

# Retrospective Analysis of the Prognostic Significance of DNA Content and Proliferative Activity in Large Bowel Carcinoma

Bert Schutte, Michèle M. J. Reynders, Theodore Wiggers, Jan Willem Arends, Lex Volovics, Fred T. Bosman, and Geert H. Blijham

Departments of Internal Medicine, [B. S., M. M. J. R., G. H. B.], Pathology [J. W. A., F. T. B.], and Statistics [L. V.], University of Limburg, P. O. Box 616, 6200 MD Maastricht, and Department of Surgery, Dr. Daniel den Hoed Cancer Center, 3008 AE Rotterdam [T. W.], The Netherlands

## ABSTRACT

In the present study we have evaluated the prognostic significance of ploidy levels and proliferative activity in 279 cases of large bowel carcinomas which were included in a surgical prospective randomized trial. Ploidy levels and proliferative activity were determined on nuclei isolated from paraffin-embedded tissues of 279 colorectal carcinoma patients, with a mean follow-up of 51.9 months. Product limit survival analysis demonstrated a borderline significant association ( $P = 0.0689$  by generalized Breslow;  $P = 0.0336$  by generalized Savage) between ploidy and survival, with a 75th quantile survival of 49.8 months for patients with diploid tumors and 35.9 months for patients with aneuploid tumors. After stratification for staging, Dukes' C cases showed a statistically significant association between tumor ploidy and survival ( $P = 0.0224$  by generalized Breslow,  $P = 0.0110$  by generalized Savage). Product limit survival analysis for proliferative activity and survival showed a similar outcome with the strongest association in Dukes' C stage of disease (75th quantile survival of 38.9 months for low proliferative and 18.0 months for high proliferative tumors).

## INTRODUCTION

Large bowel carcinoma is one of the most common neoplasms, rating second only to carcinoma of the lung. It carries a considerable mortality with an overall 5-year survival of around 50%. Adjuvant chemotherapy and radiotherapy have not been able to change survival rates significantly.

The most important prognostic factor in large bowel carcinoma is Dukes' staging. Additional features, related with prognosis, include the degree of differentiation (1) and marker profiles (2-4). Furthermore, aberrations in ploidy level are a well recognized feature of human solid tumors with an incidence of aneuploidy ranging from 50 to 90% (5). In certain tumor types (6-12), including colorectal carcinoma (13-16), analysis of DNA content appears to offer prognostic information.

In this study we have evaluated the prognostic significance of ploidy levels in a large series of large bowel carcinomas which were included in a surgical prospective randomized trial. The aim was to obtain an additional biological determinant which may be of use in segregating patients with similarly staged tumors into subgroups with different prognosis.

## MATERIALS AND METHODS

**Tissue.** Blocks of 10% neutral buffered formalin-fixed and routinely paraffin-embedded colorectal carcinoma tissue were collected from a multicenter prospective controlled surgical trial, carried out from January 1979 through January 1982. This trial was conducted to compare the value of the no touch isolation technique (17) versus conventional surgical approach. Patients with previous malignancies were excluded from the trial. For flow cytometry, cases with nonresectable tumors or distant metastases were included in the study. In 279 of 350 cases sufficient material was obtained. In all cases material from the primary tumor was used for analysis.

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**Staging.** In this trial patients were staged according to the Turnbull modification of the original Dukes' classification. Of this series of patients 22, 41, 31, and 7% were classified as Dukes' A, B, C, and D, respectively (17).

**Flow Cytometry.** The method used has been fully described elsewhere (18). Briefly, tissue was cut with a scalpel from a paraffin-embedded tissue block, dewaxed, and rehydrated in a sequence of xylene and a graded series of ethanol. The tissue was then digested with trypsin and the obtained nuclei were stained for DNA content according to the method of Vindeløv *et al.* (19). A tumor with a single  $G_1$  peak was considered to be diploid, whereas evidence of an additional  $G_1$  peak indicated the presence of aneuploidy. The DNA index was calculated as the ratio of the  $G_1$  peak with the highest DNA content to the  $G_1$  peak with the lowest DNA content. The proliferative activity was calculated by counting the number of cells between the inclination points of the descending  $G_1$  peak and the ascending  $G_2$ -M peak. In cases with less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated, without corrections for the presence of diploid S and  $G_2$ -M phase cells. Histograms with coefficients of variation less than 8% were considered of good quality.

**Statistics.** For the calculation of disease-free and overall survival, product limit survival analysis was performed using the Biomedical Computer Program P-Series (BMDP). Calculations of the significance of observed differences were made using the log rank test (Mantel-Cox) and the generalized Wilcoxon test (Breslow). Patients with adjacent organ invasion or distant metastases (Dukes' D) were included in this material for the calculation of overall survival rates, but this category of patients were excluded for the calculation of disease-free interval limiting the total number of cases to 260. To date, the mean duration of follow-up is 51.9 months (range, 44.1-60.0 months).

## RESULTS

Fig. 1 represents the frequency distribution of ploidy levels in 279 cases of large bowel carcinoma. A characteristic bimodal distribution of aneuploidy is observed with tumors having either a near diploid mode or a triploid-tetraploid mode. In a minority of cases (3.9%) multiploid tumors were observed. Since the degree of aneuploidy appeared not to be significantly correlated with survival, for further analysis tumors were classified as diploid (37.6%) or aneuploid (62.4%), the latter comprising all cases with one or more  $G_1$  peak(s) additional to the diploid  $G_1$  peak. In 194 cases we were able to calculate the percentage of S-phase cells. The frequency distribution is shown in Fig. 2. For further analysis tumors were classified as low proliferative or high proliferative with a cutoff point at 13% S-phase cells, being the mean value of normal mucosal epithelium plus 2 SD.

Product limit survival analysis demonstrated a borderline significant association between tumor ploidy and survival (generalized Breslow,  $P = 0.0689$ ; generalized Savage,  $P = 0.0336$ ). Patients with diploid tumors demonstrated a 75th quantile survival of 49.8 versus 35.9 months for patients with aneuploid tumors (Fig. 3). No statistically significant correlation between ploidy level or proliferative activity and stage was found. After stratification for staging, Dukes' C cases showed a statistically significant association between tumor ploidy and overall survival (generalized Breslow,  $P = 0.0224$ ; generalized Savage,  $P = 0.0110$ ) with a 75th quantile survival of 47.9 and 28.9 months

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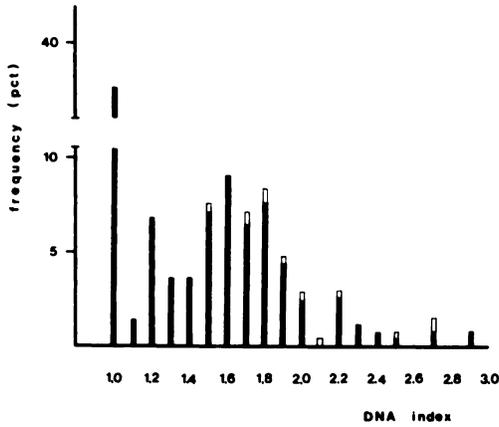


Fig. 1. Frequency distribution of DNA index in 279 cases of large bowel carcinoma. □, tumors with multiploid stemlines.

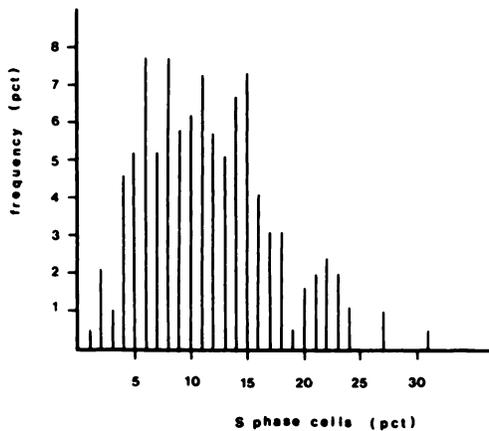


Fig. 2. Frequency distribution of the percentage (pct) of S-phase cells in 194 cases of large bowel carcinoma.

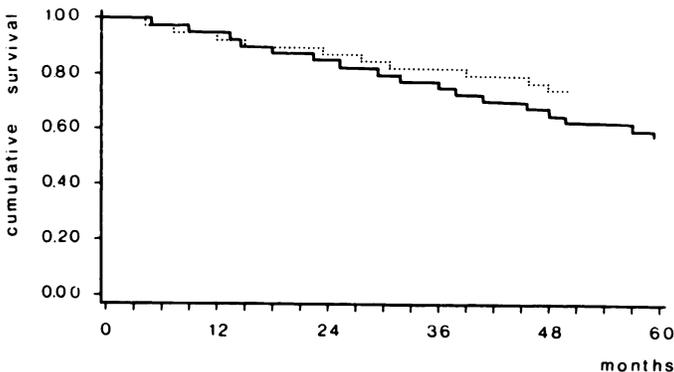


Fig. 3. Life table analysis demonstrating overall survival for patients with diploid (.....) ( $n = 105$ ) and aneuploid (—) tumors ( $n = 174$ ).  $P = 0.0689$ , generalized Breslow;  $P = 0.0336$ , generalized Savage.

for patients with diploid and aneuploid tumors, respectively (Fig. 4). In Dukes' stages A, B, and D different ploidy levels did not correlate with prognosis. A relation was also found between ploidy level and disease-free interval. Patients with diploid tumors showed a longer disease-free interval (75th quantile of 52.3 months) as compared to patients with aneuploid tumors (75th quantile of 22.2 months). This difference was significant (generalized Breslow,  $P = 0.0107$ ; generalized Savage,  $P = 0.0089$ ), even if the stages A, B, and C were taken together (data not shown).

Product limit survival analysis showed an association between proliferative activity and survival, similar to ploidy level. Overall survival for patients with low proliferative tumors was longer

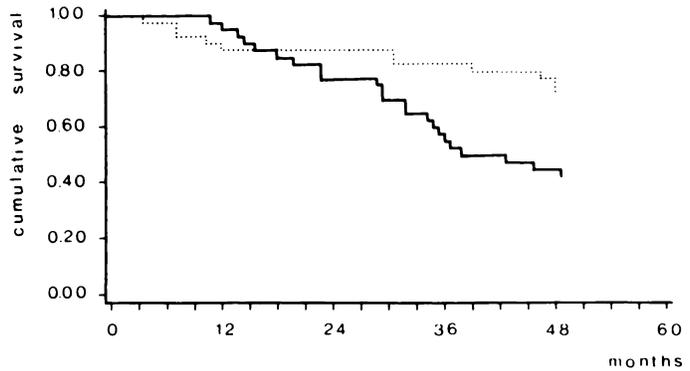


Fig. 4. Life table analysis for Dukes' C stage of disease, demonstrating survival for patients with diploid (.....) ( $n = 31$ ) and aneuploid (—) tumors ( $n = 55$ ).  $P = 0.0224$ , generalized Breslow;  $P = 0.0110$ , generalized Savage.

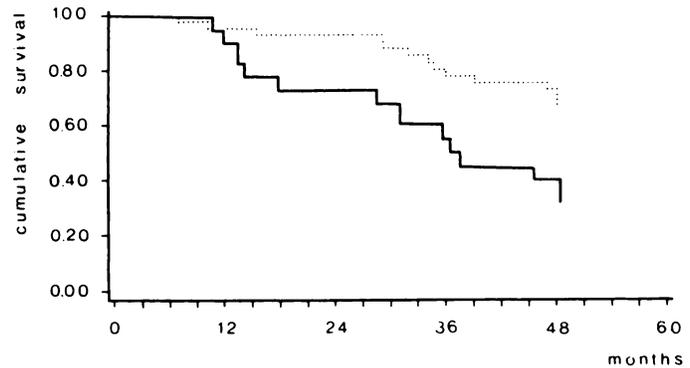


Fig. 5. Life table analysis for Dukes' C stage of disease, demonstrating survival for patients with low (.....) ( $n = 37$ ) and high (—) proliferative tumors ( $n = 19$ ).  $P = 0.0121$ , generalized Breslow;  $P = 0.0093$ , generalized Savage.

than for patients with high proliferative tumors (generalized Breslow,  $P = 0.0451$ ; generalized Savage,  $P = 0.0320$ ) (data not shown). After stratification for Dukes' stage this association was again significant only in Dukes' C disease (Fig. 5) with a 75th quantile survival time of 38.9 and 18.0 months for patients with low and high proliferative tumors, respectively (generalized Breslow,  $P = 0.0121$ ; generalized Savage,  $P = 0.0093$ ).

DISCUSSION

The DNA content of colorectal tumors cells as assessed by flow cytometry of propidium iodide-stained nuclei from paraffin-embedded tissue appears to add important prognostic information to that obtained from staging. In fact, of patients with diploid Dukes' C tumors approximately 25% expired within 4 years in contrast to 60% of the patients with a hyperdiploid DNA content, in the majority of cases due to recurrent tumor growth. Several studies have been published concerning aneuploidy in colorectal carcinoma, which generally show similar frequency distributions of aneuploid DNA content (5, 20, 21). In one report (13), specifically dealing with the relation between ploidy and prognosis, Dukes' C cases represented 21 of 33 patients. The remarkably better survival of patients with diploid tumors in that study may therefore in part be due to an overrepresentation of Dukes' C cases, in which we found a strong association between ploidy level and survival. This observation seems to be confirmed by recent publications with smaller numbers of patients (14-16).

Any effect of DNA content on the survival of Dukes' A and B patients is necessarily limited to those cases in which the tumor was not radically excised and, in view of the high cure

rate in those stages, may need very large numbers to be demonstrated. In Dukes' D patients the tumor load is high and the rapidly downhill course is apparently not appreciably influenced by variability in DNA content of the tumor cells (22). Reliable assessment of tumor extent is difficult in patients with locoregionally advanced disease. From a biological as well as a therapeutic point of view in this group of patients, stage-independent variables which might predict recurrent and therapy-resistant disease, would be useful. The DNA content appears to be one such variable (7-12). Apparently with aneuploid tumors the chance of dissemination beyond the regional lymph nodes is higher or, alternatively, such micrometastases will demonstrate a more accelerated growth. The shape of the disease-free survival curve, showing separation after 18 months which is maintained throughout the next 3 years, suggests the former explanation to be the case.

It cannot be excluded that the similar outcome of survival analysis in relation to proliferative activity and ploidy level reflects the strong correlation between the two parameters. Such a correlation can partly be explained by technical reasons, since due to the admixture of normal cells in DNA histograms of diploid tumors the percentage of S-phase cells may be spuriously low. This contention is supported by the results of Meyer *et al.* (23) in showing that [<sup>3</sup>H]thymidine-labeling indices in colorectal carcinomas are not correlated with prognosis. Cell kinetic studies, using bromodeoxyuridine incorporation *in vivo*, might clarify whether or not a difference in growth characteristics exists between diploid and aneuploid tumors and thus ascertain its potential prognostic importance.

Tumors with a diploid cellular DNA content most probably will not display a normal karyotype. Flow cytometric DNA analysis does not allow us to detect small or balanced chromosomal aberrations. Nevertheless, this technique does distinguish tumors with a normal from those with an abnormal DNA content easily and with a high success rate. Therefore, as long as a more precise determination of structural and functional abnormalities of the genome remains time consuming and cumbersome, flow cytometric DNA analysis provides the clinician with important prognostic information.

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