

Nuclear Size as Prognostic Determinant in Stage II and Stage III Colorectal Adenocarcinoma

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Abstract. *Background:* The prognostic value of morphometric nuclear features was assessed in stage II and stage III colorectal cancer (CRC). *Materials and Methods:* Primary tumors from 123 CRC patients were analyzed using an image overlay drawing system for the following nuclear size features: area, perimeter, diameter and form features. *Results:* The nuclear area (NA) was significantly different in tumors at different localizations ($p=0.029$). A large NA was a significant predictor of recurrent disease, with overall response (OR) 3.09 (1.37-6.95) ($p=0.006$). The NA was significantly larger in recurrent cases ($106.3 \mu\text{m}^2$) than in non-recurrent ones ($96.6 \mu\text{m}^2$) ($p=0.007$) and was a significant predictor of disease-free survival (DFS) in univariate (Kaplan-Meier) analysis (log-rank $p=0.0239$). However, lymph node involvement was the most powerful predictor of DFS in multivariate analysis, with OR 3.371 (95%CI 1.17-9.65) ($p=0.024$) and disease recurrence the only independent predictor of disease-specific survival (DSS), with OR 48.4 (95%CI 6.30-371.73). *Conclusion:* Quantitative measurement of the NA seems to accurately discriminate the patients, among stage II and III colorectal cancer, who are likely to develop disease recurrence. Image morphometry seems to be a useful adjunct tool in examining the subgroup of lymph node-negative patients to predict the risk of disease recurrence and indications for adjuvant therapy.

Colorectal cancer (CRC) is one of the most common malignant tumors in both sexes worldwide (1, 2). The incidence of CRC increases with advanced age (3, 4). In

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most countries, disease outcome has become more favorable during recent decades (5, 6), mainly because of improved diagnosis and novel therapeutic approaches (7, 8).

In the past, it was well established that several clinical and histopathological variables are helpful in predicting the clinical outcome of CRC patients. Such prognostic predictors include tumor stage (9, 10), histological type, tumor differentiation, ploidy, proliferative activity, p53 expression, apoptosis and vascular and lymphatic invasion. Among the most powerful prognostic determinants in CRC is the histological tumor stage, including the depth of local invasion into the bowel wall and the infiltration in the regional lymph nodes (LN) (11-13). Despite this, the clinical staging of CRC is currently based on information not obtainable by histological examination of the primary tumor, particularly when done only in biopsies, where the exact depth of tumor infiltration into the bowel wall, LN involvement and data on distant metastases cannot be obtained.

Increasing recent evidence has shown that light microscopic examination of the primary tumor by quantitative measurements could provide useful prognostic information (14). Currently, computer-assisted image analysis (nuclear morphometry) provides a new powerful tool for high-precision measurement of several variables characterizing the size and shape of the cancer cell nuclei in conventional tissue sections (15, 16). Several of these nuclear profiles seem to be useful prognostic predictors in various human malignancies (17, 18). To date, however, few studies have used morphometric measurements to determine the nuclear size and shape profiles in normal and neoplastic colorectal tissues (19). Not unexpectedly, the nuclear size is usually larger and its shape is more often irregular in cancer cells (20, 21).

Despite the fact that several well characterized morphological and clinical variables are closely associated with prognosis of CRC in large cohorts (22), the prediction of disease outcome in individual patients after curative

surgical resection is far from reproducible (23). In this respect, the potential use of nuclear morphometry to disclose prognostic predictors in CRC has not been fully exploited (24, 25). The present study was undertaken to assess whether quantitative measurement of the nuclear features were of independent prognostic value in a retrospective series of stage II and stage III colorectal adenocarcinoma.

Materials and Methods

Patients, treatment and follow-up. The study material comprised a series of 123 patients, diagnosed and treated for stage II or stage III colorectal cancer CRC at the Department of Oncology and Radiotherapy, Turku University Hospital, Finland, and six other hospitals in the same hospital district, between January 1996 and December 1997. The primary treatment was surgery in all 123 patients. Adjuvant chemotherapy was administered to 33 patients, with a mean treatment duration of 4.8 months (median 5). Adjuvant radiotherapy was given to 11 patients with rectal cancer, at a mean dose of 51.9 Gy (median 50.4).

The key clinical characteristics of the patients, in stage II or stage III, are summarized in Table I. Of the 123 patients, 95 had stage II and 28 had stage III disease. The patients were followed-up in the clinic at regular intervals until death, or still remain under continuous monitoring. At the time of writing, the mean follow-up time for the whole series was 53.9 (24.8 SD) months (median 61.3). During the follow-up, 38 (30.9%) of the patients developed a recurrent disease in a mean of 18.6 months (median 11.4) and were treated by surgery (n=14), chemotherapy (n=7), radiation (n=3) or not treated (n=14). Disease-specific survival (DSS) and disease-free survival (DFS) were calculated as usual, *i.e.*, time from diagnosis to death (due to disease) or to the date last seen alive, and time from diagnosis to the appearance of recurrent disease or the date last seen disease-free, respectively. In calculating DSS, patients who died of other or unknown causes were treated as censored events in life-table analysis.

Morphometry. All tissue samples were obtained from the primary tumor at the time of diagnosis. The samples were fixed in buffered-formalin and embedded in paraffin. Sections were cut at 5 µm and stained with hematoxylin and eosin. The nuclear profile of the cancer nuclei was measured using the Prodit morphometry program (Prodit 3.1, Promis Inc, Almere, The Netherlands); a digitized interactive image overlay system. The system includes a microscope, a personal computer (MultiSync 3D Color Monitor; NEC, Japan), a video camera (JVC TK-870U; JVC, Japan) and digitizer board (PIP 512B video digitizer board; Matrox Electronic Systems, Dorval, Quebec, Canada). Analog images of the nuclei were outlined on the monitor screen using a computer mouse. This resulted in a digitized overlay of the traced outline. The instrument was calibrated with a micrometer slide before each measurement. All measurements were carried out at the following magnification on the monitor screen (x40 objective, x10 video ocular and x2 internal magnification). On examining the sections for selection of the fields, tumor cells from the most cellular area at the periphery of the tumor were sought. Necrotic and inflammatory areas were avoided. An average of 10-15 microscopic fields was screened and 100 consecutive tumor cells with clear nuclear borders were

Table I. Key clinical characteristics of the patients.

Variables	Stage II	Stage III	Significance
Number of patients	95 (77.2%)	28 (22.8%)	
Sex			
Female	61 (64.2%)	14 (50.0%)	
Male	34 (35.8%)	14 (50.0%)	
Mean age (SD)	71.6 (12.2)	65.5 (16.3)	p=0.079*
Tumor localization			p=0.856
Rectum	18 (18.9%)	4 (14.3%)	
Right colon	41 (43.2%)	10 (35.7%)	
Left colon	6 (6.3%)	3 (10.7%)	
Transverse colon	4 (4.2%)	2 (7.1%)	
Sigmoid colon	21 (22.1%)	8 (28.6%)	
Recto-sigmoid colon	5 (5.3%)	1 (3.6%)	
Serum CEA levels (SD)			
Pre-operative	6.2 (8.1)	20.1 (26.2)	p=0.055*
Follow-up time (SD, months)	55.3 (23.8)	49.2 (27.8)	p=0.242*
Recurrent disease	26 (27.4%)	12 (42.9%)	p=0.162
Time to recurrence (SD, months)	20.5 (19.0)	14.6 (15.7)	p=0.256
Site of recurrence			p=0.005
Local	5 (16.0%)	2 (16.7%)	
Liver	3 (12.0%)	7 (58.3%)	
Lung	3 (12.0%)	1 (8.3%)	
Brain	2 (8.0%)	0 (0.0%)	
Peritoneum	0 (0.0%)	1 (8.3%)	
Multiple sites	11(44.0%)	0 (0.0%)	
Other	2 (8.0%)	1 (8.3%)	
Treatment of recurrence			p=0.061
Surgical	9 (37.5%)	4 (33.3%)	
Radiation	3 (12.5%)	0 (00.0%)	
Chemotherapy	2 (8.3%)	5 (41.7%)	
No therapy	10 (41.7%)	3 (25.0%)	
Disease-free survival (mo): (M±SD)	55.6 (26.4)	46.2 (33.1)	p=0.205*
General outcome: (n=123)			p=0.631
Alive with disease	2 (2.1%)	1 (3.6%)	
Alive free of disease	56 (58.9%)	15 (53.6%)	
Died of disease	19 (20.0%)	9 (32.1%)	
Died of other cause	12 (12.6%)	2 (7.1%)	
Died of unknown cause	6 (6.3%)	1 (3.6%)	
Disease-specific survival (mo): (M±SD)	59.5 (22.9)	52.4 (27.5)	p=0.187

*Mann-Whitney

Table II. The mean values of quantitative nuclear variables measured in the primary tumors.

Morphometric variables	Stage II	Stage III	Significance
Nuclear area (M±SD)	98.2 (21.3)	104.2 (26.2)	<i>p</i> =0.142*
Nuclear perimeter (M±SD)	37.4 (4.1)	38.5 (5.1)	<i>p</i> =0.175*
Nuclear diameter (M±SD)	11.1 (1.2)	11.4 (1.5)	<i>p</i> =0.147*
Form factor NCI (M±SD)	3.8 (0.06)	3.8 (0.07)	<i>p</i> =0.953*
Roundness (M±SD)	1.07 (0.018)	1.07 (0.014)	<i>p</i> =0.237*

M, mean; *Mann-Whitney.

outlined and measured. Overlapping nuclei were omitted. Of the morphometric variables measured by the Prodit program, the nuclear area (NA), perimeter and diameter were assessed in this study (26). All morphometric measurements were made blindly by one observer only (AB), unaware of the clinical data of the patients.

Statistical analysis. Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 12.0.1 and STATA/SE 8.2). Frequency tables were analyzed using the Chi-square test, with the likelihood ratio (LR) or Fischer’s exact test being used to assess the significance of the correlation between the categorical variables. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney) or ANOVA (analysis of variance). Logistic regression models were used to analyze the power of different covariates as predictors of the outcome variables, calculating crude overall responses (ORs) (and 95%CI) in univariate analysis. Performance indicators of NA as a marker of recurrence were calculated using the conventional contingency tables to calculate sensitivity (SE), specificity (SP) and positive (PPV) and negative predictive value (NPV), with their 95% CI, based on the F-distribution ($\pm 1.96 \times SE$). Univariate survival (life-table) analysis for the outcome measure (DSS, DFS) was based on the Kaplan-Meier method. Multivariate survival analysis was run by using Cox’s proportional hazards model in a backward step-wise manner with the log-likelihood ratio (L-R) significance test, and using the default values for entry and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log (LML) survival plots. In all tests, values of *p*<0.05 were regarded as statistically significant.

Results

The results of morphometric measurements in stage II and stage III disease are summarized in Table II. The mean NA was smaller in stage II than in stage III disease, but the difference was not statistically significant. The same was true for all the other nuclear variables measured, which had slightly higher values in stage III disease, but none were statistically significant. The median NA of the tumors in the entire series was 98.8 μm^2 (range 45.2 μm^2 –159.4 μm^2). This median NA was used as the cut-off point in further calculations to correlate the NA with the clinical parameters

Table III. Correlation of NA with clinical variables in univariate analysis.

Variables	NA above median	NA below median	OR (95%CI)	Significance
Age below or above median			1.03 (0.51-2.09)	<i>p</i> =0.536
Below	31 (50.0%)	31 (50.0%)		
Above	30 (49.2%)	31 (50.8%)		
Sex			0.64 (0.31-1.33)	<i>p</i> =0.270
Female	34 (45.3%)	41 (54.7%)		
Male	27 (56.3%)	21 (43.8%)		
CEA pre-operative (M±SD, months)	8.7 (12.4)	9.6 (17.4)		<i>p</i> =0.469*
Recurrence			3.09 (1.37-6.95)	<i>p</i> =0.006
Yes	26 (68.4%)	12 (31.6%)		
No	35 (41.2%)	50 (58.8%)		
Time to recurrence (M±SD, months)	18.5 (17.9)	19.0 (19.2)		<i>p</i> =0.753
Disease-free survival (M±SD, months)	48.5 (29.8)	58.6 (25.7)		<i>p</i> =0.122*
Disease-specific survival (M±SD, months)	54.2 (26.2)	61.9 (21.3)		<i>p</i> =0.177*
Disease-specific outcome			0.52 (0.21-1.29)	<i>p</i> =0.187
Alive	36 (48.6%)	38 (51.4%)		
Died of disease	18 (64.3%)	10 (35.7%)		

*Mann-Whitney

and disease outcome. These calculations were not repeated for the other morphometric variables, because they were closely related to the NA.

There was no relationship between age and the NA, which was identical in patients below and above the median age of 73.7 years. Similarly, the NA was not related to tumor grade. On the other hand, the NA was slightly larger (median 103.1 μm^2) in men than in women (97.3 μm^2) (*p*=0.231, Mann-Whitney). There was a significant difference in the NA between the tumors in different localizations, in that the largest NA (120.5 μm^2) was found in lesions of the transverse colon and the smallest (93.0 μm^2) in those of the left colon (*p*=0.029, Kruskal-Wallis). The NA was significantly larger

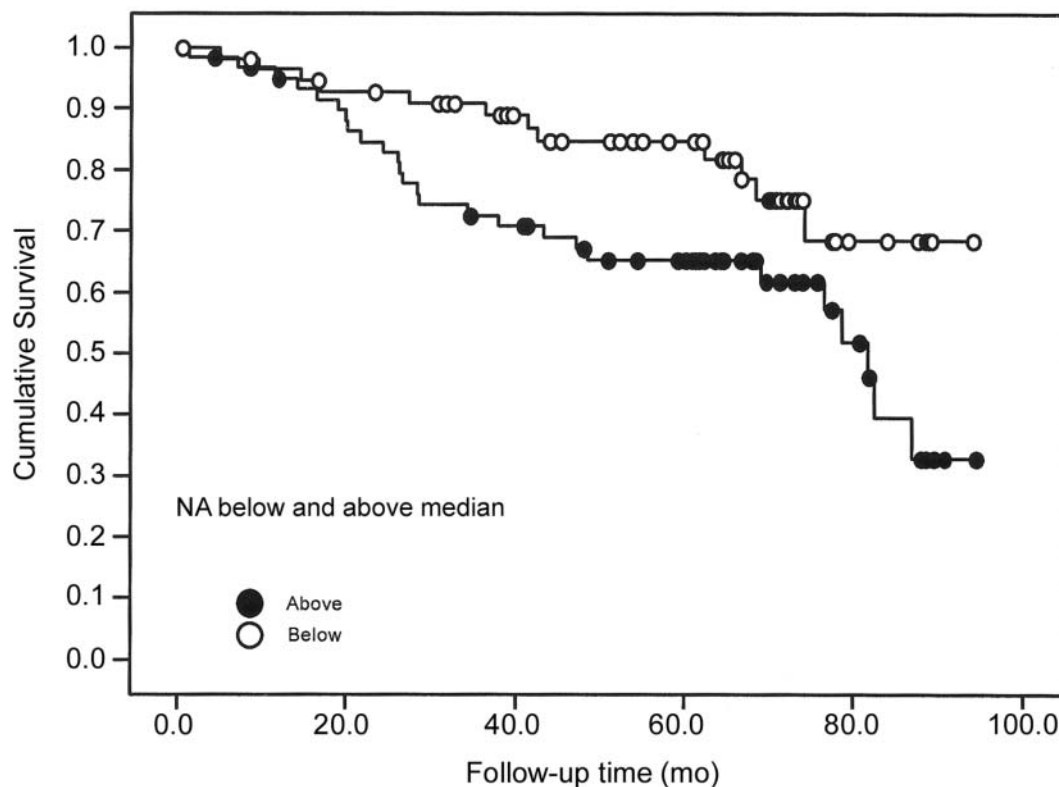


Figure 1. NA as a predictor of disease-free survival in Kaplan-Meier analysis (log-rank statistic $p=0.0239$).

in tumors which subsequently recurred ($106.3 \mu\text{m}^2$) when compared with the non-recurrent ones ($96.6 \mu\text{m}^2$) ($p=0.007$, Mann-Whitney). When analyzed separately for stages II and III disease, 70% of stage II tumors which developed recurrence had a NA above median, in contrast to 40% of those without recurrence; OR 3.37 (95%CI 1.28-8.89) ($p=0.011$). Consequently, the NA in recurrent cases of stage II was significantly larger ($105.9 \mu\text{m}^2$) than that ($95.9 \mu\text{m}^2$) in non-recurrent cases ($p=0.020$, Mann-Whitney test).

In the same way, the NA was larger in patients, who died of their disease ($105.0 \mu\text{m}^2$) when compared with those who were alive at the end of the follow-up ($99.5 \mu\text{m}^2$) ($p=0.152$).

Correlations of the NA (as cut-off point: large/small) with the clinicopathological variables and disease outcome in univariate analysis are shown in Table III. DFS was longer in node negative patients (median 55.5 months) than in those with positive lymph nodes (median 46.2 months) ($p=0.203$). The DFS was also longer in younger patients (57.3 months) than in older ones (47.9 months) ($p=0.112$) (data not in Table). The mean values of CEA (both pre- and post-operative) were not different in cases with larger or smaller NA. A large NA was a significant predictor of recurrent disease, with OR 3.09 (1.37-6.95) ($p=0.006$). As a predictor of recurrence, the NA showed a SE of 68.4%

(95%CI 53.6-83.2), a SP of 58.8% (95%CI 48.4-69.3), a PPV of 40.9% (95%CI 28.6-53.3) and a NPV of 80.6% (95%CI 70.8-90.5). In other words, patients with a NA below the median values had an 80% probability (NPV) of not having a recurrent disease. On the other hand, the NA did not bear any relationship to the time elapsed until the recurrence developed.

Univariate (Kaplan-Meier) survival analysis was used to test the value of the NA as a predictor of DFS and DSS. The NA was a significant predictor of DFS (log-rank $p=0.0239$) (Figure 1) in the whole series, and even more significant in stage II disease alone ($p=0.0129$) (Figure 2). However, the NA was of no predictive value for DSS in Kaplan-Meier analysis (log-rank $p=0.0782$) (Figure 3).

A multivariate survival (Cox) model was used to test the independent prognostic predictors of both DFS and DSS, by entering into the model the factors proven significant in univariate analysis, and controlled by the possible confounding effects of age and Dukes' stage. When the factors were entered into the model, age, Dukes' stage, LN involvement and pre-operative CEA; only the LN involvement proved to be an independent predictor of DFS, with OR 3.371 (95%CI 1.17-9.65) ($p=0.024$). In a similar analysis for DSS, only the disease recurrence proved to be a powerful independent

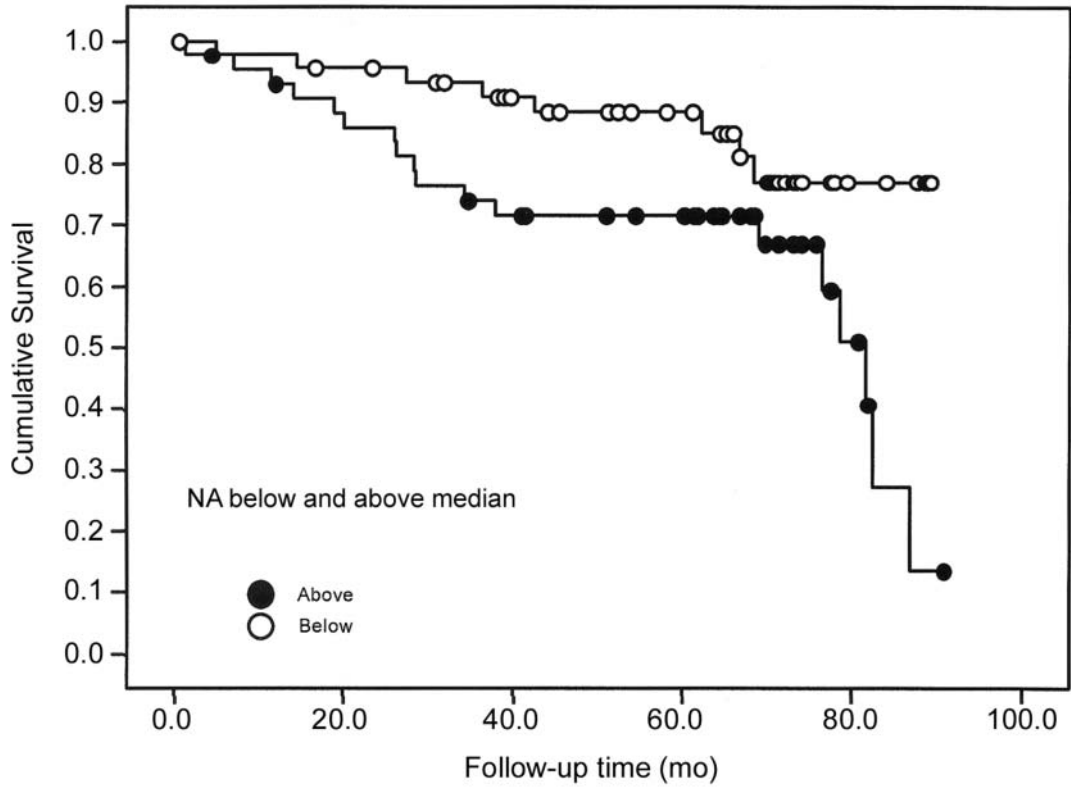


Figure 2. NA as a predictor of disease-free survival in stage II disease in Kaplan-Meier analysis (log-rank statistic $p=0.0129$).

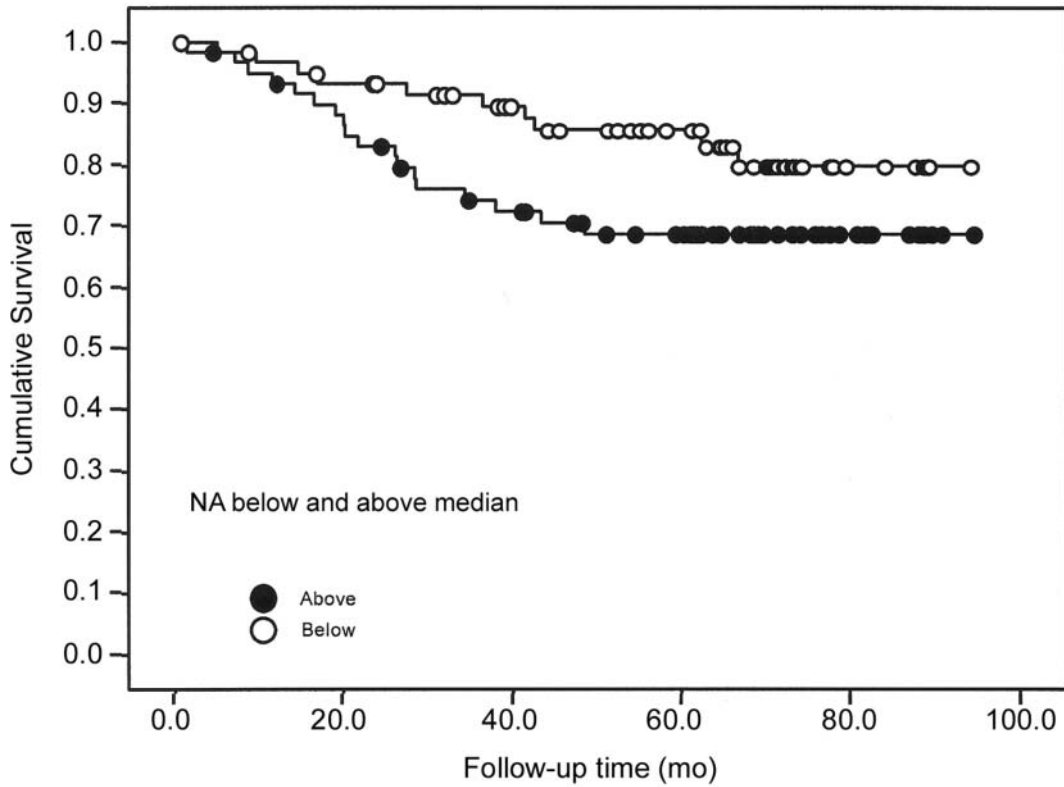


Figure 3. NA as a predictor of disease-specific survival in Kaplan-Meier analysis (log-rank statistic $p=0.0782$).

predictor of adverse clinical outcome, with OR 48.4 (95%CI 6.30-371.73). All other variables were removed from the regression model in the step-wise approach. When tested in Cox univariate mode, recurrence had OR 88.6 (95%CI 11.99-653.92) in predicting death from disease.

Discussion

The surgical cure of CRC is determined by the stage of the tumor and its biological behavior (27). It is well established that early CRC can be cured with radical surgical resection alone. Unfortunately, however, some 30% of all patients who undergo curative resection subsequently present with relapse and eventually die of their disease (28). Despite many well-defined pathological and clinical variables known to be prognostic predictors in CRC (22, 29), prediction of the disease outcome in individual patients after curative resection is still far from reliable (24). Reliable prognostication is pertinent, *e.g.*, to the decision to use adjuvant chemotherapy after curative surgical resection in patients with stage II disease (*i.e.*, in node-negative patients), which is often difficult (30). There is some hope, however, and our results already suggest, that the NA could be used to help in this decision. In addition, more rational decisions will be possible as soon the use of further markers and diagnostic tools are established for more accurate prediction of the disease outcome in individual patients.

The aim of the present study was to cast further light on issues related to the prognostication of CRC, while assessing the value of quantitative morphometric measurements of the nuclear profiles in cancer cells as independent prognostic factors. In this study, stage II and stage III disease was focused on, where molecular and other markers may help to pin-point a sub-group of patients who would eventually benefit from the use of adjuvant therapy for their disease. This important decision involves careful weighing of the risks of toxicity and complications against the potential curability of the disease. Because the 5-year survival in stage II patients is close to 70% with surgery alone (31), adjuvant therapy is not widely recommended and still no jointly agreed criteria exist as to how to select potential candidates for chemotherapy among this subgroup of CRC patients (32).

Undoubtedly, however, some stage II patients are at high risk of both local and distant recurrence and would probably benefit from adjuvant chemoradiation (33). Indeed, some recent data suggest a survival advantage for LN-negative patients receiving chemotherapy (30). The problem is how to accurately define these high-risk patients. In the present study, the performance of computer-assisted image analysis was evaluated as a potential new tool to provide such information. The instrument was used to examine a series of well-defined morphometric nuclear features of the cancer cells in stages II and III disease, to elaborate data that

would be useful in selecting the patients at high risk of recurrence, who should be subjected to adjuvant therapy. This is a novel approach, because the role of nuclear morphometry as a prognostic determinant in CRC has not previously been widely explored (25, 34).

Several interesting and important observations were made, all implicating that the quantitatively measurable morphological features of cancer cell nuclei are of significant prognostic value in stage II and stage III colorectal adenocarcinoma. Generally speaking, the NA was smaller in stage II than in stage III disease, and its median value ($98.8 \mu\text{m}^2$) proved to be a useful discriminator in some of the key clinical parameters (Table III). Interestingly, there was a significant difference in the NA between tumors in different sites of the colon, being largest in lesions of the transverse colon and smallest in tumors of the left colon ($p=0.029$, Kruskal-Wallis test). In the light of its associations with the disease outcome (see below), NA measurement in tumors of different localizations might have implications in predicting the different behavior (more or less aggressive) of the lesions at different anatomic sites. Whether this would hold true more generally needs to be further explored, in a large series with cases from all different sites of the colon.

Obviously, one of the most important observations of the present study is the one linking the NA with disease outcome, *i.e.*, the appearance of recurrence and DFS. This is clinically relevant for several reasons. Because a number of stage II patients are at high risk of recurrence, it is of paramount importance to establish reliable markers that would accurately predict those patients to be considered for adjuvant therapy. In the present series, 27.4% of stage II and 42.9% of stage III carcinomas eventually developed a recurrence within the median follow-up period of 53.3 months. This is an especially high rate, particularly for a group of LN-negative (stage II) CRC patients. Importantly, our data showed that the NA was significantly larger in tumors that subsequently recurred, as compared with the non-recurrent ones ($p=0.007$).

In univariate (Kaplan-Meier) survival analysis, the NA was also a significant predictor of DFS (log-rank $p=0.0239$). As a predictor of recurrence, NA showed a NPV of 80.6%, indicating that patients with a NA below the median values had an 80% probability (NPV) of not having recurrent disease. Not unexpectedly, the NA was also larger (far above the median) in patients who eventually died of their disease as compared with those who were alive at the completion of the follow-up period, but this difference did not reach statistical significance ($p=0.152$). Similarly, the NA was only of close to borderline significance (log-rank $p=0.0782$) as a predictor of DSS in univariate analysis.

These observations clearly implicate that LN-negative (stage II) tumors with a large NA are at high risk of local or distant recurrence and, because of its high adverse

prognostic impact (OR 48.4; 95%CI 6.30-371.73 in multivariate analysis), are also more likely to die of their disease. These patients should be appropriate candidates for adjuvant therapy. In contrast to node-negative (stage II) tumors, it is widely accepted that adjuvant therapy increases the probability of cure in surgically-treated patients with node-positive (stage III) CRC (35). Interestingly, we recently showed that, in advanced (stages III and IV) tumors, the response to adjuvant chemotherapy was markedly better in patients with a high NA in their primary tumors (36). This is also a feasible explanation of the present observations that the NA was a significant predictor of recurrence, but not necessarily of overall survival. Accordingly, patients with recurrence and high NA are also those who are likely to respond better to adjuvant therapy and, thus, probably would gain a survival advantage, which "dilutes" the adverse prognostic effect of a large NA, as recently discussed (36). Indeed, such a positive effect of adjuvant therapy on the survival gain of the patients with a high NA was even more accentuated in our recent series of advanced disease, where the DSS was significantly longer among the patients with a NA above the median ($p=0.02$) (36).

Taken together, quantitative measurement of the NA and its value above the median seem to discriminate, with high precision, the patients who are likely to develop disease recurrence (*i.e.*, end-point of DFS) among stage II and III CRC. At the same time, however, there are likely to be patients who will benefit from adjuvant chemotherapy. Indeed, our tentative data suggest that a high NA is inherent to patients who are responders to irinotecan-based chemotherapy (with improved prognosis), as distinct from those with a small NA who are non-responsive and develop progressive disease. On the basis of the present results, it can be concluded that the nuclear size features are of prognostic importance and a particularly useful adjunct tool in examining the subgroup of LN-negative CRC patients who have a high risk of local or distant recurrence and should be considered as suitable candidates for adjuvant therapy.

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