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Karyometry in recurrent superficial transitional cell tumors of the bladder

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Summary. Transitional cell carcinoma of the bladder has a high recurrence rate after local treatment. Progression to a higher stage occurs in 10–30% of the recurrent tumors, and early detection of potentially progressive tumors is important. In the current study morphometric, densitometric, and chromatin textural features of nuclei of superficial bladder tumors (pTa-T1) were studied to determine the value of karyometric features in the prediction of tumor progression. Seventy-two histological samples from 36 patients, consisting of both the primary and the first recurrent superficial tumor, were analyzed. Patients were divided into two groups: those with tumor progression, defined as an increase in tumor stage or occurrence of metastatic disease, and those without. Discriminant analysis on four karyometric features resulted in correct prediction of prognosis of 78% and 97% in the primary and recurrent tumors, respectively ($P < 0.001$). Tumor grade and stage did not offer additional information concerning prognosis. Karyometric analysis of recurrent superficial transitional cell tumors can be useful in selecting patients who need a more aggressive therapy. However, tumor characteristics of recurrent tumors varied and continuous evaluation of the karyometric features is necessary for early detection of an increase in the malignant potential of the tumor.

Key words: Karyometry – Bladder – Carcinoma, transitional cell

Superficial transitional cell carcinoma (TCC) of the bladder accounts for approximately 80% of all newly diagnosed tumors of this organ [24]. The tumor is treated by transurethral resection with or without intravesical instillation with chemo- and/or immunotherapeutic agents. However, 30%–90% of the tumors recur depending on grade, multifocality [24], and the treatment modality. Thio-TEPA resulted in a 49% recurrence rate [21]. Intravesical Epodyl therapy resulted in recurrence rates of 21–50% [18], Adriamycin showed recurrences in 40–70% [2, 15], and mitomycin C in 10–20% of cases [12]. BCG instillations after TUR reduced the recurrence rate to 22% in one study [23], but seemed of no value of reducing the number of recurrences in stage T_1 tumors. The multifocal occurrence of bladder tumors suggests a general disease of the urothelium rather than a localized process. This can explain the high recurrence rate. Although superficial at the time of diagnosis, 10–30% of these tumors become invasive or metastasize in the course of the disease [9]. In case of frequent recurrences and multiple tumors, progression rate can be as high as 83% [1].

Early identification of patients with progressive superficial bladder tumors has implications for the treatment, since a more aggressive treatment is indicated for more malignant, progressive tumors. Nuclear features of tumor cells in transitional cell tumors showed predictive value for recurrence and prognosis [4]. Several nuclear features seemed to be of importance in predicting the prognosis with bladder tumors. Nuclear profile area, its standard deviation and DNA content (2cDI and 5cER) correlated best with visual assessed tumor grade [4, 7, 10, 20, 28, 29]. Ooms et al. [20] introduced a useful quantitative grading system with measurements of superficial, large, and deep cell nuclei separately in histological samples. De Prez's group [7] described the use of an image analysis system (Samba 200) measuring morphometric, densitometric, and chromatin pattern features. These quantitative findings correlated well with visual grade. Karyometric analysis enabled subdivision of the grade-II tumors based on the 5cER [19]. Earlier flow cytometric (FCM) studies [10, 26] indicated a subdivision of grade-II tumors based on ploidy and proliferation rate, illustrating the diversity of tumors visually graded as grade II. In the current study we analyzed whether nuclear features quantitized by image analysis might play a role in predicting the prognosis in recurrent superficial bladder tumors. To test image analysis as a tool for patient follow up, the technique was applied in consecutive samples, in order to compare primary and recurrent tumor of the same patient.

Table 1. Karyometric features. For each feature, mean, standard deviation (SD) and 90th percentile (NIN) is calculated

Morphometric

Nuclear profile area (NPA)
Nuclear perimeter (PERI)
Maximal diameter (MAXD)

$$\text{Form PE (FormPE)} = \frac{\text{minimal diameter}}{\text{maximal diameter}}$$

$$\text{Form ELL (FormEll)} = \frac{4\pi\ \text{NPA}}{(\text{perimeter})^2}$$

Descriptors of smoothed Freeman difference chain (SFDC) code [3, 6].

a. MAC (mean absolute curvature) $= \frac{1}{C * N} \sum_{i=1}^{N} K(n)$

(C = number of contour pixels, N = width of smoothing operator, K(n) = SFD value in *n*)

b. MBEN (max. bending energy) (= difference between highest and lowest value in SFDC code)

c. PASS (= number of passes through threshold in SFDC code)

Densitometric

Optical density (OD)
Integrated optical density (IOD)
Variance of OD of pixels within nucleus (ODVAR)
Coefficient of variation of OD of pixels per nucleus (ODCV)
2c Deviation index (2cDI)
5c Exceeding rate (5cER)

Textural

Measurement of nuclear border staining (NBORDER) by calculating the weighted mean staining of the nucleus for which the weighing factor decreases when the pixel is more distant from the nuclear border

Markovian texture features, based on co-occurrence matrix of the pixel values after using histogram equalization (H) and linear requantization (L) for recoding pixel values in 8 value groups:

$H_1 + L_1$: entropy
$H_2 + L_2$: difference moment
$H_3 + L_3$: inverse difference moment
$H_4 + L_4$: rotation moment
$H_5 + L_5$: inverse rotation moment

Materials and methods

Patients

Thirty-six patients with superficial bladder tumors (stage Ta-T1) underwent complete transurethral resection (TUR) of the tumor and were treated with 15 intravesical instillations of Adriamycin. Primary and recurrent tumors were resected, and graded and staged by the pathologist. A mean follow up of 4.8 years (3–10 years) was available. The patients were divided into two groups: patients with progressive and those with non-progressive tumors during follow up. Tumor progression was defined as an increase in tumor stage to T2 or more or the appearance of metastases.

Materials

Paraffin-embedded, formalin-fixed TUR material was available for all patients from the primary and recurrent tumor. Four µm sections were cut, deparaffinized in xylene, rehydrated, and stained according Feulgen-Schiff (hydrolysis in 5 *N* HCl for 60 min and 30 min in Schiff reagent (Merck, Darmstadt, FRG) at room temperature)

Quantitative microscopy

Image analysis was performed with a VS100-AT framegrabber board (Imaging Technology, Woburn, USA) in a personal computer (Compaq 386s). A videocamera (HCS-CCD, MXR, Vision Technology, Eindhoven, The Netherlands) connected to an Axioskop light microscope (Zeiss, Oberkochen, FRG) was used to record the images. Hematoxylin-stained slides were used by the pathologist to mark the tumor areas of interest. Ten randomly selected images per slide were measured in these areas with a 40 times objective (pixel size 0.024 μm^2). Analysis of one image took 3 min, measuring 8 morphometric, 4 densitometric, and 11 chromatin texture features. The 5c exceeding rate (5cER) and 2c deviaton index (2cDI) were calculated, with 30–50 lymphocytes as internal reference. The 5cER represents the percentage of definitely aneuploid cells, the 2cDI the (mean square) deviation from the diploid value [5]. Software used was written in TIM (TEA, Dordrecht), an image analysis language offering several basic modules for image recording, handling, and analysis. Additional software was written in TURBO Pascal for chromatin texture analysis, Freeman chain code analysis for shape description, and data handling.

Prior to image segmentation, the image was corrected for shading and a median filter was applied. Selection of nuclei was primary based on size and values for maximal bending energy (MBEN), in order to eliminate overlapping nuclei and artefacts [3, 6]. Visual inspection of the images overlayed by contours and numbers of the selected nuclei enabled screening for out-of-focus cells or artefacts.

The nuclear features measured are described in Table 1. 27 karyometric features were statistically analyzed. Of each feature the mean, standard deviation (SD) and 90th percentile was calculated. The measurements were divided into three groups: (1) values of primary tumor; (2) values of recurrent tumor; and (3) differences in values between primary and recurrent tumor.

The SPSS/PC+ package (SPSS, Chicago, Ill.) was used for statistical analysis. Mann-Whitney U-test and discriminant analysis were applied. To reduce the number of features in the discriminant analysis a selection of features was made based on results from the Mann-Whitney U-test, results from earlier studies [19], and correlation with tumor grade. The features selected this way were: the 90th percentile of the nuclear profile area, the SD of MAC [descriptor of nuclear shape (Table 1); the standard deviation represents the degree of nuclear polymorphism], 2cDI, and the mean of Markovian feature H3, a descriptor of chromatin pattern, based on the co-occurrence matrix. These features describe the presence of very large nuclei (NIN of NPA), the presence of nuclear polymorphism (SD of MAC, standard deviation of MAC, a descriptor of nuclear shape), the variance in DNA content (2cDI), and uneven distributions in chromatin pattern.

Results

Progression and grade and stage

Twenty-three patients did not show tumor progression after the recurrent superficial tumor. Progression was found in 13 patients during follow up (33%). Histological tumor grade in the primary-tumor group was not significantly different from the recurrent-tumor group ($P > 0.05$). Whereas the tumor grade of the recurrent

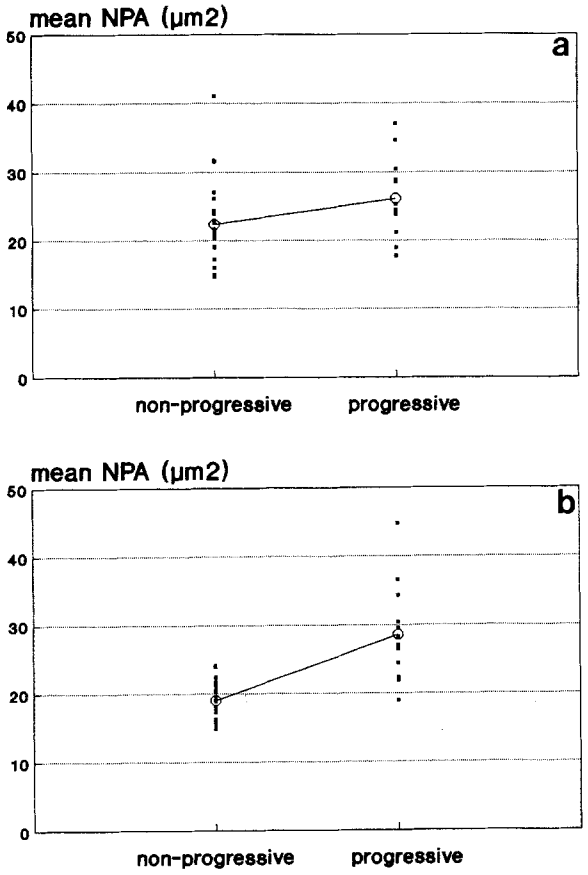

Fig. 1 a, b. Mean nuclear profile area (NPA) in non-progressive and progressive tumors (**a** primary umor, **b** recurrent tumor)

tumor was significantly higher in progressive tumors ($P < 0.01$, Chi-square test), tumor grade was not correlated with progression in the primary tumor. Like Kaubisch et al. [14], we did not find progressive tumors in patients with only grade-1 lesions as recurrent tumor, whereas only one patient with a grade-1 primary tumor showed progression. Twenty-two percent of the tumors showed an increase in tumor grade in the recurrent tumors, which is in agreement with other studies [8]. All tumors with a decrease in tumor grade between the primary and the recurrent lesion did not progress, in contrast to 4 of 8 cases which showed an increase in tumor grade ($P = 0.03$, Chi-square test).

Stage of the primary and recurrent tumors (T_a or T_1) was not significantly different. In the recurrent cases tumor stage was higher (T_1) in the tumors that subsequently progressed ($P < 0.05$, Chi-square test). Tumor stage of the primary tumor did not differ between progressive and non-progressive tumors. There was a tendency to higher tumor grade in stage-T_1 tumors as against stage-T_a, though this was significant neither in the primary, nor in the recurrent tumor.

Karyometry and grade and stage

Several karyometric features showed a correlation with histological tumor grade in the primary as well as in the recurrent tumors. Of the morphometric features, the mean nuclear profile area increased with tumor grade as did its coefficient of variation, indicating a higher anisokaryosis in the higher tumor grades. Grade-3 tumors had significantly higher values for 2cDI and 5cER, however, several cases of the high-grade tumors had values within normal range. Features in both primary and recurrent tumors showed similar correlations with tumor grade (Fig. 1). The results of the discriminant analysis resulted in 83% correct classifications. Karyometric features could not discriminate between stage-T_a and stage-T_1 tumors.

Table 2. Karyometric features that were significantly different ($P < 0.001$) between progressive and non-progressive tumors in primary and recurrent tumors expressed as *P*-values from Mann-Whitney U-test. When both primary and recurrent tumor features were not significant, the feature is not shown

	Significance of difference between N and NP in primary tumor	Significance of difference between N and NP in recurrent tumor	Significance of difference between N and NP in difference values[a]
Mean NPA	n.s.	$P < 0.0001$	$P < 0.001$
- SD NPA	$P < 0.001$	$P < 0.0001$	n.s.
- NIN NPA	n.s.	$P < 0.0001$	n.s.
- CV NPA	$P < 0.001$	$P < 0.0001$	n.s.
Mean PERI	n.s.	$P < 0.0001$	n.s.
- SD PERI	$P < 0.001$	$P < 0.0001$	n.s.
- NIN PERI	n.s.	$P < 0.0001$	n.s.
Mean MAXD	n.s.	$P < 0.0001$	n.s.
- SD MAXD	n.s.	$P < 0.0001$	n.s.
- NIN MAXD	n.s.	$P < 0.0001$	n.s.
Mean FPE	n.s.	$P < 0.001$	$P < 0.001$
Mean MBEN	n.s.	$P < 0.001$	n.s.
- SD MBEN	n.s.	$P < 0.0001$	n.s.
- NIN MBEN	n.s.	$P < 0.001$	n.s.
- SD MAC	$P < 0.001$	n.s.	n.s.
- NIN MAC	n.s.	$P < 0.001$	n.s.
- CV MAC	$P < 0.001$	$P < 0.0001$	n.s.
mtDNA	$P < 0.001$	$P < 0.001$	n.s.
mtVOLUME	n.s.	$P < 0.001$	n.s.
Mean IOD	n.s.	$P < 0.001$	n.s.
- SD IOD	n.s.	$P < 0.001$	n.s.
- NIN IOD	n.s.	$P < 0.0001$	n.s.

[a] Difference values are the differences of the karyometric feature values between primary and recurrent tumor, or, in other words, the changes in the karyometric features of the recurrent tumor compared with the primary lesion. SD, standard deviation; NIN, 90th percentile; CV, coefficient of variation (ratio of SD and mean)

Karyometry and progression

The results of the Mann-Whitney U-test showed a significant difference ($P < 0.005$) between progressive and non-progressive tumors in four of the tested karyometric features in the primary tumors and 19 features in the recurrent tumors (Table 2, Fig. 1).

Table 3. Classification result with discriminant analysis of karyometric features for prediction of tumor prognosis of samples of the recurrent tumor. (Method: Wilk's; *F*-to-enter = 3)

Standardized canonical discriminant function ($P < 0.0001$)		
Discriminant score = (0.88226 * SDMAC) + (1.11261 * 90th NPA)		
Follow-up	Predicted prognosis	
	NP	P
NP ($n = 23$)	23 (100%)	0 (0%)
P ($n = 13$)	1 (7.7%)	12 (92.3%)
Correctly predicted: 97.4% (35/36)		

Table 4. Classification result with discriminant analysis of karyometric features for prediction of tumor prognosis of samples of the primary tumors. (Method: Wilk's; *F*-to-enter = 3)

Standardized canonical discriminant function ($P < 0.001$)		
Discriminant score = (1.20846 * SDMAC) + (1.00225 * H3)		
Follow-up	Predicted prognosis	
	NP	P
NP ($n = 23$)	18 (78.3%)	5 (21.7%)
P ($n = 13$)	3 (23.1%)	10 (76.9%)
Correctly predicted: 77.78% (28/36)		

The Wilks method was used in a leave-one-out stepwise discriminant analysis (*F*-to-enter 3) to calculate a canonical linear discriminant function using the four selected feature values to discriminate between progressive and non-progressive tumors. The correct classification based on the selected nuclear features was highest in the recurrent-tumor group (97%). All non-progressive tumors ($n = 23$) and 12 of 13 (92%) progressive tumors were classified correctly (Table 3, Fig. 2a). Based on the primary tumors, a correct prediction of prognosis was obtained in 18 of 23 cases (78%) with no tumor progression (Table 4, Fig. 2b). Of 13 tumors, 10 (77%) with tumor progression were classified correctly (Table 4). This means correct classification of 78% and 97% for the primary and recurrent tumors, respectively (Fig. 2a, b). When the discriminant function found in the recurrent tumors was applied on the primary tumor group the percentage of correctly classified cases did not change (Fig. 3).

Tumor grade and stage were entered in the discriminant analysis in addition to the karyometric features. However, due to low *F*-values, tumor stage, grade, and changes in tumor grade were not selected in the stepwise analysis: i.e. there is no additional value of the classical features to the karyometric features for the prediction of tumor progression.

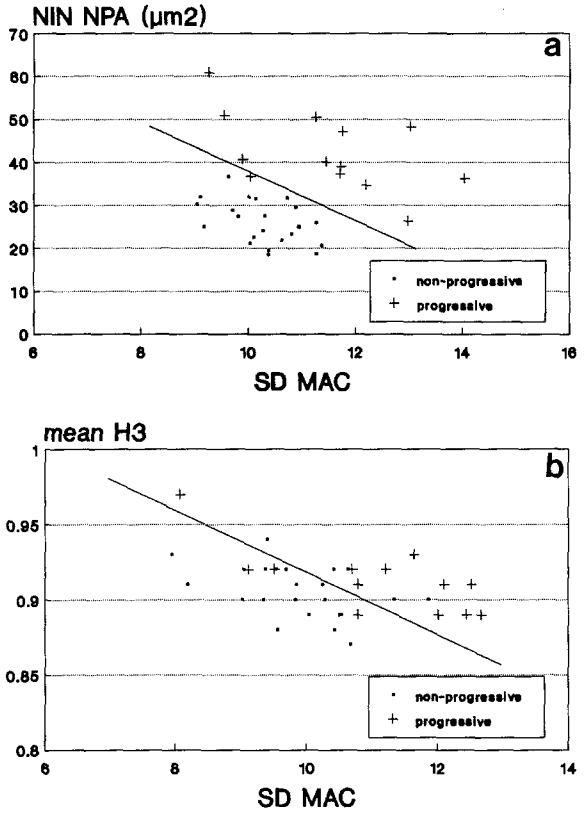

Fig. 2. a, b. Features selected in discriminant analysis on base of F-value for the primary and recurrent tumors. The line represents the zero-scores of the canonical discriminant function (**a** recurrent tumor, **b** primary tumor). Note the different karyometric features selected in the discriminant analysis for the primary and recurrent tumor group: SD of MAC was a useful feature in both groups: best classification was obtained of SD MAC in combination with NPA (nuclear profile area) in the recurrent tumors (**a**) and in combination with mean H3 (nuclear chromatin pattern) in the primary tumors (**b**)

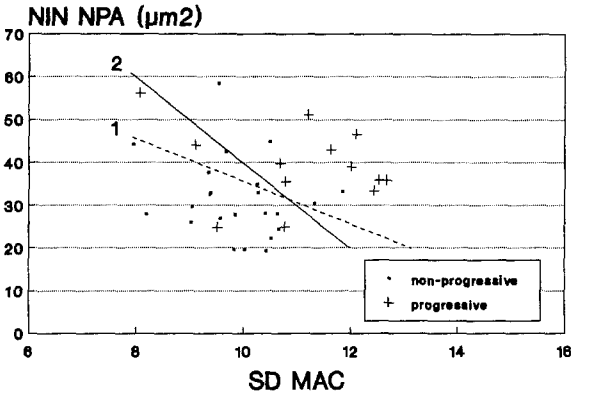

Fig. 3. Canonical discriminant function of the recurrent tumor plotted in the primary tumor group (1). *Line 2* represents the zero discriminant function scores when the analysis was done on the primary tumor group, using the two features (NIN NPA and SD MAC). In both cases (*line 1* and *line 2*) suboptimal division of progressive and non-progressive cases is obtained

All recurrent tumors and six of seven primary tumors with a 5cER higher than 10%, suggesting non-diploid tumor cells, showed progression during follow-up (Fig. 4). A less clear cut-off value was found for the 2cDI. A 2cDI

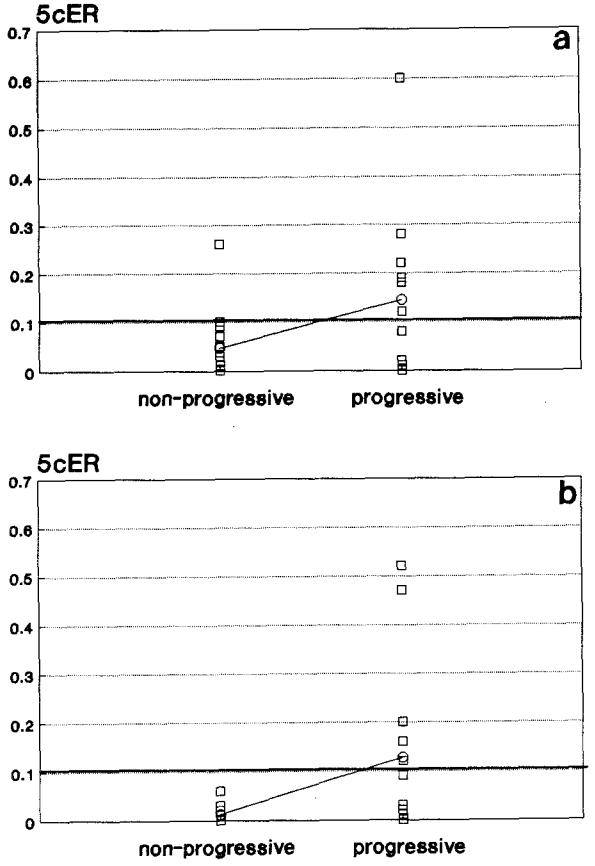

Fig. 4. 5c Exceeding rate (5cER) in non-progressive and progressive tumors (**a** primary tumor, **b** recurrent tumor). Only one of seven cases (a primary tumor) showed a 5cER value over 10% but did not progress

value over 6c was always accompanied with tumor progression in the recurrent tumor. However, 21% of tumors with a 2cDI lower than 6c also progressed.

Discussion

Superficial transitional cell tumors of the urinary bladder are characterized by a tendency to recur after resection. Gilbert et al. [8] reported a recurrence rate of 70% and found an increase in tumor grade in 25% of the recurrent tumors. Other studies reported up to 90% recurrence rate [24] depending on grade, stage, and treatment modality used [2, 11, 15, 18, 21, 23].

From a prognostic point of view the risk of invasion of a treated primary superficial tumor is more important than recurrence rate. In patients with a recurrent tumor, 10–30% progression to invasion is detected [9]. Moreover, Althausen et al. [1] reported progression rates as high as 83% in patients with frequent tumor recurrences and multiple tumors. For superficial bladder tumors, progression and recurrence rate are highest in pT1G3 tumors [11]. However, progression rates for pTaG3 and pT1G2 tumors are similar, indicating that tumor stage alone cannot predict tumor progression accurately. We found similar progression rates for these tumors. The pathological staging results from the present study, however, showed no difference in progression rate between all pTa and pT1 primary tumors. Although tumor staging in small superficial bladder tumors is often difficult [13] and might have caused the discrepancy between these findings and earlier studies, we did find different progression rates for pTa and pT1 tumors when tumor material from the recurrent tumors was staged.

In the case of superficial bladder tumor, apart from tumor stage several tumor characteristics have been investigated for prediction of tumor behavior. Tumor grade is generally used as a stage-independent indicator of tumor behavior. The subjectivity of tumor grading has led to quantitative methods to describe tumor cells and nuclei in microscopic images.

In the present study quantitative light microscopy is used to characterize cell nuclei in bladder tumors. Since only a limited number of nuclei can be analyzed, selection criteria will influence results. Contrary to other studies [4, 20] on selected nuclei, in the present study only the tumor area was selected to reduce the subjectivity. Subsequent analysis was performed in randomly chosen images within this area. This procedure was also used in an earlier study [28] and resulted in good correlation between grading and karyometric features.

Several nuclear features were significantly different between the progressive and non-progressive tumors in the primary tumor group, e.g. primary tumors containing non-diploid cells, presumably reflected by high values for 2cDI and 5cER often progressed. Although Blomjous et al. [20] found a correlation between the karyometric features of the primary tumor and progression rates, the present data showed best prediction of progression by karyometric features of the recurrent tumors. The presence of large nuclei in recurrent tumors, measured by the 90th percentile of the nuclear profile area was correlated with tumor progression. This is in agreement with earlier studies [4, 20]. Blomjous et al. [4] found increased recurrence rate and progression in patients with tumor cells with large nuclei (mean profile area $> 95\,\mu m^2$). The presence of large nuclei correlated with tumor grade in a study by Ooms et al. [20]. Unlike the present study, these histological studies [4, 20] applied criteria for selection of nuclei based on nuclear size which may given rise to reproducibility errors [19]. Reconsidering the data of these studies, it is clear that atypic cells can be characterized by karyometric analysis measuring nuclear size.

Nuclear polymorphism and atypic shape are characteristics of high-grade tumors. Montironi et al. [17] could discriminate the different tumor grades using the nuclear roundness factor (NRF). For reasons discussed elsewhere [30] the NRF is theoretically less useful in grading bladder tumors. Therefore we choose, besides the NRF a shape descriptor derived from the Freeman chain code of the nuclear profile contour. This method enables detailed analysis of convexities and concavities in the nuclear structure [6]. Although the mean NRF was significantly lower in progressive tumors, indicating more abnormal nuclear shapes in these tumors, the standard deviation of the MAC, a Freeman chain code derived feature, had

additional value to nuclear size for predicting progression and is thus preferable over the NRF.

The finding that features of the primary tumor were of less predictive value, using either the classic or the karyometric features is somewhat disappointing and illustrates the importance of regular follow up of patients with superficial bladder tumors. In seven cases the classification of the primary tumor was different from that of the recurrent tumor (in two cases this meant 'down-grading': i.e. whereas the primary tumor predicted progression, the recurrent tumor did not). In none of the seven cases was prediction based on the primary tumors correct, and only in four was the change in karyometric prediction accompanied by a change in grade or stage, which illustrates the grade-independent prognostic value of karyometric analysis.

Although the discriminant function in primary and recurrent tumors is not equal, Fig. 3 illustrates that the classification results do not improve when the function derived for the recurrent tumor is used to classify the primary tumors (see line 1 in Fig. 3).

Soloway [25] divided recurrent tumors into 'true recurrences' and 'new occurrences'. Another possible cause of recurrence is tumor implantation [25]. It is difficult to determine what cause of tumor recurrence underlies the recurrent tumors in the present study on the basis of karyometric features alone. To test the similarity of the two tumors, the differences of the karyometric feature values between primary and recurrent tumor were taken into analysis. Differences in karyometric features did not correlate with progression. Whereas these differences, in addition to the primary tumor data, were valuable in predicting progression, no more predictive value additional to the data from the recurrent tumors was shown. We therefore conclude that a change in tumor as measured with karyometric analysis cannot predict progression and that it is the data from the recurrent tumor rather than from changes in the primary tumor that determine prognosis.

In conclusion, karyometric analysis of recurrent superficial bladder tumors has a strong predictive value for tumor progression. Multivariate analysis techniques indicate additional predictive value of the features that describe the presence of extremely large (NINAR) and polymorph (SD MAC) nuclei. Visual grading of the histological slides then has no additional value. All karyometric features could be measured on routine formalin-fixed, paraffin-embedded material, and can easily be incorporated in routine diagnostic procedures. The low predictive value of the primary tumor however, indicate the need for careful karyometric follow-up for recurrent bladder tumors.

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