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# Densitometric Evaluation of DNA Content in Colorectal Cancer

**Luigi Matturri, Bruna Biondo, Franco Uggeri and Anna Maria Lavezzi**

**The aim of the present study was to provide information on the DNA content in colorectal tumours using densitometric techniques on histological samples and correlating the findings with age, sex, histological grade, stage, presence or absence of lymph-node metastasis and survival time. The distribution of DNA values was significantly related with the histological grade, Dukes' stage and infiltration of the peritumoral lymph-nodes. The distribution of DNA values was not significantly correlated with age and sex. From the data obtained in this study it can be concluded that evaluation of DNA content in colorectal adenocarcinoma can be used as a prognostic test that is complementary to histological investigation. The ploidy can provide information for classifying the degree of malignancy and can also be used to determine which tumours are biologically most aggressive.**
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## INTRODUCTION

IN THE diagnostic and prognostic evaluation of tumours, in addition to the well-known parameters such as histological type, degree of differentiation and stage, biological factors such as cell kinetics, cytogenetics, DNA content and morphometry have recently become increasingly important.

These indicators are not only of diagnostic value but can also be correlated with clinical development of the disease and survival. In the last few years many studies, using a variety of methods, have been carried out on nuclear DNA content in different types of cancer such as carcinomas of the bladder, breast, liver, stomach, ovary and lung [1–7].

These studies have demonstrated the existence of a correlation between the degree of ploidy of tumour cells and the histological characteristics and clinical development of the disease.

Various researchers have subdivided the tumours into three or four subtypes according to the percentage of cells with different DNA content. In all classifications type I, with a DNA content superimposable on that of normal cells, corresponds to diploidy whereas the last type (type III or type IV), with a wide

distribution of DNA values, corresponds to a high level of aneuploidy and a poor prognosis.

Some investigators have found indications of a relationship between the ploidy level and the prognosis [8, 9]. If a relationship were to exist, the preoperative determination of the DNA content would be of value since knowledge of the ploidy level could influence decisions regarding adjuvant therapy [10].

The aim of the present study was to provide information on the DNA content in colorectal tumours using densitometric techniques on histological samples and correlating the findings with age, sex, histological grade, stage, presence or absence of lymph-node metastasis and survival time, with a view to evaluating the potential malignancy of these tumours.

## PATIENTS AND METHODS

*Selection of patients*

We examined prospectively 58 cases of primary untreated adenocarcinoma of the colon and/or rectum in 40 male and 18 female patients. Their mean age was 61 years (range 31–88).

Cases from 1985 and 1986 were selected to allow the determination of 4 year survival statistics. Cases were excluded due to incomplete clinical follow-up, insufficient tissue availability or inadequate data.

*Pathological classification*

The routinely processed tumour specimens were classified with regard to their degree of differentiation (well: 13 cases; moderately: 26 cases; and poorly differentiated: 19 cases) and according to Dukes' classification (stage A: 5 cases; stage B: 26 cases; and stage C: 27 cases).

The histological subdivision was as follows: 37 cases of tubular adenocarcinoma and 21 cases of tubule-papillary adenocarcinoma.

The tumour location was 32 cases from descending colon and 26 cases from rectum.

*Tissue preparation*

The specimens were fixed in buffered formalin 10% and embedded in paraffin. Sections from tumour samples of four micron thickness were stained with haematoxylin–eosin for histological examination and the method of Feulgen–Coleman was used for densitometric analysis of DNA. The Feulgen staining technique includes acid hydrolysis of DNA, which removes purine bases and thereby unmasks the aldehyde groups of deoxyribopentose molecules [11].

Decolourised Coleman–Feulgen solution was allowed to react with the aldehyde groups and was thereby converted into its coloured form and covalently bound to DNA. We used hydrolysis in 5 mol/l HCl, 22°C for 60 minutes and stained in Coleman–Feulgen solution for 1 h at room temperature.

*DNA determination*

Densitometric analysis was performed blind of the clinical and pathological parameters using a Zeiss–Kontron IBAS 2000 image analyser and a programme of semi-automatic cariometry applied. This programme was based on an interactive menu for the automatic valuation of grey levels.

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*Table 1. DNA content related to histological grade*

| Histological grade | | Type I | Type II | Type III |
|---|---|---|---|---|
| Well differentiated (G1) | ($n$ = 13) | 54% | 38% | 8% |
| Moderately differentiated (G2) | ($n$ = 26) | 19% | 46% | 35% |
| Poorly differentiated (G3) | ($n$ = 19) | 11% | 47% | 42% |

$\chi^2 = 9.766, P = 0.045$.

Measurements are performed by evaluating the light transmission per pixel and transforming these values into values representing the optical density of the nuclei, then integrating over the area of the particle (IOD = integrated optical density).

For each case 100 tumour cells from different areas were examined. The diploid values of reference (2C) was determined by evaluating the DNA content of 100 lymphocytes. The use of internal standards minimised the possibility of staining differences due to variations in fixation.

The histograms of ploidy distribution were subdivided into three types, according to the classification of Haraguchi *et al.* [4]: type I, over 90% of cells with DNA content under 4C (diploidy); type II, more than 10% of cells with DNA content over 4C and/or under 10% of cells with more than 6C (low aneuploidy); and type III, over 10% of cells with DNA content above 6C (high aneuploidy).

*Statistical analysis*

The types of DNA content observed were related to age, sex, histological grade, pathological stage and presence or absence of peritumoral lymph-node infiltration.

The relationships between the types of DNA content and other clinical and pathological parameters that have been useful in prognostication for colorectal adenocarcinoma were tested using the modified Student's $t$ test.

$P$ values $<0.05$ were considered statistically significant.

To evaluate the prognostic relevance of DNA content, stage and histological grade survival was computed from the time of surgery until death or date of last clinical observation. Univariate analyses were done with the Kaplan–Meier product-limit method and the logrank test was used to assess differences between subgroups.

The multivariate analysis was not assessed due to the small number of cases analysed prospectively. Instead, trend for further evaluation with a larger number of cases were identified.

## RESULTS

Of the 58 colorectal adenocarcinoma examined, 14 were of type 1, 26 of type II and 18 type III. DNA content did not appear to have any significant relation to age and sex of patients.

The significant relation ($P$ = 0.045) between DNA content and histological grade accounted for a prevalence of type 1 (54%) in a well differentiated subset. The moderately differentiated tumours were almost equally distributed among all three types and the poorly differentiated tumours belonged predominantly to types II (47%) and III (42%) (Table 1).

DNA content was also related to the peritumoral lymph-node involvement: cases without peritumoral positive lymph-node belonged predominantly to type II (52%), while those with lymph-node infiltration were prevalent in type III (52%) ($P$ = 0.004) (Table 2).

*Table 2. DNA content related to lymph-node metastasis*

| Lymph-node metastasis | Cases | Type I | Type II | Type III |
|---|---|---|---|---|
| No | 31 | 35% | 52% | 13% |
| Yes | 27 | 11% | 37% | 52% |

$\chi^2 = 11.289, P = 0.004$.

*Table 3. DNA content related to Dukes' stage*

| Dukes' stage | Cases | Type I | Type II | Type III |
|---|---|---|---|---|
| A | 5 | 60% | 40% | 0% |
| B | 26 | 31% | 54% | 15% |
| C | 27 | 11% | 37% | 52% |

$\chi^2 = 13.273, P = 0.010$.

A shifting in the prevalence of DNA content from type I to type II was observed in relation to tumour stage (Table 3). This correlation was statistically significant ($P = 0.01$); in fact the proportion of cases with DNA aneuploidy (type III) ranged from 0% in stage A to 52% in stage C.

In this series of patients, sex was not of prognostic relevance, while age was significantly related to survival at 4 years. ($\chi^2 = 27.883; P = 0.0001$).

The analysis of the histological grade showed only a poor relationship with the survival ($\chi^2 = 5.863$; $P = 0.0533$) (Fig. 1).

Moreover, Duke's stage appeared a significant indicator of survival ($\chi^2 = 40.912$; $P = 0.0001$). In fact, the probability at 4 years was 80%, 48% and 6% for stages A, B and C, respectively (Fig. 2).

Survival time was significantly affected by DNA content ($\chi^2 = 9.012$; $P = 0.011$): in fact the percentage of survivors decreased from type I (45%), to type II (36%) to type III (13%) (Fig. 3).

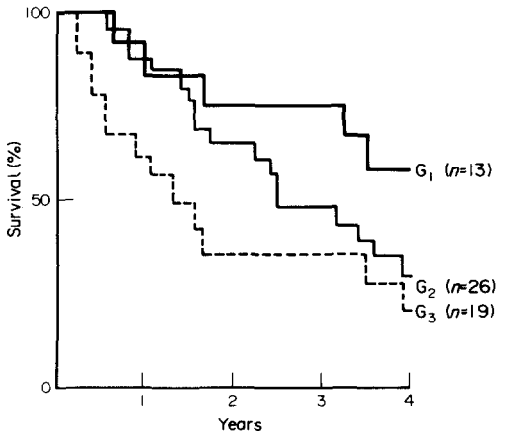


**Fig. 1. Survival related to histological grade. $\chi^2 = 5.863$, $P = 0.0533$.**

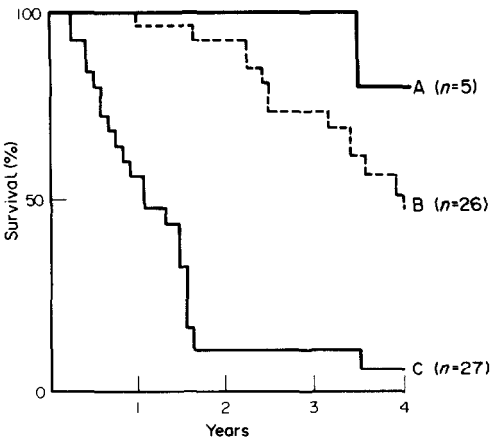


**Fig. 2. Survival related to Dukes' stage. $\chi^2 = 40.912$, $P = 0.0001$.**

## DISCUSSION

Studies carried out so far to determine the ploidy of tumour cells have mainly used flow cytometric techniques on isolated cells obtained from fresh solid tumour samples, or from paraffin embedded material following mechanical disaggregation and treatment with proteolytic enzymes.

Different studies [12, 13] have compared the DNA values provided by flow cytometry on fresh tissue with those ones from paraffin samples and have shown a good correspondence between the two approaches. This suggests that it may be possible to obtain information on the ploidy from archived specimens. It should, however, be pointed out that flow cytometry leads to a selection of the analysable nuclei and the neoplastic component may therefore be diluted, particularly if this is localised, with normal and inflammatory cells.

In this study in order to evaluate the DNA content of colorectal tumour cells we used static cytometry on histological sections stained by the Feulgen technique.

An advantage of this method, in comparison with flow cytometry, is that it can be applied directly on the material used for histological analysis, including biopsy samples; also it allows a precise correlation with the morphological features of the tumour.

The disadvantages of this technique, as compared with flow cytometry, are the small number of cells analysed, which, however, are still statistically representative of the sample, and the longer time and greater care required for each evaluation.

In the present study, the distribution of DNA values was

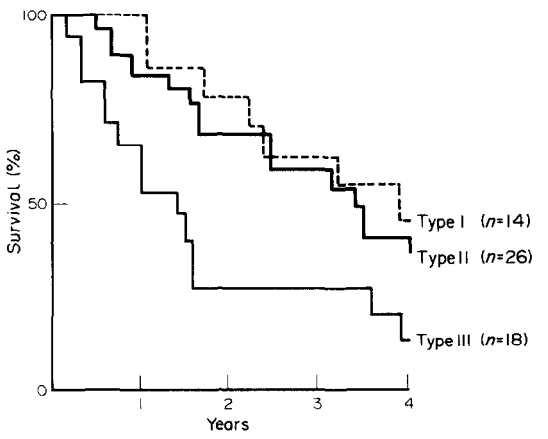


**Fig. 3. Survival related to DNA content. $\chi^2 = 9.012$, $P = 0.0011$.**

significantly related with the degree of histological differentiation and with the Dukes' stage, contrary to the findings of two studies on colorectal carcinomas by Goh and Hiddemann [14, 15].

Our results show that the evaluation of the ploidy can be used as prognostic test in addition to histological investigation. In fact the quantitative evaluation of DNA in colorectal adenocarcinoma can provide complementary information for classifying the degree of malignancy and can also be used to determine which tumours are biologically most aggressive.

A close association was also demonstrated between DNA content and infiltration of the peritumoral lymph-nodes [16]. In fact, type III was observed especially in those cases where metastasis was present while the cases where lymph-nodes were unaffected belonged with prevalence to types I and II.

Survival time was significantly related with the ploidy. In fact the survivors' percentage reduced from 45.45% in the cases of type I to 13.45% in the cases of type III, in agreement with previous studies [17–20]. The survival curves demonstrate that patients with tumours characterised as DNA type II constitute a group with prognosis comparable with those of diploid type (type I) (Fig. 1).

A multiple regression analysis would provide a precise assessment of the relative contribution of ploidy to prognosis also in the presence of other prognostic variables. However, we believe that the relatively low number of cases precludes a powerful definition of prognostic factors.

From the data obtained in this study it can be concluded that evaluation of DNA content in colorectal adenocarcinomas cannot be considered to be an absolute predictive test, but it is nevertheless an important parameter for indicating the degree of malignancy and it can be, particularly in association with other prognostic indicators such as cell kinetics, cytogenetics and morphometry, an improvement to define the biological behaviour of these tumours.

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