Image cytometry for DNA analysis in endometrial carcinoma correlated with other prognostic parameters

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Abstract. We evaluated by static cytometry DNA ploidy parameters in 30 stage I-IV endometrial carcinomas and correlated these data with standard clinical-pathological features and disease-free period. We observed a direct correlation between either non-diploid DNA content and deeper myometrial invasion ($p<0.02$) or D.I.≥1.2 and M2-M3 tumors ($p<0.009$). The Kaplan Meier survival curves illustrate a more rapid relapse of disease associated with non-diploidy, high 5cExR, high level of proliferation and D.I.≥1.2. while Cox regression model gave relative hazards for disease recurrence of 4, 6, 3.7 and 2.1 for non-diploidy, D.I.≥1.2, high 5cExR and high level of proliferation respectively. This prospective study confirmed the prognostic value of DNA Index, and its usefulness in clinical practice especially in stage I cases, otherwise characterised by favourable standard prognostic factors, is discussed.

Introduction

There are many surgical-pathological features which are important for the prognosis of endometrial carcinoma. Salient ones include: grade of histological differentiation, histotype, myometrial invasion and lymph node involvement (1,2). More recently, other elements have been reported as having possible prognostic value, for example, various alterations at the genetic-molecular level such as aneuploidy (3-10), altered expression of p53, of *erb*B2/*neu*, and of growth factors and their receptors (11-13).

Some authors report that ploidy evaluation is the most significant parameter for prognosis of early stage tumors (14,15).

Most studies which have analyzed ploidy in endometrial cancer used flow cytometry techniques. Recently, however, image analysis procedures have been introduced which allow ploidy analysis as well as the study of other features closely related to the quantitative distribution of DNA in the tumor population examined. These techniques are sufficiently rapid and accurate (13,16).

The aim of this prospective study was to correlate known traditional clinical and pathological prognostic factors with ploidy and other parameters related to DNA distribution in the tumor population, evaluated by image analysis. The prognostic significance of these latter parameters has also been related to the disease-free survival time.

Materials and methods

Patients and sample collection. Our study is based on 30 selected cases of endometrial carcinoma treated in the II Institute of Obstetric and Gynecology of the University of Rome 'La Sapienza', between 1990 and 1994. The basis for selection was the possibility of taking fresh surgical specimens from the primary tumor site during surgery from patients who had adequate therapy and follow-up.

Patients underwent surgical treatment via laparotomy, peritoneal washing, total hysterectomy (with bilateral salpingoophorectomy) upper third colpectomy and lastly pelvic lymph-adenectomy. In one case, the latter excision was excluded because of obesity.

Patients were aged between 47 and 83 years. Only 4 were in pre-menopause. The subdivision in pathological stages was the following: 19 stage I, 5 stage II, 5 stage III and I stage IV. The most common histotype observed was endometrial adenocarcinoma (21 cases); the remaining cases included 6 adenoacanthomas, 2 squamous adenocarcinomas and 1 clear cell carcinoma. In 12 cases the tumor was well differentiated (G1), in 14, moderately differentiated (G2), and in 4 cases poorly differentiated (G3). In 6 cases the myometrium was not infiltrated, 16 presented with limited infiltration of the inner third and in 8 cases the tumor extended beyond this boundary.

Only in 4 cases were the pelvic lymph nodes involved and in one case the internal iliac lymph nodes. Peritoneal washing revealed no neoplastic elements. The surgical-pathological features of the patients are shown in Table I. Adjuvant therapy was prescribed for all operated patients with poor prognoses. The medium follow-up period was 22 months (range 3-49 months). One patient died of post

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Table I. Pathological variables.

Case No.	Pathological state	Grade	Myometrial invasion	Lymph nodes	Histotype
1	I A	G1	M0	N^-	AK
2	I A	G1	M0	N^-	AA
3	I A	G1	M0	N^-	AK
4	I A	G1	M0	N^-	AK
5	I A	G1	M0	N^-	AK
6	I B	G1	M1	N^-	AA
7	I B	G1	M1	N^-	AK
8	I B	G1	M1	N^-	AA
9	I B	G2	M2	N^-	AK
10	I B	G2	M1	N^-	AK
11	I B	G2	M1	N^-	AK
12	I B	G1	M1	N^-	AK
13	I B	G1	M1	N^-	AA
14	I B	G1	M1	N^-	AK
15	I B	G2	M1	N^-	AK
16	I B	G2	M2	N^-	AA
17	I B	G3	M1	N^-	AS
18	I B	G2	M1	N^-	AK
19	I C	G2	M2	N^-	AK
20	II B	G2	M1	N^-	AK
21	II B	G2	M2	Nx	AS
22	II B	G2	M2	N^-	AK
23	II B	G2	M3	N^-	AK
24	II B	G3	M3	N^-	Clear cells AK
25	III B	G3	M1	N^-	AK
26	III C	G3	M2	N^+	AK
27	III C	G2	M2	N^+	AK
28	III C	G2	M3	N^+	AK
29	III C	G1	M1	N^+	AA
30	IV B	G2	M0	N^+	AK

AK, adenocarcinoma; AA, adenocanthoma; AS, adenosquamouscarcinoma.

Image analysis. After the removal of the uterus neoplastic material was obtained by touch imprint and then immediately fixed in buffered formalin (10%). The specimens were then stained applying a modified Feulgen method by using a kit (DNA staining kit 102300-01 Becton Dickinson, Elmhurst, Illinois). A CAS 200 was used for image analysis and the following parameters were analyzed: DNA index (D.I.) which gives the relationship between the modal value of DNA contained in tumor cells and that contained in normal cells, DNA ploidy which is the state of DNA in the main tumor cell clone, the 5c exceeding rate (5cExR) which is the percentage of elements which exceed by two and a half times the normal amount of DNA and lastly the level of proliferation, considered as the sum of elements corresponding to the S and G2 phases.

Rat hepatocytes were used as an external control population to set the system. Once the system is 'calibrated' the DNA index of the elements under study are automatically defined. The DNA index of each tumor was calculated and defined as the D.I. of the primary clone. The cases were subsequently stratified on the basis of their D.I. into low (<1.2) or high DNA index (≥1.2). For each case we evaluated 30 diploid cells (epithelial cells or lymphocytes) as internal control.

Tumors whose DNA index differed by less than 10% from the internal control population were defined as diploid while all the others were defined as non-diploid (near diploid, tetraploid, polyploid, and clearly aneuploid). If there were two peaks, one diploid and the other aneuploid, the tumor was considered non-diploid.

The 5cExR, or number of elements in which the DNA content exceeded 5c was evaluated as a percentage. Above 4% of the elements present in the sample was considered high and low in other cases (≤4%).

The level of proliferation was considered low if less than or equal to 20% and high if greater than 20%.

Statistical analysis. We used the X^2 test with Yates correction and Fisher's exact test (17) to examine the relationship between the pathological variables (histological grading, myometrial invasion, pathological staging and lymph node involvement) and the variables related to DNA parameters in the population under study to see if they were dependent on each other.

The survival curves were calculated using the Kaplan-Meier method and compared with the log rank test (18). The influence of the variables on survival was investigated by using the Cox regression model (19). The analysis were carried out using the EPI-INFO package and S plus.

Table II. Image analysis results.

Case No.	Ploidy	DNA index	5cExR	Prolif. level
1	D	1.09	L	L
2	n.D.	1.02	L	E
3	n.D.	1.14	E	L
4	D	0.97	L	L
5	D	0.97	L	L
6	D	0.96	L	L
7	D	1.0	L	L
8	D	1.14	L	L
9	n.D.	1.23	L	L
10	n.D.	1.03	E	E
11	D	1.08	E	E
12	n.D.	1.0	L	E
13	D	1.07	L	E
14	D	1.13	L	E
15	D	1.01	L	E
16	n.D.	1.19	L	E
17	D	1.08	L	E
18	n.D.	2.39	E	E
19	D	1.0	L	L
20	D	1.1	L	L
21	n.D.	1.4	E	E
22	D	1.02	L	E
23	n.D.	1.2	L	N.D.
24	n.D.	1.39	E	N.D.
25	D	1.06	L	E
26	n.D.	1.2	E	L
27	n.D.	1.02	L	E
28	n.D.	1.14	L	E
29	D	1.07	L	L
30	n.D.	0.9	E	E

D, diploid; n.D., non diploid; L, low; E, elevated; N.D., not done.

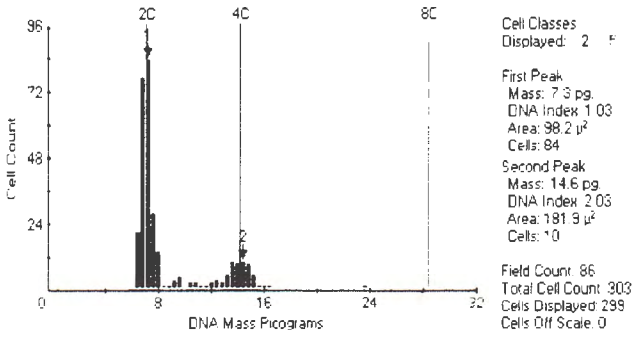

Figure 1. Distribution of DNA mass. Case 7 diploid histogram.

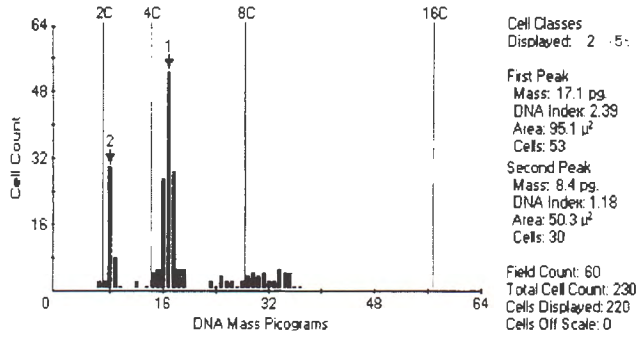

Figure 2. Distribution of DNA mass. Case 18 aneuploid histogram.

Results

The results are summarized in Table II. On the basis of pathology staging, tumors were divided into early stage (19 stage I) and advanced stages (11 stage II-IV); using myometrial invasion as a parameter the cases were divided into initially infiltrating forms (20 cases M0/M1) and advanced infiltrating ones (10 cases M2/M3). Similarly, the cases were divided into well differentiated forms (12 G1) and less differentiated ones (18 G2/G3).

The different surgical pathological parameters were correlated with the X^2 test corrected by Yates and Fisher's exact test. This correlation was always statistically significant ($p<0.02$). In particular, there was a close correlation between histological grading and myometrial invasion ($p<0.002$).

The internal control population was always diploid with a D.I. between 0.91 and 1.04 (median 0.99) and the coefficient of variation (CV) was between 2.7 and 6.1% (median 4.3%).

Of the 30 cases studied 16 (53%) were considered diploid (Fig. 1) and 14 (47%) non-diploid (Fig. 2). Of the latter, one was near diploid, 1 tetraploid, 5 polyploid and 7 clearly aneuploid.

Fourteen out of 20 M0/M1 tumors were diploid and 8 out of 10 M2/M3 tumors were non diploid. Thus, it is clear that ploidy is directly correlated to myometrium invasion ($p<0.02$).

On the other hand, the correlation between ploidy and grading is not demonstrated even though 11 out of 14 non-diploid cases were partially or poorly differentiated ($p=0.11$).

The same correlation was observed for stage and ploidy: diploid forms prevail in stage I (12 out of 19) and of the 11 cases in advanced stage 7 were non-diploid; however this is statistically insignificant.

If we observe the correlation between lymph node involvement and ploidy, 15 out of 24 negative lymph node cases were diploid while only 1 out of 5 with lymph node involvement was diploid ($p=0.14$).

The DNA index of the main tumor population ranged between 0.96 and 2.39 with a CV between 1.2 and 10.4% (median 6.1%). In relation to our cut-off, 24 cases had a low D.I. and 6 a high D.I.

All of the well differentiated tumors presented a D.I.<1.2 while partially or poorly differentiated ones had a D.I. equal to or above 1.2 in 6 cases and inferior in 12 cases. This result is at the limit of statistical significance ($p=0.05$).

Nineteen of the 20 cases confined to the inner third of the myometrium had a D.I.<1.2 and 5 out of 10 of the extended tumors had a high D.I. The only case without myometrial invasion but with a high D.I. was a stage IV which had invaded the ovaries and pelvis because of contiguity (case 30). Hence, these parameters are closely correlated ($p<0.000$).

Nevertheless, there does not seem to be a correlation between stage and D.I. or between D.I. and lymph node involvement (p=1.0).

In 7 diploid tumors there were no elements with a DNA content above 5c. In 15 cases this value was higher than 0% and less than 3.6% and in 8 it was higher than 8% (high 5cExR); there were no tumors with intermediate values so our cut-off was chosen as 4%. On the basis of this sub-division only one diploid tumor had a high 5cExR while the other 7 tumors with a high 5cExR were non-diploid. This variable is statistically correlated to ploidy (p=0.01) and to D.I. (p<0.03).

Only one case of well differentiated tumor had a high 5cExR and this was a polyploid tumor with a low level of proliferation; the other 11 well differentiated adenocarcinomas had a low or zero 5cExR. On the other hand, the moderately or poorly differentiated tumors can be divided into ones with a high and ones with a low number of elements exceeding 5c. This tendency, however, did not acquire statistical significance (p=0.09).

The stage appears to have no correlation whatsoever even with this ploidy parameter. Nor do myometrial invasion and 5cExR show any correlation.

Proliferation level was another parameter studied. Also in this case, as with 5cExR, tumors seemed to be stratified in two distinctive groups: the first with a level of proliferation between 2 and 16% and the second between 24 and 42%; 20% was chosen as a cut-off to sub-divide the lesions into low and high proliferating lesions. In 2 policlonal cases the presence of peaks made it impossible to evaluate the level of proliferation. On the basis of this cut-off 12 cases presented a low level of proliferation and 16 a high one. Most of the scarcely proliferating tumors were found among the diploid forms (9/12). Nevertheless, we also found high levels of proliferation in neoplasms with a DNA content within the norms.

Statistical studies showed no correlation between this parameter and ploidy (p=0.2) or the D.I. (p=1.0) or 5cExR (p=0.7). What does seem to emerge is a correlation between the level of proliferation and grading even though this does not reach a statistical significance (p=0.06); This could be because of the small number of cases considered. In fact, of the 7 diploid cases with high levels of proliferation as many as 5 are moderately or poorly differentiated. The same occurs among non diploid tumors where out of a total of 9 cases with high proliferation 7 are G2/G3.

The level of proliferation was generally low in well differentiated forms (8/12 cases) and high in the others (12/16 cases).

In 3 cases out of 12 with low levels of proliferation and 5 out of 16 with high levels the myometrium had been extensively invaded.

In advanced stages (II-IV) the rapidly proliferating ones prevailed, however, as many as 10 out of 19 tumors in stage I had a high level of proliferation.

The correlations between the various parameters are shown in Tables III and IV.

Table III. Statistical correlations between DNA ploidy and clinico-pathological variables.

	Ploidy D vs n.D	D.I. <1.2 vs >1.2	5cExR low vs elevated	Proliferation level low vs elevated
Stage				
I vs II-IV	n.s.	n.s.	n.s.	n.s.
Grade				
G1 vs G2-G3	n.s.	p=0.05	p<0.1	n.s.
Myometrial invasion				
M0-1 vs M2-3	p<0.02	p<0.01	n.s.	n.s.

D, diploid; n.D, non diploid; D.I., DNA index; n.s., not significant.

Table IV. Statistical correlations between ploidy parameters.

	Ploidy D vs n.D	D.I. <1.2 vs >1.2	5cExR low vs elevated
D.I. <1.2 vs >1.2	p=0.005	-	-
5cExR low vs elevated	p=0.01	p<0.03	-
Proliferation level low vs elevated	n.s.	n.s.	n.s.

D, diploid; n.D, non diploid; D.I., DNA index; n.s., not significant.

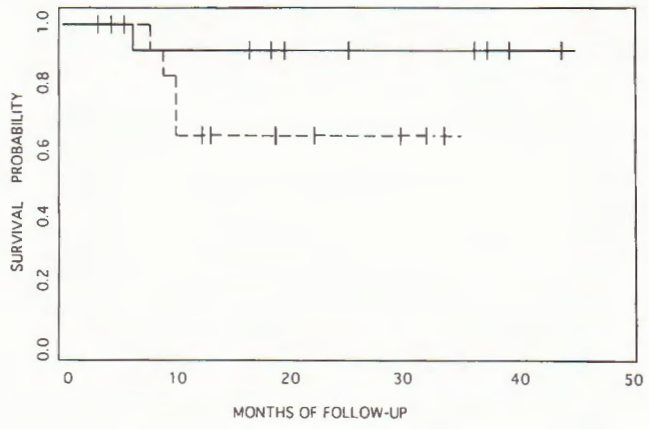

Figure 3. Kaplan Meier survival to recurrence for diploid (——) versus non diploid (---) tumor patients. Log rank test p=0.17.

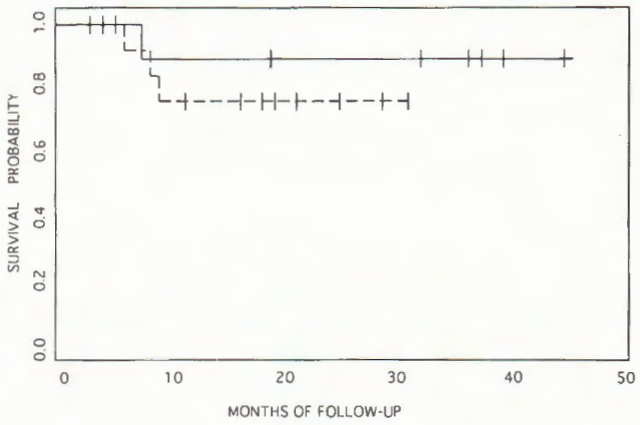

Figure 5. Kaplan Meier survival to recurrence curve for low (——) versus high (- -) level of proliferation tumor patients. Log rank test p=0.5.

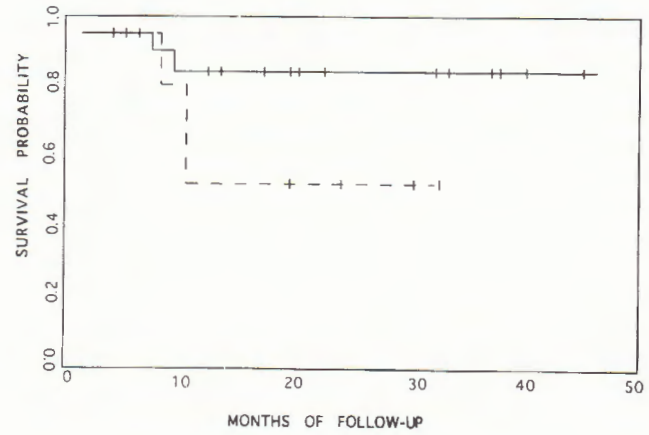

Figure 4. Kaplan Meier survival to recurrence curves for low (——) versus high (---) 5cExR tumor patients. Log-rank test p=0.12.

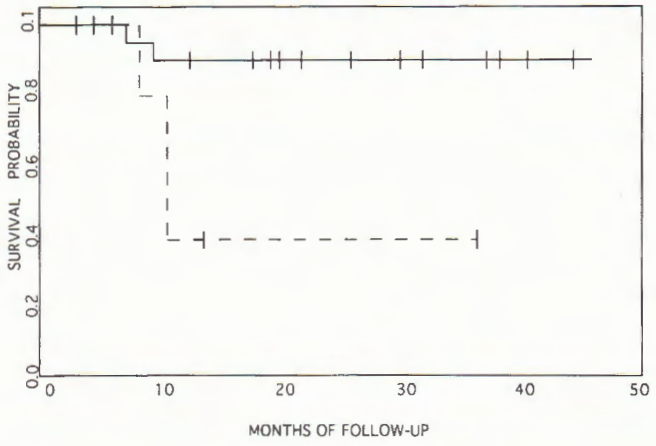

Figure 6. Kaplan Meier survival to recurrence curve for D.I.<1.2 (——) versus D.I.>1.2 (---) tumor patients. Log rank test p=0.02.

We also evaluated our parameters in relation to disease free survival time. The disease-free interval curves relative to ploidy, 5cExR, level of proliferation and D.I. are shown in Figs. 3, 4, 5 and 6 respectively.

For each parameter evaluated a difference between the two curves can be observed, but the log rank test revealed a statistical significance only in the case of D.I. (p=0.02).

We therefore proceeded to analyse the data with Cox's regression model. Patients with non diploid tumors had a more than four times greater risk of recurrence compared to patients with diploid neoplasms. This risk was even higher when the DNA index was taken into account. This revealed that women with tumor forms, where the D.I. was greater than or equal to 1.2 (compared to the forms where it was less than 1.2), had a risk 6 times greater of recurrence and this was statistically significant ($p<0.05$).

In a similar way the risk doubles when there is a high level of proliferation and when 5cExR is high the relative risk

Discussion

In the case of endometrial carcinoma our results confirm the correlation between some of the most commonly used prognostic parameters (grading and myometrial invasion; grading and surgical stage; surgical stage and myometrial invasion) as reported by others (1,2). While women with endometrial carcinoma confined to the uterine corpus could theoretically be cured by hysterectomy alone, recurrence rates up to 30% have been reported (15,20). It is therefore particularly important to be able to identify other independent prognostic parameters to put alongside the existing ones. This need led us to analyze certain intrinsic biological parameters which are characteristic of tumors, and especially those related to DNA content and its indexes (ploidy, 5cExR, DNA index, and proliferation levels).

Ploidy has been the subject of numerous studies. Most authors, however, used flow cytometry techniques (3-10)

Our work appears to arrive at results which are overlapping, in spite of the relatively limited follow-up period. What is more important, it uses a static cytometry system which lends itself more easily to routine analysis because of the low cost benefit ratio, on one hand, and the small quantity of material required on the other.

Both flow (3-10) and static cytometry (13,16,21) have demonstrated the prognostic value of ploidy. Authors who have carried out multivariable analysis have seen that it is an extremely important independent prognostic parameter whether it be considered alone or in parallel with surgical stage, histological grade and myometrial invasion (13), stage and myometrial invasion (9), depth of myometrial invasion and mean AgNOR area (22) or grade and S phase (SPF) (7). For some authors it is the single most important independent prognostic parameter (14-16). Lukes *et al* (13) showed that among molecular-genetic prognostic factors, DNA ploidy was the strongest predictor of persistent or recurrent disease.

Our study correlates ploidy and probability of survival in terms of disease-free time as well, with a relative risk of disease relapse in non-diploid forms which is four times greater than diploid ones. However, this data does not reach statistical significance, perhaps because of the limited number of cases studied.

Although the degree of proliferation, similar to the S phase, is probably an important parameter in evaluating the speed of neoplastic growth, in our study it appears as a less significant prognostic parameter. Nor was it closely correlated to the other pathological parameters investigated. For some authors (3,23,26) the S phase seems to have an important prognostic role while in other studies (24,25) the data are comparable to ours. It is worthwhile to underline that this parameter did not correlate either with other DNA variables analyzed or with standard features and therefore could provide additional biological information of tumor behaviour that could be particularly useful in stage I neoplasia. In our study 53% of the early stage tumors had high level of proliferation.

The 5cExR proved to be of particular clinical relevance, especially in borderline forms and in early stage tumors of the ovaries (27). Its importance in endometrial carcinoma, however, has not yet been sufficiently investigated. Our studies would seem to indicate agreement with Koher *et al* (28) showing that it has a certain prognostic value.

From a prognostic point of view the D.I. appears to be particularly significant. This variable was also studied by Lukes *et al* (13) and Symond (21). In these studies a cut-off value of 1.25 (13,29) and 1.5 (9,16,21) was used to distinguish low and high ploidy tumors.

This parameter compared to ploidy has the advantage of being more easily reproducible. Various authors use different ranges to define ploidy or introduce terminology such as near diploid (6) or tetraploid which is not used by others.

In our study using image analysis, we observed many tumors with low D.I. that include even tetra or polyploid elements, with the primary clone within diploid range, and the prognostic interpretation of these patterns is controversial. In our study these tumors followed a course which overlapped that of the diploid forms but our results need to be confirmed with a longer follow-up.

The DNA index was significantly correlated with histological grading, with myometrial invasion and with disease-free interval but not with surgical stage. Thus it may enable us to distinguish, within tumors of the same stage, and particularly stage I tumors which have a generally favourable prognosis, those with a high D.I. which can be at risk for recurrence even in the very long-term. It would therefore be possible to set up a more individualized therapeutic program. Coleman *et al* (8), who used this parameter as a continuous variable, showed that it was inversely proportional to survival.

Although the prognostic importance of ploidy has been widely demonstrated, its purely biological significance in endometrial carcinoma has not been sufficiently explored. Bocking *et al* (30) have postulated that initially the neoplastic cell has limited genetic alterations (translocations, small deletions and amplifications of small chromosomal segments) and remains within the diploid or near diploid range. Subsequently a chromosomal duplication without cellular division would seem to transform the primary clone into a tetraploid or near tetraploid one. Finally, because of the loss of some chromosomes, or the preferential duplication of others, a clear aneuploid set of chromosomes is found. Bishop (31) has demonstrated that these gross structural changes are tied to oncogene alterations. The aneuploid cell with its genetic patrimony which is no longer balanced would thus be particularly unstable due to its accumulation of a higher number of genetic defects and a higher number of modifications to regulatory/metastatic genes compared to diploid carcinomas. Endometrial carcinoma would seem to reflect this evolutionary picture. It is, probably, less aggressive than other tumors (e.g. breast cancer) (32) and in most cases (60-70%) it is diploid or near-diploid. We, as others (13,16,21), have demonstrated that when the DNA index is still low (D.I.<1.2) the prognosis is better, perhaps because the tumor is still in its early stages of natural history. We have also demonstrated, in accord with Lukes *et al* and Goodman *et al* (3,13), that diploid tumors are particularly numerous in stage I.

It is well known that when a tumor is clinically detected many years have gone by since onset of neoplastic transformation; indeed, quite often one has arrived at the last stages of a long tumor progression. Hence, for therapeutic purposes it is important to know not only at what moment we have arrived in the evolutionary history at the time of diagnosis (this is adequately described by the surgical pathological features and stage) but also its future 'evolutionary potential'. As Coleman *et al* (8) demonstrated, this is best represented by ploidy. They have shown that advanced stage tumors are aneuploid in more than two thirds of cases even though they appear to be confined in the uterus (clinical stage I). Furthermore, they have shown how the ploidy of the primary tumor (and not that of an eventual metastatising clone) is determinant for the patient's prognosis. Heterogeneity and tumor instability, which are associated with aneuploidy, can determine the evolution of the disease. All this would explain why ploidy could have such an important prognostic role (7,24,25) which is greater than that of other more specific genetic modifications (13)

in as much as it reflects the overall degree of genetic damage, and this is particularly the case in endometrial carcinoma.

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