Virchows Archiv A Pathol Anat (1989) 415:421–428

Virchows Archiv A
Pathological Anatomy and Histopathology

Comparison of quantitative and classic prognosticators in urinary bladder carcinoma

A multivariate analysis of DNA flow cytometric, nuclear morphometric and clinicopathological features

C.E.M. Blomjous[1], N.W. Schipper[1], W. Vos[1], J.P.A. Baak[1], H.J. de Voogt[2], and C.J.L.M. Meijer[1]

From the [1] Department of Pathology, Free University Hospital, De Boelelaan 1117, 1007 MB Amsterdam, The Netherlands and [2] Department of Urology, Free University Hospital, Amsterdam, The Netherlands

Summary. The prognostic value of nuclear morphometry and DNA flow cytometry of paraffin embedded material of 58 patients with primary and untreated transitional cell carcinoma of the bladder was compared with that of histological grade (WHO-system), tumour stage (TNM-classification), tumour size, multiplicity and ulceration. Small nuclear size (mean nuclear area $\leq 95\ \mu m^2$) ($n=25$) and DNA diploidy ($n=28$) indicated a favourable outcome (5-year survival 95.8% and 92.2%); large nuclei (mean nuclear area $>95\ \mu m^2$) ($n=33$) and DNA aneuploidy ($n=30$) indicated a worse prognosis (5-year survival 61.4% and 62.5%) (Mantel-Cox: $p=0.002$ and $p=0.007$). The quantitative techniques had the advantage over subjective histological grading that distinguishment of an intermediate patient group (WHO-system: grade 2; $n=32$) with heterogeneous outcome (5-year survival 78%) was avoided. Multivariate analysis showed tumour stage as the most important prognosticator of survival. Neither the quantitative techniques, nor the other classic features added significantly to the prediction. The additional value of the quantitative techniques was however shown in superficial carcinoma (TNM-classification: stage Ta and T1; $n=37$): large nuclei (mean nuclear area $>95\ \mu m^2$) ($n=15$) and aneuploid DNA peaks ($n=13$) were associated with progressive recurrent tumour ($n=7$) (Mantel-Cox: $p=0.03$ and $p=0.0004$). The quantitative methods thus indicate which patients are at risk for progression and may enable more appropriate treatment at an earlier stage of disease.

Introduction

Therapy and prognosis of urinary bladder carcinoma mostly depend on the clinicopathological stage of the tumour, and to a less marked degree on histological grade, tumour size, multiplicity and ulceration. Superficial carcinomas (TNM-classification: stage Ta and T1) (Harmer 1978) are commonly treated by transurethral resection, whether or not followed by intravesical chemotherapy; advanced tumours, invading into or beyond the muscular wall (stage T2 to T4), are preferentially treated by radical cystectomy.

However, establishment of the depth of invasion by physical examination may be difficult (Schmidt and Weinstein 1976), and pathological investigation may be biased by the fear of the urologist to biopsy deeply. Other prognosticators independent of invasive growth may thus be helpful.

A considerable number of patients with superficial carcinoma (stage Ta and T1) develop recurrence after transurethral resection of their initial tumour, part of them with progression to high grade (WHO-classification: grade 3) or advanced stage (stage T2 to T4) (Heney et al. 1982; Lutzeyer et al. 1982; Barnes et al. 1985). These new occurrences may result from mucosal field alterations adjacent to the primary tumour. Criteria to identify these patients at risk for progression may be helpful in therapeutic strategy.

Histological grade has been regarded as an important predictive feature next to tumour stage. Tumour grading is however impaired by a considerable lack of intraobserver and interobserver agreement (Busch et al. 1975). Pathologists may furthermore be inclined to classify many tumours as intermediate grade carcinoma (WHO-system: grade 2). The heterogeneous behaviour of these intermediate lesions has been emphasized in various studies (Wijkstroem et al. 1984; Jordan et al. 1987). The concept is gaining acceptance that the majority of transitional cell carcinomas exists either as non-aggressive low grade lesions or aggressive high grade carcinomas (Jordan et al. 1987). From this point of view, there is a need for meth-

Offprint requests to: C.E.M. Blomjous

ods that can reduce the number of intermediate cases.

The need of other predictive criteria in addition to tumor stage and grade has stimulated the examination of several cellular surface antigens and nuclear DNA content by various techniques (Lamb 1967; Fossa et al. 1977; Pauli et al. 1978; Wiley et al. 1982). Nuclear morphometry and DNA flow cytometry have thus far been shown to be particularly valuable (Tribukait et al. 1982; Gustafson et al. 1982; Ooms et al. 1983a; Blomjous et al. 1988a, b).

The purpose of this retrospective study is to evaluate the clinical usefulness of both quantitative techniques applied to material from the initial and untreated bladder tumour, and to investigate their additional prognostic value over the classic prognosticators, using multivariate analysis.

Materials and methods

Of 121 consecutive patients who had been submitted to transurethral resection of a primary and untreated transitional cell carcinoma of the urinary bladder between September 1974 and August 1979, 58 were selected for this study. Patients were included only when clinical follow up was available. Further criteria were that archival paraffin blocks of formalin fixed tumour specimens still were present and that cauterisation artefacts were not abundant. There were no differences in age, sex, and tumour stage between the original and selected group. One representative tissue block was chosen from each specimen and stained with haematoxylin and eosin. Histological grade was assessed according to the World Health Organization classification (Mostofi et al. 1973) by two independent pathologists, in addition to the examination by routine diagnostic procedures. Complete agreement between these three observations was obtained for 72% of the cases and no differences of more than one grade occurred. In case of disagreement the prevailing grade was assessed. Tumour stage was assigned according to the TNM classification (Harmer 1978).

Clinical features. Forty four patients were male and 14 female. Age ranged from 46 to 88 years, median and mean 69.5 and 68.9 years. All 37 patients with superficial carcinoma (stage Ta and T1) had been initially treated by transurethral resection alone. Treatment of the 21 patients with advanced disease (stage T2 to T4) varied, depending on general health condition and likelihood of cure. Treatment consisted of local radium implantation (3), radical cystectomy (7), conservative radiotherapy (9); 2 patients remained untreated.

Morphometric analysis was performed with a projection microscope, equipped with a 100× oil immersion objective, projecting at a graphic tablet (MOP-Videoplan, Kontron, Munich, FRG) with an optical magnification of 2000×. The nuclear areas of selectively sampled nuclei were measured, according to a previously described morphometric method (Blomjous et al. 1988c). The method essentially entails the selection of the most atypical area of the histological slide (size: approximately 5 low power fields 10×), and subsequent measurement of 10 nuclei that are selected on account of their large size. This method has shown satisfactory intraobserver and interobserver reproducibility (correlation coefficients of mean nuclear areas and standard deviation >0.90; regression coefficients between 0.75 and 1.25). Moreover, from a variety of other morphometric procedures, this selective method has proven to be the most suitable one to discriminate between different tumour grades, resulting to a considerable percentage of 93.2% correctly classified cases from a learning set of bladder tumours (Blomjous et al. 1988c).

DNA ploidy in paraffin embedded specimens was determined using the method described by Hedley et al. (1983), with slight modifications. Sections cut at 50 µm from selected tissue blocks were dewaxed in xylene, rehydrated, and enzymatically dispersed by incubation with protease (Sigma, St. Louis, Missouri, USA; P-5255, 0.05% in 0.9% buffered saline, pH = 7) for 30 min at 37° C, with vigorously intermittent vortex mixing. After mechanical detachment by repeated syringing (21 gauge needles) and filtration through a 50 µm nylon gauze, the cell suspension was centrifugated, washed in Tris-hydrochloride acid buffer, and recentrifugated. The cells were finally stained with the DNA fluorochrome 4′,6′-diaminido-2-phenyl-indole dihydrochloride (DAPI, Sigma D-1388), final concentration 2 µg DAPI/100 ml Tris-hydrochloride acid buffer.

The cell suspensions were analysed with the PAS II flow cytometer (Partec, Arlesheim, Switzerland), using excitation light at 350 nm. Formalin fixed mouse thymocytes served as an external standard for instrument setting. The DNA index was determined from the histograms. The first modal cell peak was considered to represent the G0/G1 peak of the diploid cell population. Samples were regarded as aneuploid when in addition to the G0/G1 and G2/M peaks, one or more other peaks were detected. Furthermore, samples of which the proportion of peritetraploid cells (DNA indices between 1.9 and 2.1) exceeded 10% of the whole cell population were considered as peritetraploid.

Statistical analysis was carried out using the BMDP package (Dixon et al. 1981).

To study the prognostic value of the classic and quantitative features separately, univariate survival analysis according to Kaplan-Meier was performed. Features having continuously varying values were made discrete for this analysis in 2 distinct categories, as indicated in table 2. Differences between the survival (Kaplan-Meier) curves were analysed using the Mantel-Cox statistic. For the evaluation of combined features, multivariate survival analysis was performed using Cox's regression model. These analyses take into account the time at risk from first presentation to either death or last follow up.

The value of the quantitative features to the prediction of progression was also analysed according to Kaplan-Meier, using the Mantel-Cox statistic to evaluate the differences between the curves.

P-values below 0.05 were regarded as significant.

Results

At first presentation 33 patients had a single primary tumour, 17 patients had 2 or 3 tumours, and 8 presented with more than 3 primary lesions. The size of the primary tumour or, in case of multiple lesions, of the largest tumour ranged from less than 1 cm to 12 cm diameter. Eighteen patients presented with tumours up to 3 cm diameter, 17 with lesions from 3 to 5 cm and 23 patients had tumours of more than 5 cm. Ulceration of the tumour was observed at cystoscopic examination in 13 cases.

Table 1 shows the distribution of histological

Table 1. Distribution of tumour grade and stage

	Stage					
	Ta	T1	T2	T3	T4	Total
Grade 1	14	2	0	0	0	16
Grade 2	7	12	7	1	5	32
Grade 3	0	2	5	1	2	10
Total	21	16	12	2	7	58

grade and tumour stage. Sixteen carcinomas were classified as grade 1, 32 as grade 2 and 10 as grade 3. Thirty seven patients had superficial tumours (stage Ta and T1) and 21 advanced lesions (stage T2 to T4).

Nuclear morphometry yielded mean nuclear areas ranging from 49.07 to 269.60 μm^2 (mean and standard deviation: 114.49 and 50.75 μm^2) and standard deviations of 4.0 to 80.89 μm^2 (mean and standard deviation: 15.86 and 16.53 μm^2). Flow cytometry showed 28 tumours to be diploid and 30 aneuploid, of which 13 were peri-tetraploid and 4 had multiple aneuploid peaks. The majority of the superficial tumours (stage Ta and T1) were diploid (24 of 37 cases) and almost all advanced carcinomas (stage T2 to T4) had aneuploid peaks (17 of 21 cases). The mean coefficient of variation of the G0/G1 peak amounted to 6.5% (standard deviation: 2.3%). The distribution of the mean nuclear area and DNA ploidy, as expressed by the DNA index, is shown in Fig. 1.

Table 2 summarizes the differences in survival between subclasses of the features in univariate analysis. Of the classic features, increased tumour size and the presence of ulceration at cystoscopy corresponded with worse survival. Obviously, advanced tumour stage and higher histological grade also had a worse prognosis. The 5-year survival of the patient groups with superficial (stage Ta and T1) and advanced (stage T2 to T4) tumours came to 97% and 42.9% (Fig. 2a), and of the patients with grade 1, 2 and 3 carcinoma to 93.8%,

Table 2. The differences in survival between subclasses of different features

Feature	Number of patients	% 5-year survival	Mantel-Cox value	Probability of no difference
Tumour size				
≦3 cm	18	100	8.73	$p=0.02$
3–5 cm	17	80.2		
>5 cm	23	55.9		
Multiplicity				
$n=1$	33	74.6	0.48	$p=0.8$
$n=2$ or 3	17	81.9		
$n>3$	8	75		
Ulceration				
no	45	86.8	18.48	$p<0.0001$
yes	13	46.2		
Histologic grade				
grade 1	16	93.8	8.71	$p=0.01$
grade 2	32	78		
grade 3	10	50		
Tumour stage				
Ta and T1	37	97	19.44	$p<0.0001$
T2 to T4	21	42.9		
Mean of nuclear area				
≦95 μm^2	25	95.8	9.69	$p=0.002$
>95 μm^2	33	61.4		
SD of nuclear area				
≦10.5 μm^2	31	89.10	5.87	$p=0.02$
>10.5 μm^2	27	63.14		
DNA-ploidy				
diploid	28	92.2	7.31	$p=0.007$
aneuploid	30	62.5		

SD: standard deviation

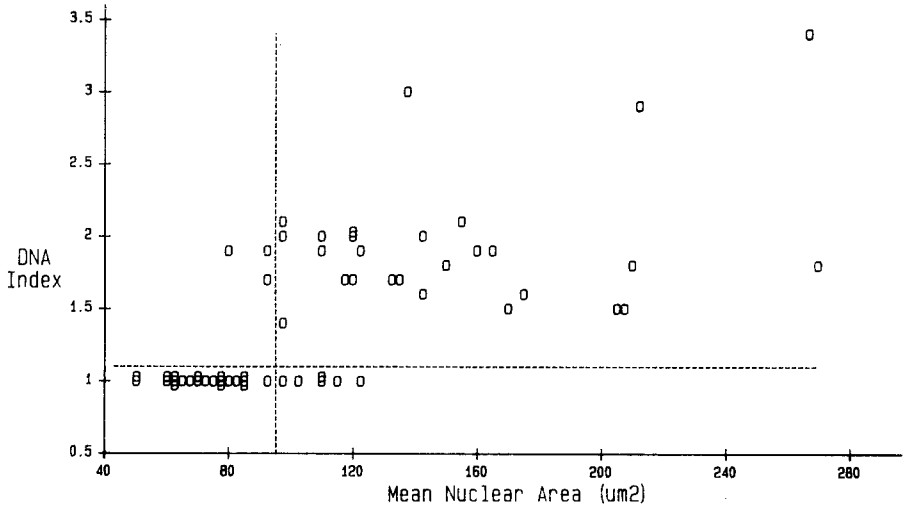

Fig. 1. Distribution of the mean nuclear area and DNA ploidy as expressed by DNA index. *Horizontally dotted line* separates the diploid ($n=28$) from the aneuploid ($n=30$) tumours. *Vertically dotted line* divides the tumours with small nuclei (mean nuclear area ≦95 μm^2) ($n=25$) from those with large nuclei (mean nuclear area >95 μm^2) ($n=33$)

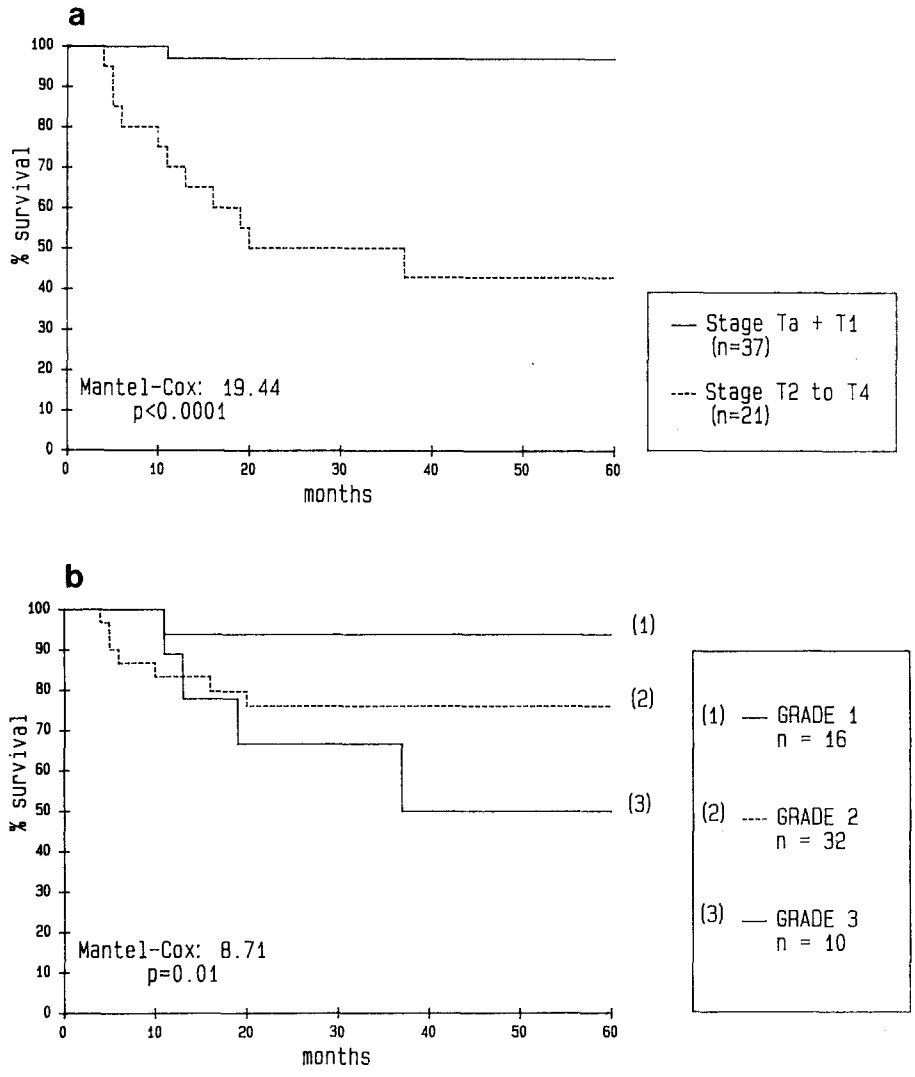

Fig. 2a, b. The Kaplan-Meier survival curves for patients with superficial (stage Ta and T1) and advanced (stage T2–T4) tumours (**a**), and for patients with grade 1, 2 and 3 carcinomas (**b**)

78% and 50% (Fig. 2b). Only the presence of multiple lesions at first presentation was insignificant for the prediction of survival.

Of the quantitative features, an increased nuclear size (mean nuclear area >95 μm^2) concurred with a considerably worse prognosis in comparison with small nuclei (5-year survival 95.8% and 61.4%) (Fig. 3a). The same was true for the standard deviation. Also the survival rate of patients with aneuploid primary tumours was significantly worse than of those with diploid lesions (5-year survival 92.2% and 62.5%) (Fig. 3b).

Tumour stage, being the best single prognosticator of patient survival, was the first variable entered in the multivariate model. Stepwise selection pointed to tumour ulceration as the second variable, and none of the remaining features contributed to the model. Apparently, all the other features, including histological grade as well as the quantitative features, were completely explained by tumour stage and ulceration.

To analyze the prognostic value of the combined features, a prognostic index (PI) was made up, using the coefficients of the proportional hazards model:

$$\begin{aligned}PI = {} & 2.1399 \text{ (tumour stage: Ta} = 1, \text{ T1} = 2, \text{ T2} = 3,\\ & \text{T3} = 4, \text{ T4} = 5)\\ & + 1.1687 \text{ (ulceration: absent} = 1, \text{ present} = 2).\end{aligned}$$

Dichitomization of the patients into subgroups with low and high prognostic scores ($PI \leqq 3.00$; $n = 35$ and $PI > 3.00$; $n = 23$) yielded no substantial improvement of the prognostication, however (5-year survival 96.0% and 48.5%; Mantel-Cox: 20.09; $P < 0.0001$) when compared with the survival prediction by tumour stage alone (see Fig. 2a).

To study the separate value of the combined quantitative techniques, analysis was also carried

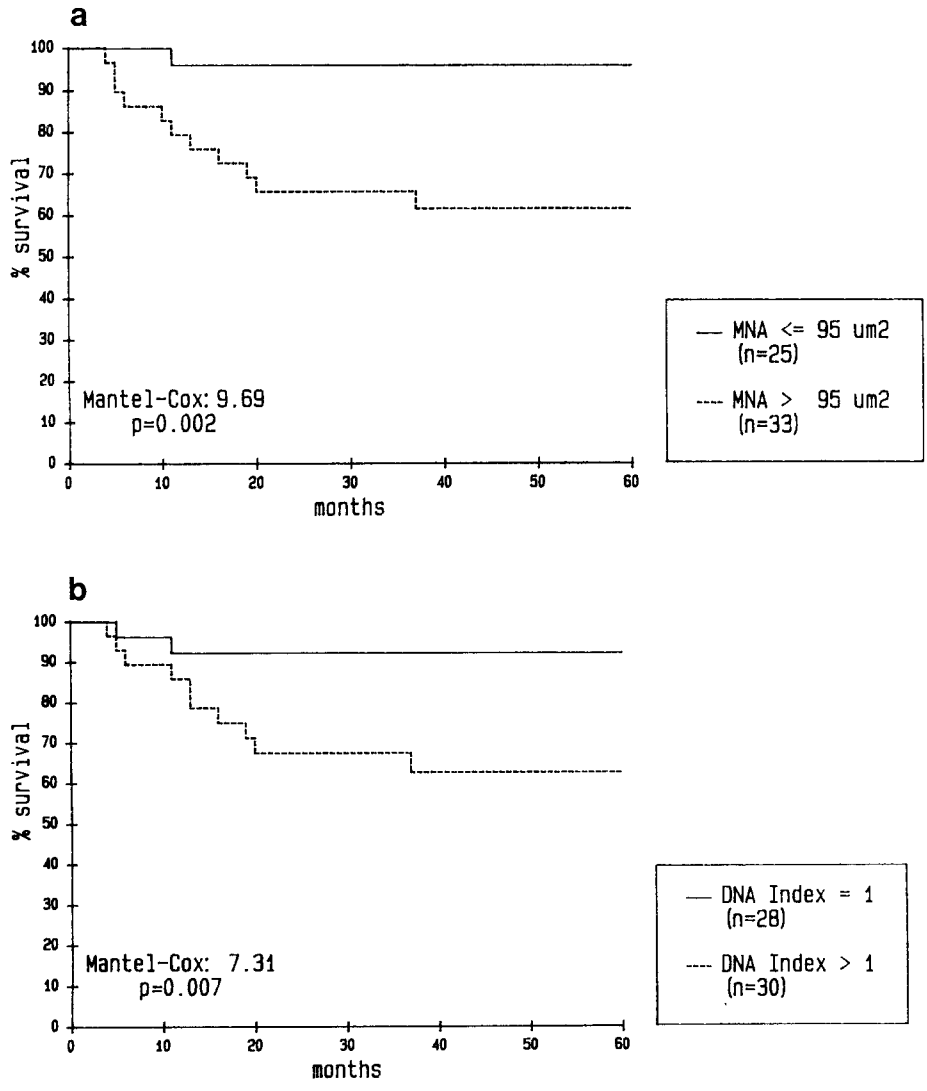

Fig. 3a, b. The Kaplan-Meier survival curves for patients having tumours with small (mean nuclear area $\leq 95\ \mu m^2$) and large (mean nuclear area $>95\ \mu m^2$) nuclei (**a**), and for patients with diploid and aneuploid tumours (**b**)

out leaving the classic features out of consideration. After the entrance of ploidy as the first variable in the multivariate model, the morphometric features did not contribute significantly to the model.

Although the quantitative features apparently had no additional value to tumour staging for the prediction of survival, their significance was expressed by the prediction of progression in superficial disease (stage Ta and T1). Seven of 37 patients developed progressive recurrence. Two of them had progression from low (grade 1 or 2) to high grade (grade 3) carcinoma, in one case accompanied by flat carcinoma in situ. One of them underwent cystectomy because of multiple superficial recurrences and postoperatively manifested with abdominal metastases. Four others suffered muscle invasive recurrent tumour.

Nuclear morphometry and flow cytometry both were valuable predictors of progressive recurrence. Five of the 15 patients with large nuclei (mean nuclear area $>95\ \mu m^2$) developed progression, against only 2 of the 22 patients with small nuclei (mean nuclear area $\leq 95\ \mu m^2$) (Mantel-Cox: 4.78; $p=0.03$), see Fig. 4a. Furthermore, 6 of the 13 patients with aneuploid tumours had progressive disease, opposed to only 1 of the 24 patients with diploid carcinoma (Mantel-Cox: 12.55; $p=0.0004$), see Fig. 4b.

Discussion

Whereas subjective histological grading results in observer disagreement of 20% to 45% (Busch et al. 1975; Ooms et al. 1983b), nuclear morphometry has the advantage of reproducibility (Blomjous et al. 1988c). In a recent study, we have worked out a sensitive, reliable and practical morphomet-

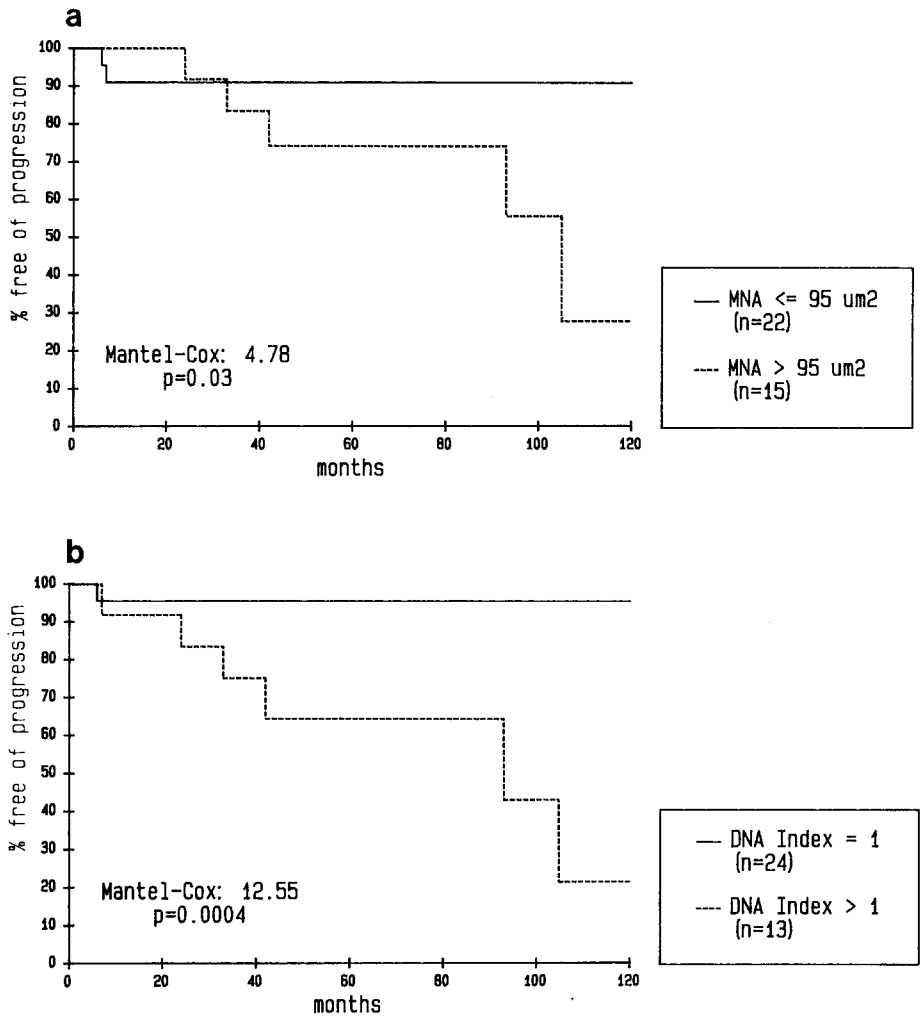

Fig. 4. The Kaplan-Meier curves of progression of superficial carcinoma (stage Ta and T1) for patients having tumours with small (mean nuclear area $\leqq$ 95 μm^2) and large (mean nuclear area >95 μm^2) nuclei (**a**), and for patients with diploid and aneuploid tumours (**b**)

ric method based on selective sampling of nuclei (Blomjous et al. 1988c). This selective method of sampling is responsible for our two to threefold higher values of nuclear areas when compared with the literature (Bjelkenkrantz et al. 1982; Helander et al. 1984; Montironi et al. 1985).

In recent years, flow cytometry of paraffin embedded archival material has become an established method to compare ploidy status of tumours with clinical outcome (Hedley et al. 1983; Frierson 1988). Ploidy data from paraffin embedded specimens are comparable to those obtained from fresh tumour samples, although some difficulties may be encountered (Hedley et al. 1983; Frierson 1988). In particular, peridiploid aneuploid peaks may be overlooked because of wide G0/G1 peaks. However, in a previous study we have found that diploid cases with coefficients of variation below and over 5.5% did not show any difference in histological grade, tumour stage or clinical outcome (Blomjous et al. 1988a). The peridiploid cell populations possibly masked by wide diploid peaks had apparently no clinical relevance.

Cytogenetic analysis has shown that (peri)diploid tumours, in addition to aneuploid carcinomas, frequently also have an abnormal chromosome pattern (Granberg-Oehman et al. 1980; Wijkstroem et al. 1984). Their clinical course is nevertheless usually more benign than of aneuploid lesions (Tribukait et al. 1982; Blomjous et al. 1988a), as also shown in the present study. Increased aggressiveness is apparently especially related to gross quantitative DNA abnormalities (Wijkstroem et al. 1984). Although the underlying mechanism is not fully understood, abnormal DNA content obviously reflects a deeply disturbed cell-biological function that may be translated into more aggressive behaviour.

The current study demonstrates a strong correlation between DNA aneuploidy and increased nuclear size (mean nuclear area >95 μm^2). This explains why combination of both quantitative tech-

niques does not improve the prognostication in comparison with the single methods. Combined employment seems thus unprofitable for diagnostic purposes. The single methods are well matched, although multivariate analysis points to flow cytometry as a slightly more sensitive technique. Nevertheless, morphometry is a fairly simple and less expensive alternative, which can be applied in any routine laboratory.

Histological grading in the current series illustrates the well known propensity of pathologists to classify relatively few lesions as low grade (grade 1) or high grade (grade 3) carcinoma (Jordan et al. 1987). The lack of detailed guidelines apparently results in a large number of intermediate cases (grade 2); in our series coming to approximately half of the tumours.

Using quantitative techniques the patients are separated into only 2 subgroups. The clinical outcome of patients with small nuclei (mean nuclear area $\leqq 95\ \mu m^2$) and diploid DNA histograms (5-year survival 95.8% and 92.2%) approaches that of the grade 1 patient group (5-year survival 93.8%). A similar tendency is found for the subgroups with large nuclei (mean nuclear area $>95\ \mu m^2$) and aneuploid tracings (5-year survival 61.4% and 62.5%) with regard to the grade 3 patient group (5-year survival 50%). When compared with histological grading, the quantitative methods thus hold the advantage that no intermediate group (grade 2: $n=32$) with heterogeneous outcome (5 year survival 78%) is distinguished.

The current study shows that tumour stage is the most significant prognosticator of survival. Ulceration is selected as the second variable by multivariate analysis, but this feature is found in relatively few cases ($n=13$). The prognostication is, furthermore, not substantially improved by combination of both features. The quantitative techniques do not contribute at all in the multivariate prognostic index. They yield apparently no prognostic information in addition to the classic features, and their importance thus consists merely in the objectivation of these features.

The additional value of the quantitative techniques finds especially expression among the patients with superficial carcinoma (stage Ta and T1), as the techniques indicate if a given patient is at risk to develop progressive recurrence. This practically implicates that 'ab initio' a more aggressive treatment may be considered if a patient has a superficial tumour with large nuclei (mean nuclear area $>95\ \mu m^2$) or an aneuploid DNA histogram.

The results of our analyses should be understood in light of the limitations of the material. Our data were obtained from primary and untreated bladder tumours. The effects of subsequent therapy varied however inevitably, as treatment was individualized depending on characteristics of the initial tumour and subsequent recurrences. Our observations are thus related to the tumour behaviour in treated patients rather than to the natural history of transitional cell carcinoma.

In conclusion, nuclear morphometry and DNA flow cytometry are important objective methods to predict survival. Increased nuclear size (mean nuclear area $>95\ \mu m^2$) and DNA aneuploidy are strongly correlated with aggressive tumour behaviour, and their impact contrasts favourably with histological grade. However, tumour stage still remains the most important predictor of survival, as shown by multivariate analysis.

The finding of increased nuclear size or DNA aneuploidy in superficial carcinoma should be regarded as a sign of aggressive behaviour of future tumours, and demands consideration of more aggressive treatment than currently given.

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Received February 27, 1989 / Accepted June 1, 1989