DNA densitometry of colorectal cancer

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Abstract

DNA analysis was assessed by densitometry for 281 cases of colorectal adenocarcinoma. Detection of aneuploidy in a single case rose from 65% if one, to 92.5% when three or more sections, were analysed. Although aneuploid tumours had significantly larger nuclear areas than near diploid tumours ($p=0.009$), densitometric measurements showed no association with clinicopathological variables. DNA content determined by densitometry was compared with that from flow cytometry on 465 tissue sections from 241 cases. Aneuploidy assessed by flow cytometry was significantly associated with that determined by densitometry ($p<0.01$ for all comparisons), ploidy state being similar in 381 sections (82%, $\kappa=0.63$, $p<0.001$), and 187 cases (77.6%, $\kappa=0.57$, $p<0.001$). Univariate survival analysis showed that DNA densitometric variables had no significant association with survival in (a) all cases, (b) cases without lymph node metastases, or (c) cases without distant metastases. Multivariate regression analysis of densitometric and clinicopathological variables identified Dukes's stage, patient age, and tumour differentiation as the combination of variables most closely related to survival. Densitometric measurement of DNA content could not significantly improve on the prognostic model containing these three variables. It is concluded that, although the assessment of DNA content by densitometry is comparable with that of flow cytometry, conventional histological variables remain the best predictors of prognosis in colorectal cancer.

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prognostic value of tumour DNA content in colorectal cancer are conflicting, some reports implying that it is of major prognostic importance,⁸⁻¹³ others that it is of little value relative to Dukes's stage.¹⁴⁻¹⁹

In contrast with flow cytometry, DNA densitometry, determining the DNA content of small numbers of tumour cells from stained microscope sections, is potentially more specific. DNA content assessed by this technique is claimed to be related to survival in several tumours,²⁰⁻²⁵ including colorectal cancer.²⁶ The prognostic value of densitometry in colorectal cancer, however, requires verification in a larger series of cases. A study was therefore performed to establish the role of nuclear DNA content, as determined by DNA densitometry, compared with currently accepted prognostic criteria in colorectal adenocarcinoma.

Materials and methods

PATIENTS, CLINICOPATHOLOGICAL, AND FLOW CYTOMETRIC DATA

All available paraffin wax embedded histological material from 312 patients having surgical resection of colorectal adenocarcinoma at two teaching hospitals between January 1974 and December 1983 was studied. All material was reviewed by a single pathologist for Dukes's stage² and the histopathological variables comprising Jass' classification.³ Clinical information, including patient age, sex, tumour site, symptom duration, liver function tests, and intestinal obstruction was also determined. Flow cytometry, allowing the assessment of the ploidy state and proportion of cells in each phase of the cell cycle, was performed on paraffin wax embedded material from each case²⁷ (Table I).

DNA DENSITOMETRIC STUDY

Five micron sections were cut from all paraffin wax embedded material and stained using the Feulgen reaction in a method that has been found to be reliable.²⁸⁻³¹ Integrated optical density (IOD), which reflects Feulgen staining of a nucleus relative to its size, was used to measure nuclear DNA content.

The Feulgen stained sections were examined under oil with an Olympus microscope and the image transmitted by a high resolution CCD camera to a VIDAS image analyser (Kontron). A minimum of 25 lymphocyte nuclei were traced per section, using a stylus pen and graphic tablet. After correction for background absorption, the mean IOD of the lymphocytes was taken as the value for the near diploid population of the section and used to set the 2c value on the DNA content scale.

Current prognostic criteria for colorectal cancer are not entirely satisfactory; the TNM classification¹ is complex, modifications of Dukes's classification² have led to confusion, while Jass' classification³ is poorly reproducible.⁴ Combining these with assessments of tumour behaviour, such as DNA content,⁵ might result in a better prediction of prognosis. Generally, two techniques have been developed for the cytological measurement of nuclear DNA content. Early reports measuring DNA content from section preparations using light microscopy (densitometry) proved promising,⁶ but cumbersome.⁷ Emphasis therefore shifted to flow cytometry, in which large numbers of cells can be rapidly analysed for their nuclear DNA content. Flow cytometry, however, may fail to detect small numbers of aneuploid tumour cells, containing abnormal amounts of DNA, if most of the specimen contains cells with normal (near diploid) DNA content. Consequently, flow cytometric studies assessing the independent

TABLE I DNA densitometric, flow cytometric, and clinicopathological variables assessed

Densitometric	
2c deviation index	(absolute value)
5c exceeding rate	(%)
Mean c value	(absolute value)
Histogram type	I, II, III, IV
Nuclear area	(microns ²)
Flow cytometric	
Percentage of cells in the phases of the cell cycle (G ₀ /G ₁ , S, G ₂ /M)	
Proliferative index	(sum of the S and G ₂ /M phases)
Ploidy state	(Diploid v non-diploid) (Diploid v aneuploid v tetraploid)
Pathological	
Dukes's stage	A, B, C, D
Tumour type	tubular, papillary, mucinous
Differentiation	well, moderate, poor
Tubule configuration	simple, complex, irregular, none
Fibrosis	little, moderate, marked
Growth pattern	expanding, infiltrating
Nuclear polarity	well, moderately, poorly preserved
Lymphocytic infiltration	little, moderate, marked
Clinical	
Age	
Sex	
Site	right, left, sigmoid, rectum
Symptom duration	0–3, 3–6, 6–12, >12 months
Liver function tests	normal, abnormal
Intestinal obstruction	present, absent

The IOD of a minimum of 50 tumour nuclei was then determined for each section, corrected according to the lymphocyte standards, and plotted as a frequency histogram.

DNA DENSITOMETRY VARIABLES

The histogram of c value distribution for each section was classified according to established criteria²⁹: type I: only a small number of cells deviate more than a few per cent from the 2c (near diploid) value; type II: two well defined peaks around the 2c and 4c regions or a distinct modal value in the near tetraploid (4c) region; type III: peaks around the 2c and 4c regions with a sizeable number of cells with c values similar to those of lymphocytes in DNA synthesis; type IV: very irregular DNA aneuploidy, with DNA amounts ranging from 2c to beyond 6c.

Near diploid cases were taken as those in which all sections showed type I histograms. Cases showing type II histograms were classified as tetraploid, those with type III or IV histograms as aneuploid.

By convention, DNA aneuploidy may also be expressed as the proportion of cells with values above 5c (the 5c exceeding rate). Similarly, the more abnormal the DNA content, the greater the number of cells that deviate from the 2c value (– that is, the greater the 2c deviation index). In addition to these assessments of DNA content, mean nuclear area was derived for each section (Table I). All analyses were performed for both the mean and maximum values of each variable per case.

In accordance with the clonal concept of tumour development, the ploidy state of the entire tumour was taken as that of the most aneuploid specimen comprising that tumour.

PILOT STUDIES

Nuclei sample size – Two sections were randomly chosen from each of 20 randomly selected cases and 50 tumour nuclei measured from each of three randomly selected fields per section.

Analysis of variance for a nested design determined the components of variance from nucleus to nucleus within a field, from field to field within a section, and from section to section within a subject. The numbers of nuclei, fields, and sections that gave a subject mean with the greatest precision (– that is, the lowest standard error) were then calculated to give the number of nuclei which should be measured per section.³²

Well and poorly differentiated tissue – A study was performed to determine if DNA densitometry differs significantly between well and poorly differentiated colorectal tumour mucosa. Twenty five cases in which three independent observers identified the tumour as being uniformly well differentiated were randomly selected and compared with a similar number of uniformly poorly differentiated tumours, using Student's paired samples *t* and the χ^2 tests.

DNA DENSITOMETRIC AND CLINICOPATHOLOGICAL VARIABLES

The relation between densitometric and the clinicopathological variables was investigated using Pearson correlation coefficients and Student's independent samples *t* test. To reduce the risk of chance agreement in the large number of analyses involved (type I error), significance was taken at the 1% value.

DNA DENSITOMETRIC AND FLOW CYTOMETRIC VARIABLES

Ploidy state obtained by flow cytometry (near diploid, tetraploid, aneuploid) was compared with that from densitometry (histogram types I–IV) for each section and for each case using the χ^2 test and Cohen's κ statistic.³³ Comparison of flow cytometric ploidy state with the other densitometric variables was determined by the Student's *t* test. The relation between each of the densitometric and the other flow cytometric variables was investigated using Spearman correlation coefficients.

SURVIVAL ANALYSIS

Univariate – The relation between each of the densitometric variables and survival was assessed by the log rank test. Analysis was initially performed for all deaths, irrespective of cause, as the causes of death recorded at certification may be inaccurate. The analysis was then repeated for deaths where the underlying cause was registered as colorectal cancer at death certification. Including patients with metastases might obscure a significant relation with survival in better prognosis cases and therefore the relation of densitometric variables to survival was repeated for cases (a) without nodal metastases (Dukes's stages A and B) and (b) for cases without distant metastases (Dukes's stages A, B, and C combined). Significance was taken at the 1% value, to reduce the risk of type I errors. Before analysis, continuous variables were categorised into three equal sized groups using tertiles and DNA histogram type analysed for individual types and their combinations.

Multivariate – To determine if DNA densi-

tometry is of a prognostic value independent from known prognostic variables such as Dukes's stage, the densitometric and clinicopathological variables were entered into a Cox's proportional hazards regression analysis. This allowed derivation of hazard ratios for categorical variables and for a 1% increase in continuous variables. The likelihood ratio test statistics resulting from the proportional hazards analysis³⁴ were referred to the χ^2 distribution, to determine the combination of variables that best predicted prognosis in our dataset. Analysis was performed both for all deaths and for colorectal cancer deaths.

Results

PILOT STUDIES

Nuclei sample size – Densitometric DNA analysis varied little when more than 10 nuclei per field, or four fields per section were measured. A total of 50 tumour nuclei, 10 cells from each of five randomly selected fields, was therefore chosen as representative of each section in this study.

Well and poorly differentiated tissue – There was no significant difference in any densitometric variable between well and poorly differentiated colorectal mucosa.

DNA DENSITOMETRIC STUDY

Clinicopathological data were obtained for all 312 patients. Suitable histological material was available for DNA densitometry on 281 of these (86.8%), while flow cytometry was successful on 275. The mean number of sections analysed by densitometry was 2.02 per case, range one to six. The proportion of DNA aneuploid cases rose from 65% if one section was analysed to 86.3% if two sections, and 92.5% when three or more sections, were assessed per case.

Forty nine patients (17.4%) showed type I histograms in all samples, the numbers of cases classified as type II, III, and IV histograms being 80 (28.5%), 85 (30.3%), and 67 (23.8%), respectively. The proportion of cases classified as near diploid, tetraploid, and aneuploid by flow cytometry were 39.4%, 13.3%, and 47.3%, respectively.

DNA DENSITOMETRIC AND CLINICOPATHOLOGICAL VARIABLES

There was no significant association between DNA densitometric and any clinicopathological variable.

DNA DENSITOMETRIC AND FLOW CYTOMETRIC VARIABLES

Direct comparison of densitometry and flow cytometry was possible on 465 sections and 241 cases. The coefficient of variation of the mid point of the G_0/G_1 peak (of all the flow cytometry specimens was 7.3%. Ploidy state agreed between the two techniques in 381 sections ($\chi^2=177.04$, $df=1$, $p<0.001$) and 187 cases ($\chi^2=54.7$, $df=1$, $p<0.001$). The corresponding κ values were 0.63 and 0.57,

TABLE II Association of flow cytometric ploidy state with densitometric variables

	Mean value	95% Confidence limits	p Value
Nuclear area (mean, microns ²)			
Diploid	35.6	34.4 to 36.8	0.009
Tetraploid	35.9	32.8 to 39.0	
Aneuploid	38.3	37.1 to 39.5	
2c deviation index (absolute value)			
Diploid	1.59	1.39 to 1.79	0.002
Tetraploid	1.66	1.22 to 2.09	
Aneuploid	2.13	1.91 to 2.36	
5c exceeding rate (percentage)			
Diploid	10.2	7.3 to 13.1	0.016
Tetraploid	12.1	6.2 to 17.9	
Aneuploid	16.6	13.3 to 19.9	
Mean c value (absolute value)			
Diploid	2.96	2.77 to 3.16	0.008
Tetraploid	2.99	2.56 to 3.41	
Aneuploid	3.41	3.20 to 3.62	

representing substantial and moderate levels of agreement, respectively.³⁵ Progression from diploid to aneuploid tumours assessed by flow cytometry was significantly associated with increasing values of nuclear area, its standard deviation, 2c deviation index, 5c exceeding rate, and mean c value (Table II). Both 2c deviation index and 5c exceeding rate were positively correlated to increasing proportions of cells in the G_2/M phase of the cell cycle ($p<0.005$ and $p<0.001$, respectively).

SURVIVAL ANALYSIS

Univariate – Analysis for all causes of death and colorectal cancer deaths produced similar results. Neither the maximum nor mean values of the DNA densitometric variables, nor any combination of histogram types, was significantly related to survival (Table III). Similarly, no densitometric variable was significantly related to survival for cases without nodal or without distant metastases.

TABLE III Number of deaths, hazard ratio, and significance values obtained from survival analysis (tertiles) of all causes of death (maximum value per case)

	Observed deaths	Expected deaths	Hazard ratio	p Value
2c deviation index (absolute value)				
<1.15	57	58.3	1	0.97
1.15–1.97	60	58.8	1.04	
>1.98	57	56.7	1.02	
5c exceeding rate (percentage)				
<3.9	58	58.9	1	0.91
4.0–12.5	57	58.6	1.01	
>12.5	59	56.5	1.01	
Mean c value (absolute value)				
<2.6	61	58.7	1	0.87
2.6–3.3	55	57.9	1.09	
>3.3	58	57.4	1.03	
Nuclear area (mean, microns ²)				
<33.6%	58	56.9	1	0.53
33.7–38.9	53	59.1	1.14	
>39.0	63	57.9	1.06	
Ploidy (histogram type)				
I	24	25.2	1	0.18
II	35	37.1	1.02	
III	43	42.1	1.07	
IV	34	31.8	1.11	

TABLE IV Hazard ratios obtained from multivariate survival analysis corrected for age and Dukes's stage. The significant pathological variables are shown for comparison

	Hazard ratio	95% Confidence limits	p Value
DNA Densitometry			
2c	0.99	0.78 to 1.30	0.75
5c	1.05	0.85 to 1.12	0.62
Mean c	1.02	0.96 to 1.04	0.81
Nuclear area	1.00	0.98 to 1.02	0.75
Ploidy (histogram type)			
I v II+III+IV	1.16	0.60 to 1.34	0.58
I+II v III+IV	1.09	0.65 to 1.29	0.67
I+II+III v IV	1.03	0.59 to 1.24	0.75
Pathology			
Differentiation			
Well v poor	0.51	0.34 to 0.80	0.003
Moderate v poor	0.49	0.30 to 0.81	
Polarity			
Well v poorly preserved	0.62	0.44 to 0.89	0.005
Moderate v poorly preserved	0.78	0.65 to 0.91	
Configuration			
Complex v irregular/none	0.50	0.33 to 0.73	0.003
Simple v irregular/none	0.67	0.46 to 0.98	
Infiltration			
Marked v little	0.69	0.45 to 1.07	0.048
Moderate v little	0.70	0.55 to 0.86	

Multivariate – The results derived from analysis of all deaths were again similar to those of colorectal cancer deaths. Cox's multivariate regression analysis identified increasing patient age and Dukes's stage as the clinicopathological variables most significantly related to poor survival. Some pathological variables, but no densitometric variables, were independently related to survival after age and Dukes's stage were taken in account (Table IV). When included in continuous (uncategorised) form, no densitometric variable could add significantly to a model consisting of terms for age, Dukes's stage, and tumour differentiation, the combination of variables which was found to be best at predicting survival in colorectal cancer in our dataset.

Discussion

Although early densitometric reports claimed that aneuploidy may be detected from as few as three cells,⁶ this study agrees with most recent reports in showing that assessing 50 nuclei permits a satisfactory representation of the DNA content of the section as a whole. Reports on colorectal tissue using interactive techniques similar to this study have measured 50 or 100 cells,^{36–38} while reports using automatic machines have assessed 200 to 1000 cells per case.^{26 39–42} This suggests that, compared with flow cytometry, densitometry is comparatively more efficient at detecting DNA abnormalities.

Studies comparing the two techniques support this conclusion. The significant association in the assessment of ploidy between densitometry and flow cytometry noted in this study has been previously recognised,^{21 43–47} with correlation coefficients of 0.83 being reported.⁴⁶ It seems that DNA densitometry, however, may detect abnormal DNA content more readily than flow cytometry. Bauer *et al* noted excellent correlation between the two techniques in 92 human solid tumours, although in nine cases, an aneuploid population was detected by DNA

image, but not by flow cytometric analysis.⁴⁸ A comparison of 31 colorectal carcinomas by flow and image cytometry found that, whereas 70% of the cases were non-diploid by image analysis, only 56% were similarly classified by flow cytometry.³⁷ Comparable results from this study were 82.6% and 60.7%, respectively.

Variation of DNA content within the same specimen (DNA heterogeneity) is well recognised in flow cytometry and has been noted in densitometric reports.^{6 49} Bocking *et al* recently reported an 81% agreement in the 2c deviation index between different sections from the same tumour,⁵⁰ while it is recognised that by densitometry, 40% of colorectal cancers are homogeneously and 60% heterogeneously aneuploid.³⁸ In our study aneuploidy increased progressively with the number of sections analysed, reaching 92.5% when three or more sections were assessed per case. This emphasises the utmost importance in studies of this type that sufficient samples be analysed to improve the detection of DNA aneuploidy.

Flow cytometric reports comparing well and poorly differentiated tissue have produced conflicting results, primarily because they related the overall worst grade of the entire specimen to the worst ploidy state of the entire specimen. In contrast, this study, although small, directly compared tumour nuclei in areas of specifically identified well or poorly differentiated tissue and should more accurately reflect the relation between ploidy and histological grade. It suggests that there is no relation between ploidy state and histological grade in colorectal cancer. As histological grade is considered to be of prognostic value, this finding may partially explain the failure of DNA ploidy to be related to survival in this study.

It might be anticipated that, because DNA aneuploidy represents increased amounts of nuclear DNA, aneuploid tumours might have larger nuclear areas. This study confirms such an assumption and suggests a strong relation between DNA ploidy and nuclear area⁵¹; the larger the nucleus the more likely it is to be aneuploid.

There is evidence that diploid cells may progress to tetraploidy and aneuploidy and that such aneuploid cells would have a biological advantage related to both genome and cell function.⁵² Some densitometric studies on colorectal cancer suggest that ploidy may be of prognostic value. One study suggested that the prognostic difference in rectal cancer between the races is contributed to by a significant difference in ploidy state.⁵³ Other reports, on small numbers of cases and without the use of survival analysis, have found diploid tumours to have a better prognosis than non-diploid tumours.^{37 49}

Albe *et al* recently assessed the prognostic value of DNA image cytometry on 211 cases of colorectal adenocarcinoma.²⁶ Univariate survival analysis suggested that DNA ploidy might be related to patient outcome. Multivariate survival analysis, however, showed that no ploidy category was significantly related to survival. The findings of this study are similar, in that none of the DNA densitometric variables, or categories of ploidy, were significantly related to

survival, either on univariate or multivariate survival analysis. This agrees with most large flow cytometric studies using multivariate survival analysis,¹⁶⁻¹⁹ including our own,⁵⁴ which found that flow cytometry was not of prognostic value in colorectal cancer. Therefore, both flow cytometric, and potentially more specific densitometric studies, suggest that DNA content is of little prognostic benefit in relation to currently accepted criteria in colorectal cancer.

It therefore seems unlikely that DNA analysis using densitometric or flow cytometric methods will play a significant prognostic part in the management of colorectal cancer patients, probably because they can only detect comparatively large changes in tumour DNA content. Densitometry may yet prove of benefit in the diagnosis of premalignant conditions,⁵⁵⁻⁵⁸ because its morphometric features may be used interactively to classify any given cell and therefore replace large numbers of flow cytometric parameters. The future assessment of DNA content will probably lie in gene probes, which can detect changes in chromosome structure more subtly than DNA densitometry.^{59,60} In the meantime, it seems that conventional histological variables remain the best predictors of prognosis in colorectal cancer.

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