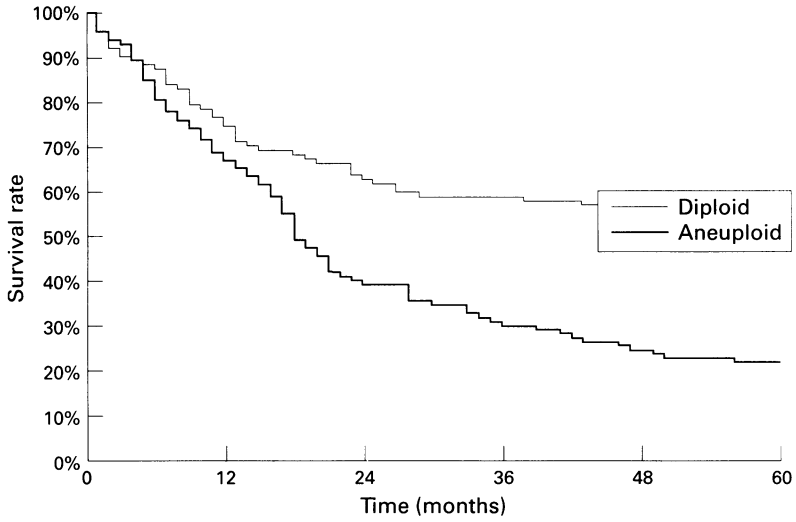

Figure 4 Association of survival with DNA index ($p < 0.00001$).

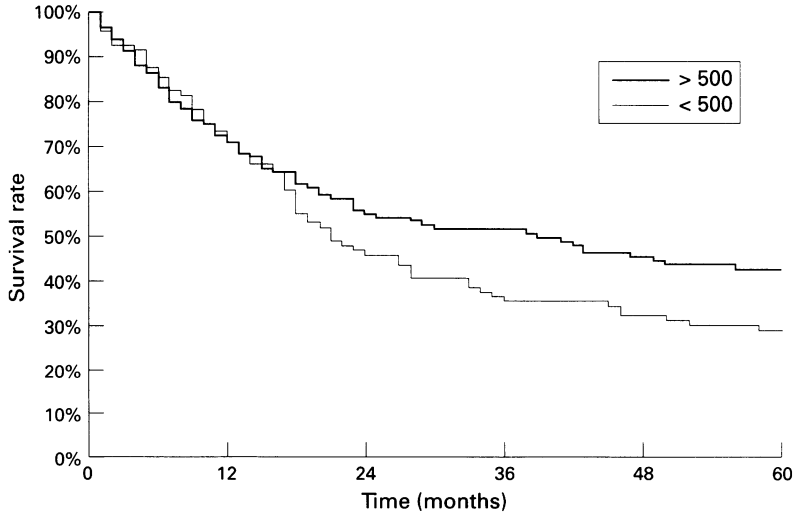

Figure 5 Association of survival with $p62^{c\text{-}myc}$ ($p = 0.09$).

As there are five tumour type categories, four histological grades, and seven disease stages, this gives a theoretical total of 140 possible combinations of these three variables. Moreover, there are a several additional variables to be considered, namely DNA index (two extra categories), completeness of surgery (three extra categories), and $p62^{c\text{-}myc}$ levels (two extra categories). The number of patients in this study is inadequate to consider all possible combinations of variables. Hence, as a prelude to both the univariate and multivariate analysis, a detailed statistical analysis of the various subcategories was carried out to ascertain whether reduction in the number of variables could be achieved by combining groups which were not statistically different. This type of approach has been used previously in ovarian cancer where, for example, all stage 1 subcategories (a, b, and c) and both stage 2 subcategories (a and b) have been grouped into stages 1 and 2 respectively.[46 47]

A practical and therapeutic classification of direct relevance to clinical management is:

- Tumour believed to be confined to ovary(ies)
- Tumour spread beyond the ovary but macroscopically removed
- Visible tumour burden remains after surgery.[36]

Similarly, some publications combine tumour grade categories (for example, Rodenburg and colleagues[29]) who group together grades 2 and 3. This approach is perfectly valid as a parameter reduction procedure but there should be both clinical and statistical justification for combining categories.

The association between DNA index and survival is shown in fig 4, where it can be seen that patients with diploid tumours (D1 < 1.1) have a significantly increased probability of survival, $p<0.00001$.

The $p62^{c\text{-}myc}$ nuclear content was associated with survival at a probability of only 0.09 (fig 5), which cannot be regarded as significant. A higher $p62^{c\text{-}myc}$ content was found in well differentiated than in moderately plus poorly differentiated tumours, with medians of 704 and 482 respectively (Kruskal-Wallis $\chi^2 = 6.48$, $p < 0.001$). The $p62^{c\text{-}myc}$ levels did not differ significantly across either stage or DNA index but the highest $p62^{c\text{-}myc}$ levels were found in the "biopsy only" patients.

MULTIVARIATE ANALYSIS

The multivariate analysis used the Cox regression model,[45] into which all the factors listed in table 6 were entered, and in addition the $p62^{c\text{-}myc}$ levels were considered as continuous variables. Using a forward stepwise variable selection procedure, stage was the first variable to enter the model and tumour grade the second. Using a significance level of 0.05 for inclusion, no further variables added to the prediction of survival afforded by this model although completeness of surgery was of borderline significance ($p < 0.09$). The results are given in table 7.

Table 7 Results of the multivariate analysis

	Variable	*χ^2 Statistic to enter model*
Step 1	FIGO stage	97.6 on 1 df, $p < 0.0001$
Step 2	Grade	15.4 on 1 df, $p = 0.0001$

Table 8 Summary of results for significance of DNA index versus survival in univariate and multivariate analyses, DIu and DIm respectively, with number of patients in the various stage and grade categories

	p Value		*Stage*				*Grade*			
Author (ref)	*DIu*	*DIm*	*1*	*2*	*3*	*4*	*1*	*2+3*	*n*	*Year*
Rodenburg (29)	<0.0002	<0.05	0	10	50	14	15	56	74	1987
Friedlander (51)	<0.0001	0.001	0	0	96	31	13	107	120	1988
Klemi (46)	<0.0001	0.001	26	20	54	34	35	99	134	1989
Vergote (50)	<0.0004	0.004	290	0	0	0	121	169	290	1993
Erba (17)	NS	NS	0	0	75	15	13	77	90	1909
Barnabei (47)	<0.0400	NS	17	13	61	24	17	98	115	1990
Current (all)	<0.0001	NS	79	61	73	10	89	134	223	1995
Current (inv)†	<0.0001	NS	56	58	71	10	61	134	195	1995
Current (bln)	NS	NS	23	3	2	0	28	0	28	1997

†The overall results from the current study (all) are subdivided into invasive (inv) and borderline (bln) tumours.

Table 9 Individual paired comparisons of the frequencies in each stage category from the various studies cited; χ^2 is shown above and to the right of the diagonal with its associated p value below and to the left

	Rodenburg	*Friedlander*	*Klemi*	*Vergote*	*Erba*	*Barnabei*	*Current*
Rodenburg	–	15.27	20.01	348.29	11.20	10.80	45.70
Friedlander	0.0016	–	53.31	409.05	1.46	33.64	113.96
Klemi	0.0002	<0.0001	–	303.16	45.71	3.16	32.22
Vergote	<0.0001	<0.0001	<0.0001	–	370.36	314.50	281.44
Erba	0.0107	0.6926	<0.0001	<0.0001	–	26.86	82.39
Barnabei	0.0129	<0.0001	0.3672	<0.0001	<0.0001	–	34.59
Current	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–

References: Rodenburg *et al*[29]; Friedlander *et al*[51]; Klemi *et al*[46]; Vergote *et al*[50]; Erba *et al*[17]; Barnabei *et al*[47]; Current, current study excluding borderline tumours.

Table 10 Individual paired comparisons of the frequencies of grade 1 with grade 2+3 tumours; χ^2 is shown above and to the right of the diagonal with its associated p value below and to the left

	Rodenburg	*Friedlander*	*Klemi*	*Vergote*	*Erba*	*Barnabei*	*Current*
Rodenburg	–	3.00	0.39	9.45	0.81	0.83	2.16
Friedlander	0.0832	–	8.68	0.02	0.33	0.51	16.16
Klemi	0.5345	0.0032	–	11.45	3.69	4.15	0.79
Vergote	0.0021	0.8994	0.0007	–	21.21	25.42	4.99
Erba	0.3674	0.5656	0.0546	<0.0001	–	0.02	8.23
Barnabei	0.3608	0.4769	0.0416	<0.0001	0.8957	–	9.60
Current	0.1420	<0.0001	0.3742	0.0255	0.0041	0.0019	–

References: Rodenburg *et al*[29]; Friedlander *et al*[51]; Klemi *et al*[46]; Vergote *et al*[50]; Erba *et al*[17]; Barnabei *et al*[47]; Current, current study excluding borderline tumours.

Discussion

In this paper we present results of both univariate and multivariate analyses in carcinoma of the ovary, where the relations among DNA index, p62$^{c\text{-}myc}$, FIGO stage, histological type, tumour grade, completeness of surgery, and patient survival were examined in detail.

The findings in the univariate analyses, where stage, surgical completeness, tumour grade, and DNA index were all associated with survival at $p < 0.00001$, were to be expected. Histological type was less strongly associated with survival, at $p = 0.003$. However, p62$^{c\text{-}myc}$ showed no significant association with survival ($p = 0.09$). This last finding was of no surprise as this protein is now known to be associated with cellular proliferation states, non-malignant as well as malignant.

Two further p62$^{c\text{-}myc}$ findings deserve comment. First, there were significantly higher levels in well differentiated than in moderately plus poorly differentiated tumours ($p = 0.001$), a similar result to that found in both testicular[39] and cervical cancer.[3. Second, the highest p62$^{c\text{-}myc}$ levels were found in the biopsy-only patients.

This protein has a short half life[49] and it is possible that the quantity of protein per nucleus is related to the time between ligation of the blood supply and fixation. In the biopsy-only patients the time between sampling and fixation will have been very short, whereas the time taken for a major surgical extirpation is likely to have been considerably longer, which might have resulted in protein degradation.

MULTIVARIATE ANALYSIS

In the multivariate analysis significant associations of survival were found only with disease stage and tumour grade (at or below $p = 0.00001$).

Completeness of surgery as an independent prognostic variable was of borderline significance ($0.05 < p < 0.09$. This was not surprising as completeness of surgery is somewhat "softly" defined and will always show some dependence on extent of local tumour spread and hence stage. Survival in those cases not cured by surgery will depend on effective chemotherapy, when results may be better with a lower tumour burden.

The most striking and unexpected finding was the failure to show that DNA index was an independent prognostic variable ($p > 0.05$). In our univariate analysis the DNA index was very highly associated with survival ($p < 0.00002$), in agreement with the majority of other univariate analyses.[2 12 13 16 18-22 24 30] Certain multivariate studies have reported previously that DNA index represents a powerful independent prognostic factor in ovarian cancer.[19 29 46 50] However, Erba *et al*[17] and

Barnabei *et al*[47] both reported that DNA index was not an independent prognostic variable in multivariate analyses.

Possible reasons for these discrepancies should be considered, and table 8 summarises results extracted from the multivariate analyses[17 29 46 47 50 51] together with those from this study. This gives the significance of DNA index versus survival in univariate and multivariate analyses (DIU and DIM respectively), with the numbers of patients in the various stage and grade categories.

It is quite obvious that the studies cited are not all directly comparable as there are wide differences in the numbers of patients in the various stage categories and, for example, we should not be comparing the results of Vergote *et al*[50] with those of Friedlander *et al*[51] and Erba *et al*,[17] as the study designs were different. If we now compare all individual pairs of studies, excluding the borderline tumours from our work, and if we take the conventional cut off value of $p = 0.05$, we can see that we are only justified in comparing the results of Friedlander *et al*[51] with Erba *et al*[17] ($p = 0.6926$), and of Klemi *et al*[46] with Barnabei *et al*[47] ($p = 0.3672$) (table 9).

The results in table 10 show that we are justified in comparing the results of Friedlander *et al*[51] with those of Erba *et al*[17] ($p = 0.5656$) but not of Klemi *et al*[46] with Barnabei *et al*[47] ($p = 0.0416$). As stage and grade are independent variables we can combine these statistically to give a χ^2 value of 1.86 with 3 degrees of freedom ($p = 0.6011$) for stage and grade comparison of Friedlander *et al*[51] with Erba *et al*.[17] Hence in statistical terms it is only the latter two sets of results which can be compared directly, as they represent comparable stage and grade samples. The former shows that DNA index is an independent prognostic variable in both the univariate and multivariate analyses and the latter shows that it is not a significant prognostic variable in either. If we relax our statistical criteria a little and compare the results of Klemi *et al*[46] with those of Barnabei *et al*,[47] we find that DNA index is a significant prognostic variable in both the univariate and multivariate analyses in the former work but that it is a barely significant prognostic variable in only the univariate analysis of the latter ($p = 0.04$).

CONCLUSION

An important point is raised by the above finding which concerns the overall philosophy of carrying out DNA index determinations. Because DNA index was not an independent prognostic variable in these studies it must, by definition, be dependent on—or secondary to—disease stage or tumour grade, both of which were again shown to be independent variables at $p < 0.0001$, a very high degree of significance. Hence DNA index did not provide any additional prognostic information in comparison with these two well tried and tested "classical" determinants. The latter should be carried out rigorously in all cases as a matter of routine (which was the case here) and this begs the conclusion that there should be no need to perform a DNA index in ovarian cancer if staging and histological grading are carried out correctly. The significance of this observation needs further scrutiny.

The smallest DNA index detectable by either flow cytometry or image analysis techniques is about 1.05, and for this to be reliable a coefficient of variation on the G1 peak of less than 4% would be needed. Most studies do not achieve this, particularly those obtained using flow cytometry with paraffin embedded biopsies. Hence a 5% increase in DNA index cannot be detected reliably; however, this represents a massive abnormality at the genetic level. Such amplifications simply cannot be detected by flow cytometric or simple image analysis techniques measuring total DNA content. Furthermore, some tumours are associated with translocations, rearrangements, and deletions,[52-58] some of which are manifest as chromosomal aberrations[59] with little or no change in total DNA; these, again, cannot be detected by measuring total DNA.

DNA index changes actually detectable by flow and image analysis techniques measuring total DNA represent late change in genetic pathology. One mechanism postulated for the development of an increased DNA index is endoreduplication giving rise to 4C DNA content cells equivalent to a DNA index of 2.0. Intermediate DNA indices between 1 and 2 could then be formed by loss of chromosomes from the 4C cells. However, a DNA index greater than unity, whatever the mechanism for its production, is undoubtedly a manifestation that the cells are "sick" and this is more likely to be observed the longer the pathology has been present. Hence it is most likely to be seen in the more advanced stages[18 24 50 60] in the least differentiated lesions,[5-8] which is exactly the pattern revealed by this multivariate analysis. Although this interpretation may be simplistic, the results reported here do very strongly suggest that we should employ the null hypothesis that the DNA index is not an independent prognostic variable until proven to be so using multivariate statistical analysis techniques. It is only by performing such studies that we will be able to be sure that DNA indices give us not just data but reliable and useful clinical information that could not be obtained by simpler and less expensive means.

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1 Hiddeman W, Schumann J, Andreef M, *et al.* Convention on nomenclature for DNA cytometry. *Cancer Genet Cytogenet* 1984;**13**:181–3.
2 Atkin NB. Modal DNA value and chromosome number in ovarian neoplasia. A clinical and histopathological assessment. *Cancer* 1971;**27**:1064–73.
3 Atkin NB, Kay R. Prognostic significance of modal DNA value and other factors in malignant tumours, based on 1465 patients. *Br J Cancer* 1979;**40**:210–21

4 Coulson PB, Thornthwaite JT, Woolley TW, *et al.* Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer patient survival. *Cancer Res* 1984;**44**:4187–96.
5 Friedlander ML, Hedley DW, Taylor IW. Clinical and biological significance of aneuploidy in human tumours. *J Clin Pathol* 1984;**37**:961–74.
6 Hedley DW. Flow cytometry using paraffin-embedded tissue: five years on. *Cytometry* 1989;**6**:229–41.
7 Johnson TS, Williamson KD, Cramer MM, *et al.* Flow cytometric analysis of head and neck carcinoma DNA index and S-phase fraction from paraffin-embedded sections: comparison with malignancy grading. *Cytometry* 1985;**6**: 461–70.
8 Frankfurt OS, Slocum HK, Rustum YM, *et al.* Flow cytometric analysis of DNA aneuploidy in primary and metastatic human solid tumors. *Cytometry* 1984;**5**:71–80.
9 Frankfurt OS, Arbuck SG, Chin JL, *et al.* Prognostic applications of DNA flow cytometry for human solid tumors. *Ann NY Acad Sci* 1986;**468**:276–90.
10 Volm M, Mattern J, Sonka J, *et al.* DNA distribution in non-small cell lung carcinomas and its relationship to clinical behaviour. *Cytometry* 1985;**6**:348–56.
11 Zimmerman PV, Hawson GAT, Bint MH, *et al.* Ploidy as a prognostic determinant in surgically treated lung cancer. *Lancet* 1987;ii:530–3.
12 Berchuck A, Boente MP, Kerns BJ, *et al.* Ploidy analysis of epithelial ovarian cancers using image cytometry. *Gynecol Oncol* 1992;**44**:61–5.
13 Blumenfeld D, Braly PS, Ben-Ezra J, *et al.* Tumour DNA content as a prognostic feature in advanced epithelial ovarian carcinoma. *Gynecol Oncol* 1987;**27**:389–402.
14 Brescia RJ, Barakat RA, Beller U, *et al.* The prognostic significance of nuclear DNA content in malignant epithelial tumors of the ovary. *Cancer* 1990;**65**:141–7.
15 Chadha S, Cornelisse CJ, Schaberg A. Flow cytometric DNA ploidy analysis of ovarian granulosa cell tumors. *Gynecol Oncol* 1990;**36**:240–5.
16 Christov K, Vassilev N. Flow cytometric analysis of DNA and cell proliferation in ovarian tumors. *Cancer* 1988;**61**: 121–5.
17 Erba E, Ubezio P, Pepe S, *et al.* Flow cytometric analysis of DNA content in human ovarian cancers. *Br J Cancer* 1989; **60**:45–50.
18 Friedlander ML, Taylor IW, Russell P, *et al.* Ploidy as a prognostic factor in ovarian cancer. *Int J Gynecol Pathol* 1983;**2**:55–63.
19 Friedlander ML, Hedley DW, Taylor IW, *et al.* Influence of cellular DNA content on survival in advanced ovarian cancer. *Cancer Res* 1984;**44**:397–400.
20 Friedlander ML, Taylor IW, Russell P, *et al.* Cellular DNA content: a stable feature in epithelial ovarian cancer. *Br J Cancer* 1984;**49**:173–9.
21 Hamaguchi K, Nishimura H, Miyoshi T, *et al.* Flow cytometric analysis of cellular DNA content in ovarian cancer. *Gynecol Oncol* 1990;**37**:219–23.
22 Iversen OE, Skaarland E. Ploidy assessment of benign and malignant ovarian tumors by flow cytometry: a clinicopathologic study. *Cancer* 1987;**60**:82–7.
23 Kaern J, Trope C, Kjorstad KE, *et al.* Cellular DNA content as a new prognostic tool in patients with borderline tumors of the ovary. *Gynecol Oncol* 1990;**38**:452–7.
24 Kallioniemi OP, Punnonen R, Mattila J, *et al.* Prognostic significance of DNA index, multiploidy, and S-phase fraction in ovarian cancer. *Cancer* 1988;**61**:334–9.
25 Kuhn W, Kaurfmann M, Feichter GE, *et al.* DNA flow cytometry, clinical and morphological parameters as prognostic factors for advanced malignant and borderline ovarian tumors. *Gynecol Oncol* 1989;**33**:360–7.
26 Lage JM, Weinberg DS, Huettner PC, *et al.* Flow cytometric analysis of nuclear DNA content in ovarian tumors. *Cancer* 1992;**69**:2668–75.
27 Murray K, Hopwood L, Volk D, *et al.* Cytofluorometric analysis of the DNA content in ovarian carcinoma and its relationship to patient survival. *Cancer* 1989;**63**:2456–60.
28 Padberg BC, Arps H, Franke U, *et al.* DNA cytophotometry and prognosis in ovarian tumors of borderline malignancy. A clinicomorphologic study of 80 cases. *Cancer* 1992;**69**: 2510–4.
29 Rodenburg CJ, Cornelisse CJ, Heintz PAM, *et al.* Tumor ploidy as a major prognostic factor in advanced ovarian cancer. *Cancer* 1987;**59**:317–23.
30 Sahni K, Tribukait B, Einhorn N. Flow cytometric measurement of ploidy and proliferation in effusions of ovarian carcinoma and their possible prognostic significance. *Gynecol Oncol* 1989;**35**:240–45.
31 Schueler JA, Cornelisse CJ, Hermans J, *et al.* Prognostic factors in well-differentiated early-stage epithelial ovarian cancer. *Cancer* 1993;**71**:787–95.
32 Seidman JD, Norris HJ, Griffin JL, *et al.* DNA flow cytometric analysis of serous ovarian tumors of low malignant potential. *Cancer* 1993;**71**:3947–51.
33 Volm M, Bruggemann A, Gunther M, *et al.* Prognostic relevance of ploidy, proliferation, and resistance-predictive tests in ovarian carcinoma. *Cancer Res* 1985;**45**:5180–5.
34 Zangwill BC, Balsara G, Dunton C, *et al.* Ovarian carcinoma heterogeneity as demonstrated by DNA ploidy. *Cancer* 1993;**71**:2261–7.
35 Watson JV, Curling OM, Munn CF, *et al.* Oncogene expression in ovarian cancer. A pilot study of the c-myc oncoprotein in serous papillary ovarian cancer. *Gynecol Oncol* 1987; **28**:137–50.
36 Hudson CN, Potsides P, Curling OM. An audit of surgical treatment of ovarian cancer in a Metropolitan health region. *J R Soc Med* 1991;**84**:206.
37 Evan GI, Lewis GK, Ramsay G, *et al.* Isolation of monoclonal antibodies specific for human c-myc proto-oncogene products. *Mol Cell Biol* 1985;**5**:3610–16.
38 Niman HL, Houghten RA, Walker LE, *et al.* Generation of protein-reactive antibodies by short peptides in an event of high frequency: implications for the structural basis of immune recognition. *Proc Natl Acad Sci USA* 1983;**80**: 4949–53.
39 Sikora K, Evan G, Stewart J, *et al.* Detection of the c-myc oncogene product in testicular cancer. *Br J Cancer* 1985;**52**:171–6.
40 Sikora K, Chan S, Evan G, *et al.* c-myc Oncogene expression in colorectal cancer. *Cancer* 1987;**59**:1289–95.
41 Hedley DW, Friedlander ML, Taylor IW, *et al.* Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983;**31**:1333–5.
42 Watson JV, Sikora KE, Evan GI. A simultaneous flow cytometric assay for c–myc oncoprotein and DNA in nuclei from paraffin-embedded material. *J Immunol Methods* 1985;**83**:179–92.
43 Watson JV. Enzyme kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. *Cytometry* 1980;**1**:143–51.
44 Watson JV. A method for improving light collection by 600% from square cross section flow cytometry chambers. *Br J Cancer* 1985;**51**:433–5.
45 Cox DR. Regression models and life tables. *J Stat Soc (B)* 1972;**34**:187–223.
46 Klemi PJ, Joensuu H, Maenpaa J, *et al.* Influence of cellular DNA content on survival in ovarian carcinoma. *Obstet Gynecol* 1989;**74**:200–4.
47 Barnabei VM, Miller DS, Bauer KD, *et al.* Flow cytometric evaluation of epithelial ovarian cancer. *Am J Obstet Gynecol* 1990;**162**:1584–92.
48 Hendy-Ibbs P, Cox H, Evan GI, *et al.* Flow cytometric quantitation of DNA and c-myc oncoprotein in archival biopsies of uterine cervix neoplasia. *Br J Cancer* 1987;**55**: 275–82.
49 Rabbitts PH, Watson JC, Lamond A, *et al.* Metabolism of c-myc gene products: c-myc mRNA and protein expression in the cell cycle. *Embo J* 1985;**4**:2009–15.
50 Vergote IB, Kaern J, Abeler VM, *et al.* Analysis of prognostic factors in stage I epithelial carcinoma: importance of degree of differentiation and deoxyribonucleic acid ploidy in predicting relapse. *Am J Obstet Gynecol* 1993;**169**:40–52.
51 Friedlander ML, Hedley DW, Swanson C, *et al.* Prediction of long term survival by flow cytometric analysis of cellular DNA content in patients with advanced ovarian cancer. *J Clin Oncol* 1988;**6**:282–90.
52 Baker SJ, Fearon ER, Nigro JM, *et al.* Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989;**244**:217–21.
53 Benedict WF, Murphree AL, Banerjee A, *et al.* Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene. *Science* 1983;**219**:973–5.
54 Fearon ER, Cho KR, Nigro JM, *et al.* Identification of a chromosome 182 gene that is altered in colorectal cancers. *Science* 1990;**247**:49–56.
55 Masuda H, Miller C, Koefler H, *et al.* Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc Natl Acad Sci USA* 1987;**84**:7716–19.
56 Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;**244**:707–12.
57 Wolf D, Rotter V. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. *Proc Natl Acad Sci USA* 1985;**82**:790–4.
58 Yunis JJ, Ramsay N. Retinoblastoma and sub-band deletion of chromosome 13. *Am J Dis Child* 1978;**132**:161–3.
59 Gilbert F. Chromosome aberrations and oncogenes. *Nature* 1983;**303**:475.
60 Haapasalo H, Atkin NB, Collan Y, *et al.* Tumour ploidy, morphometry, histological grading and clinical features in ovarian carcinoma: mutual relations. *Ann Cell Pathol* 1991; **3**:261–71.