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Clinical importance of DNA content in rectal cancer measured by flow cytometry

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SUMMARY The DNA content of 369 rectal cancers was measured by flow cytometry. One hundred and four (28%) were diploid, 252 (68%) were aneuploid, and 13 (3·5%) were tetraploid. Diploid cancers were associated with an improved 5 year survival ($p < 0·001$) and were more likely to present at an early stage. DNA content, however, did not confer independent prognostic information in a Cox model based on four discrete pathological variables. Patients were classified by a new system of prognostic grouping and those with a very good or a very poor outlook were removed leaving 137 prognostic group III patients. No further substratification of this group by DNA content or by four additional pathological variables could be achieved. As the new prognostic system is not improved by the addition of ploidy, routine adoption of flow cytometry in the assessment of rectal cancer cannot be recommended.

Flow cytrometry offers a simple and objective method for classifying tumours according to their DNA content.[1] In a series of colorectal cancers the DNA content follows a bimodal distribution with about one third of tumours being near-diploid and the remainder being aneuploid.[2,3] The technique can not resolve minor changes in karyotype which may characterise near-diploid tumours, nor will it detect very small populations of aneuploid cells. Theoretically, these goals are mutually exclusive,[4] but greatly improved resolution may be achieved in the future through the technique of flow karyotyping.

Given the fact that aneuploidy has been shown to be a marker of poor prognosis for a variety of tumours, it might be hoped that DNA flow cytometry would provide an objective guide to prognosis in patients with large bowel cancer, but published data have yielded conflicting findings. Some reports describe an association between aneuploidy and advancing stage[5–7]; others find no such association.[2,8–13] Survival curves for near-diploid and aneuploid large bowel cancers have been remarkably similar in different series.[3,9,10,12] A notable exception is a study describing a five year survival of 100% for diploid cancers.[6] On the other hand, major differences emerge when the magnitude of the independent contribution of DNA distribution to survival is calculated. Some studies find ploidy to have little independent prognostic importance in the presence of other variables[3,9]; others consider ploidy to be as important or even more important than Dukes stage.[6,7]

If ploidy is of prognostic importance then it is difficult to understand how it could be totally unrelated to stage. It should be appreciated, however, that the Dukes classification provides only a limited guide to prognosis.[14] The ABC system of Dukes supplies some information on the extent of spread within a surgical specimen, and it is conceivable that other variables might add further, independent prognostic information. This could be resolved by studying the distribution of DNA content in a large and well documented series of large bowel cancers.

It has been suggested that the main factor that determines outcome in patients undergoing "curative" surgery for large bowel cancer is the presence or absence of occult hepatic metastases.[15] If this were true then prediction of the existence of occult hepatic metastases and prediction of long term cancer-related death would be essentially similar exercises. A new prognostic classification seems to be better than other systems at predicting death due to cancer (and presumably occult hepatic metastases) in patients undergoing "curative" surgery for rectal cancer.[16] This classification identifies 30% of patients with an

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excellent prognosis (group I), 30% with a good prognosis (group II), 20% with a fair prognosis (group III), and 20% with a poor prognosis (group IV). If aneuploidy were associated with an adverse outcome one would expect to find significant differences in the distribution of DNA content according to prognostic grouping. Analysis of the discrete variables that are used to derive the prognostic classification would determine which, if any, predicted aneuploidy. Finally, the predictive value of DNA analysis might be increased by removing cases having either a poor or a very good prognosis. Thus once cancers have spread to distant sites degree of ploidy becomes redundant.[17] Conversely, cases with no positive lymph nodes and little or no direct extension beyond the bowel wall will have an excellent prognosis, regardless of degree of ploidy.

Material and methods

Most of the material used in this study was derived from a consecutive series of 331 patients undergoing "curative" surgery for rectal cancer and used previously to develop a new prognostic classification (table 1).[16] One hundred and fifty patients formed the basis of an earlier report on DNA flow cytometry.[3] Fifty six of these were retested as a preliminary exercise to assess the reproducibility of the procedure. Only 299 of the 331 cases used to derive the new prognostic grouping could be studied because tissues from the remainder had been fixed in a mercury based fixative and gave unacceptable results. We wished to consider a particular prognostic group in isolation (group III) and therefore enlarged the number of group III cases to a total of 137 by adding 70 further cases from a second data set used to test the new prognostic classification.[16] All specimens used in this study (a final total of 369) were subjected to meticulous pathological examination and patients were followed up for at least 10 years or until death. Details of methods used in histopathological reporting have been published previously.[18]

A single formalin fixed, paraffin wax embedded block was selected from each specimen, ensuring that this included the deepest point of direct spread in continuity. A suspension of single nuclei was prepared from a 50 μm section, using the method pioneered by Hedley.[19] An adjacent 4 μm section was stained with haematoxylin and eosin and examined to confirm the presence of tumour and ensure that the type and grade accorded with the pathological records. The nuclei were stained with propidium iodide. Flow cytometry was performed with a FACS I cell sorter using an argon ion laser tuned to the 488 nm line at a light power of 200 mW. Fluorescence was measured using a 610 nm long-pass filter and at least 20 000 nuclei were analysed for each case. Aneuploidy was defined as the presence of a population of nuclei giving a profile distinct from the diploid pattern (figs 1a and b). A peak with a DNA index of 2·00 and comprising at least 10% of the nuclear population was defined as tetraploid. Because one study has stated that diploidy confers a highly favourable prognosis,[7] we elected to preserve

Table 1 *Derivation of new prognostic classification*

Prognostic groups derived from total score for each patient:	
Total score	*Prognostic group*
0–1	I
2	II
3	III
4–5	IV

Number of positive lymph nodes: 0 = 0, 1–4 = 1, 5+ = 2.
Spread: within wall = 0, beyond wall = 1.
Character of invasive margin: expanding = 0, infiltrating = 1.
Peritumoural lymphocytic infiltration: conspicuous = 0, other = 1.

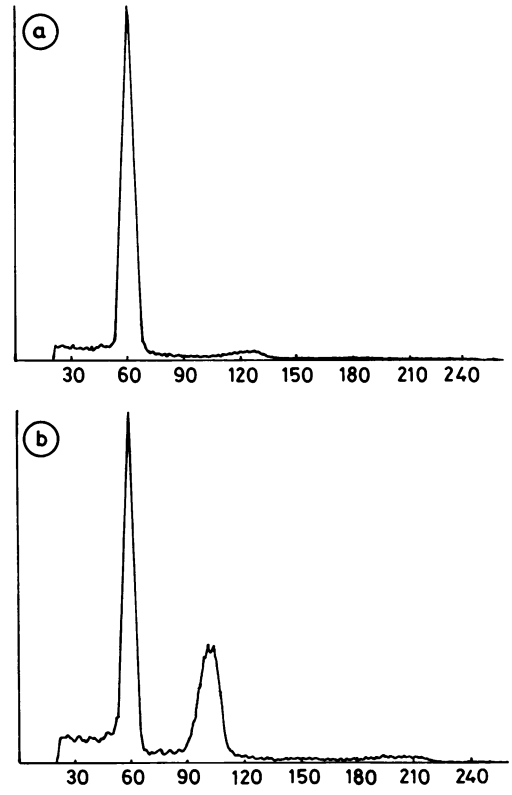

Fig 1 *Diploid (a) and aneuploid (b) DNA histograms. Vertical axis measures number of nuclei and horizontal axis channel number or fluorescence intensity. There is an aneuploid G1/0 peak at channel number 100 (b).*

Table 2 *Relation between discrete pathological variables and ploidy*

Variable and grade	*Diploid*	*Aneuploid/ tetraploid*	χ^2	*df*	*p value*
Nodal state:					
0	79	112			
1–4	17	121	34·9	2	<0·001
5+	8	32			
Invasive margin:					
Expanding	84	138			
Intermediate	5	49	26·5	2	<0·001
Infiltrating	15	78			
Lymphocytic infiltration:					
Conspicuous	41	41			
Intermediate	30	101	24·9	2	<0·001
Little/none	33	123			
Direct spread:					
None	25	24			
Slight	64	163	19·3	2	<0·001
Extensive	5	78			
Differentiation					
Well	24	37			
Moderate	68	196	4·5	2	NS
Poor	12	32			
Extramural venous spread:					
No	82	205	0·03	1	NS
Yes	22	60			

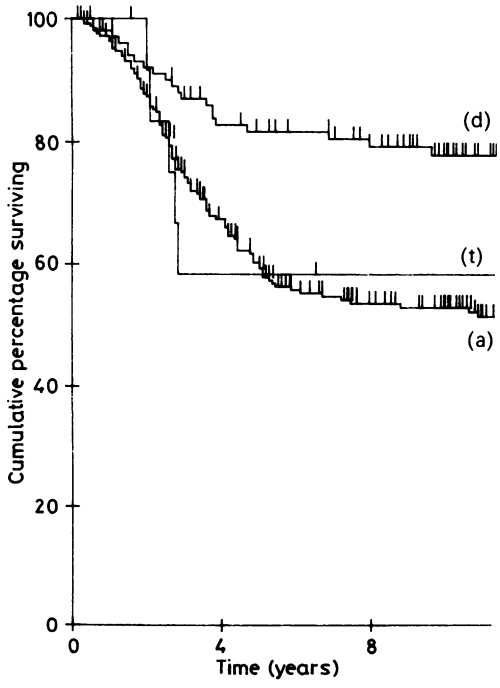

Fig 2 *Kaplan-Meier survival curves for diploid (d), aneuploid (a) and tetraploid (t) rectal cancers.*

Table 3 *DNA distribution by new prognostic groups*

	I	*II*	*III*	*IV*
	No (%)	*No (%)*	*No (%)*	*No (%)*
Diploid	44 (55·0)	32 (36·4)	17 (12·4)	11 (17·2)
Aneuploid/tetraploid	36 (43·0)	56 (63·6)	121 (87·6)	53 (82·8)
Total	80	88	137	64

χ^2 3df = 52·0 ($p < 0·001$)
χ^2 for trend 1df = 51·4 ($p < 0·001$).

the predictive value of this group by labelling all doubtful cases as aneuploid. In measuring the DNA content of what is assumed to be a uniform population of nuclei the adequacy of performance is given by the coefficient of variation (quotient of the standard deviation and the mean value of measured distributions).[4] The mean coefficient of variation was 4·92%. Further details on the preparation of nuclear suspensions and flow cytometry are published elsewhere.[20]

DNA ploidy was added to the clinical and pathological database stored in a mainframe computer. Kaplan-Meier survival curves were constructed, deaths from causes other than cancer being treated as censored observations at the time of death. Patients dying within 30 days of surgery were excluded from the analysis. Thus the intended clinical end point was death due to cancer. The distribution of DNA content was determined in cases stratified by a new prognostic classification (table 1)[16] and by the more familiar Dukes system.[21] Cox regression (multivariate) analysis was undertaken using the BMDP2L program.[22] The prognostic contribution of DNA ploidy was examined in two models. Variables entered into the first model were discrete pathological observations shown previously to be of independent prognostic importance: number of positive lymph nodes (0, 1–4, 5+), penetration of bowel wall (no/yes), diffusely infiltrating invasive margin (no/yes) and conspicuous peritumoral lymphocytic infiltration (yes/no).[16] Because the data set was not identical with that analysed previously, the assumption that the independent prognostic variables would be the same as before was tested by submitting all variables with individual

Table 4 *DNA distribution by Dukes stage*

	A	*B*	*C1*	*C2*
	No (%)	*No (%)*	*No (%)*	*No (%)*
Diploid	24 (52·2)	55 (37·7)	19 (12·0)	6 (31·6)
Aneuploid/tetraploid	22 (47·8)	91 (62·3)	139 (88·0)	13 (68·4)
Total	46	146	158	19

χ^2 3df = 40·0 ($p < 0·001$).

Table 5 *Summary of univariate survival analysis*

Variable	*df*	*Logrank* χ^2	*p value*
No of positive nodes (0, 1–4, 5+)	2	93·7	<0·001
Dukes stage (A, B, C1, C2)	3	82·6	<0·001
Invasive margin (expanding, intermediate, infiltrating)	2	75·5	<0·001
Differentiation (well, moderate, poor)	2	63·2	<0·001
Lymphocytic infiltration (conspicuous, intermediate, little/none)	2	52·9	<0·001
Direct spread (none, slight, extensive)	2	42·2	<0·001
DNA ploidy (diploid, aneuploid, tetraploid)	2	17·6	<0·001
Extramural venous invasion (absent/present)	1	6·0	0·02
Type (adenocarcinoma, mucinous carcinoma, signet ring cell)	2	0·3	NS
Sex	1	0·4	NS

Table 7 *Cox regression model for Dukes stage, differentiation, and ploidy*

Variable with coding	*Coefficient*	*Standard error*	*Likelihood ratio* χ^2	*df*	*p value*
Dukes 1	2·34	1·02			
Dukes 2	3·09	1·01	51·8	3	<0·001
Dukes 3	4·06	1·03			
Differentiation 1	1·31	0·34			
Differentiation 2	0·97	0·21	21·8	2	<0·001
Ploidy	0·63	0·25	7·0	1	0·008

Dukes 1–1 = Dukes A, 2 = Dukes B.
Dukes 2–1 = Dukes A, 2 = Dukes C1.
Dukes 3–1 = Dukes A, 2 = Dukes C2.
Differentiation 1–1 = well, 2 = poor.
Differentiation 2–1 = moderate, 2 = poor.
Ploidy–1 = diploid, 2 = aneuploid/tetraploid.

prognostic significance to Cox regression analysis. A second model based on Dukes stage and grade of differentiation was also constructed.

Survival analysis of prognostic group III cases alone was undertaken. Patients in this group can not be given a confident prediction of likely outcome and for this reason the possibility that other, hitherto uninvestigated variables might be of importance was studied. These included size of tumour (<3 cm, 3–5 cm, >5 cm), appearance of tumour (protuberant, ulcerating/circumscribed, and ulcerating/diffuse), rectal subsite (lower middle, upper, rectosigmoid), and extent of disease in bowel circumference (<2 quadrants, 2–3 quadrants, circumferential).

Results

Five of the 56 retests gave different results: three previously aneuploid became diploid and two previously diploid became aneuploid. It was assumed that these cancers were heterogeneous with an aneuploid subpopulation and all five were regarded as aneuploid for the purposes of this study.

Of the 369 patients studied, 136 were recorded as dying of rectal cancer. There were 104 (28%) diploid, 252 (68%) aneuploid, and 13 (3·5%) tetraploid cancers. Associations were found between ploidy and other discrete pathological variables: nodal state, direct spread, character of invasive margin and lymphocytic infiltration (table 2). In a logistic regression analysis, however, only nodal state, invasive margin, and lymphocytic infiltration had independent relations with ploidy (data not shown). Differentiation and venous invasion were not related to ploidy (table 2). In univariate survival analysis ploidy was a highly significant prognostic variable (logrank = 17·6; $p < 0·001$) (fig 2). Five year survival for diploid and aneuploid cases was 81·6% and 59·8%, respectively. Distribution of DNA ploidy for cases stratified by a new prognostic classification (table 3) and by the Dukes classification (table 4) was skewed with diploid cancers being more common in prognostic group I and Dukes A cases. Because there were few tetraploid cancers and these were similar to aneuploid cases in terms of survival, aneuploid and tetraploid cancers are grouped together in these and most of the following analyses.

Although the association between ploidy and survival is significant, its logrank value was considerably lower than that of other variables (table 5). Furthermore, Cox regression analysis showed that the survival advantage conferred by ploidy lacked independence in a prognostic model containing four discrete prognostic variables (table 6) and retained

Table 6 *Effect of adding ploidy to Cox regression model into which lymph node invasion, direct spread, invasive margin and lymphocytic infiltrate have been forced*

Variable with coding	*Coefficient*	*Standard error*	*Likelihood ratio* χ^2	*df*	*p value*
Positive lymph nodes (1 = none, 2 = 1–4, 3 = 4+)	0·89	0·12	50·2	2	<0·0001
Invasive margin (1 = other, 2 = infiltrating)	0·92	0·18	24·9	1	<0·0001
Lymphocytes (1 = conspicuous, 2 = other)	0·90	0·34	8·6	1	0·004
Spread (1 = within wall, 2 = beyond wall)	2·11	1·01	9·1	1	0·003
Ploidy (1 = diploid, 2 = aneuploid + tetraploid)	0·24	0·24	1·1	1	0·3 (NS)

When all variables with individual prognostic importance (table 5) were entered into a Cox regression model without forcing in selected variables the first four variables shown above were in the final model.

Table 8 *Logrank test for ploidy and other pathological variables in 137 prognostic group III cases*

Variable	*df*	*Logrank* χ^2	*p value*
Ploidy (diploid, aneuploid tetraploid)	2	1·86	NS
Size (< 3, 3–5, > 5 cm)	2	1·51	NS
Gross appearance (protuberant, ulcerating/circumscribed, diffuse)	2	1·53	NS
Circumferent involvement (< 2, 2–3 quadrants, circumferential)	2	0·24	NS
Subsite (lower. middle, upper rectum, rectosigmoid)	3	1·57	NS

Multivariate analysis confirmed that no variable was related to survival.

only a relatively small independent effect in the presence of Dukes stage and differentiation alone (table 7).

Neither ploidy nor any additional variable could be shown to influence survival in prognostic group III patients (table 8).

Discussion

This study succeeds in explaining certain reported inconsistencies regarding the role of flow cytometry in the assessment of large bowel cancer. The distribution of diploid and aneuploid cancers and their Kaplan-Meier survival curves do not differ appreciably from reports by others.[2 10 12] Most authors, however, failed to show an association between DNA ploidy and Dukes stage,[2 8–13] whereas DNA content has been reported to provide independent prognostic information.[6 7 10–12] Our data contradict both of these conclusions. Thus the distribution of DNA content is related to Dukes stage (table 4) and to a new system of prognostic grouping (table 3) and confers no independent prognostic information in a model based on four discrete pathological variables (table 6). A small and clinically unimportant independent effect is seen in the presence of Dukes stage and differentiation alone (table 7). Thus the relatively small regression coefficient for ploidy limits the size of its independent contribution in this model. Furthermore, the exclusion of other independent prognostic variables, on the grounds that they are not as familiar as Dukes stage and differentiation, can not be justified. Although the assessment of lymphocytic infiltration and invasive margin is subjective, the exercise is simple and acceptable levels of intraobserver[18] and interobserver[23] agreement have been recorded. Failure by others to show an association between ploidy and stage may be explained by the small size of most series, the confounding effects introduced when pathological data may not have been collected with meticulous care, and the relatively weak prognostic effect of ploidy.

We considered that any small, independent contribution by ploidy might be increased by removing cases in which clinical outcome can be predicted with a high level of confidence. Thus survival analysis by ploidy was studied in prognostic group III cases alone (table 8). The fact that this did not turn out to be the case is not especially surprising as an independent effect by ploidy would have been detected by Cox regression analysis even if this had applied only to a particular subgroup. It remains to be explained, however, why ploidy should influence prognosis in univariate analysis of the entire series. The answer to this question lies in the pronounced weighting of diploid cancers in prognostic group I. Diploid tumours may be heterogeneous and include a group with a near-normal chromosomal constitution and a low biological aggressiveness—that is, a limited capacity for direct, lymphatic, and distant spread. Such a subgroup would be expected to be over-represented within prognostic group I.

In a new system of prognostic classification group III is the least satisfactory because clinical outcome cannot be predicted with any measure of confidence. It is noteworthy that further substratification of this group could not be achieved by four additional variables (gross appearance, size, circumferential spread and rectal subsite) as well as by ploidy (table 8).

Claims that DNA flow cytometry might be of value in prognosis and indeed in patient management must be viewed with caution. While the technique provides prognostic information in univariate analysis and might be expected to contribute to the asssessment of biopsy material, its value for the patient is limited. Thus flow DNA cytometry does not assist in the identification of patients with an excellent prognosis or with a poor prognosis. Given the regression coefficient of 0·24 (table 6), the risk of cancer-related death in diploid patients compared with aneuploid/tetraploid patients is 79%. Not only is the survival difference for individual patients very small, but the 95% confidence interval is sufficiently wide (49%–126%) to allow for an improved prognosis in aneuploid cases. The existence of tumour heterogeneity in relation to DNA content is well documented.[24 25] In our series of 56 retests five gave differing results. The existence of heterogeneity limits the value of DNA flow cytometry further. One study describes a 100% five year survival for diploid colorectal cancer and regards DNA flow cytometry as superior to Dukes staging.[6] This conclusion, however, was based on a small and highly selected series that included only three diploid Dukes C cases. We suggest that the differing estimates of the magnitude of the independent effect of DNA content on survival may reflect the expected variation between small series and different standards of pathological reporting.

In conclusion, measurement of DNA content within

tissue samples from a surgically removed rectal cancer confers no independent prognostic information and its use for this purpose can not be justified. As ploidy is an important prognostic determinant when considered in isolation, however, the technique could have a role in the interpretation of forceps biopsy material, though this requires further critical study. Other areas in which flow cytometry may retain a role are in the study of tumour heterogeneity and progression. Here the improved resolution offered by flow karyotyping holds promise for the future.[26]

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