Virchows Arch A Pathol Anat (1990) 416:521–525

Virchows Archiv A
Pathological Anatomy and Histopathology

The reproducibility of cytomorphometrical grading of bladder tumours

H.G. van der Poel[1], M.E. Boon[1], E.A. van der Meulen[2], and A. Wijsman-Grootendorst[1]

[1] Leiden Cytology and Pathology Laboratory, Leiden, The Netherlands
[2] Department of Mathematics, University of Groningen, Groningen, The Netherlands

Received May 31, 1989 / Received after revision November 2, 1989 / Accepted December 29, 1989

Summary. The reproducibility of cytomorphometrical nuclear area measurements on transitional cell carcinoma is studied. The inter-individual consistency is low ($r=0.55$, $P=0.0005$) when nuclei for measurement are chosen at random. When we use a systematic analysis based on cell situation and cytological features of malignancy to select the 50 nuclei for measurement per slide, the consistency between two technicians appears to be significantly higher ($r=0.90$, $P=0.015$). Not only inter-individual consistency increases using the selection, but the correlation with histological tumour grade also improves significantly for both technicians. The results show that cytomorphometry is a method of grading bladder carcinoma when an accurate selection of nuclei to measure is used. Cytomorphometrical studies should contain a clear description of the way the nuclei for measurement are chosen.

Key words: Bladder tumour – Grade – Cytomorphometry – Reproducibility

Introduction

Bladder neoplasms rank in the ten most common causes of cancer death in many countries (Silverberg 1985). The tumour grade correlates with prognosis (Jordan et al. 1987; Koss 1985) and nuclear area significantly correlates with tumour grade (Colpaert et al. 1987; Kalnins et al. 1970; Montironi et al. 1985; Ooms et al. 1982; van der Poel et al. 1988). These facts make morphometry a valuable method in the grading of bladder tumours.

The grading of bladder cancer is based on the parts of the tumour with the most pronounced anaplasia (Colpaert et al. 1987). The urine of patients with bladder tumours often contains a mixture of high-grade tumour cells, lower-grade tumour cells and normal urothelial cells (Colpaert et al. 1987). When measuring exfoliated cells in urine it is important to measure only those cells that represent the true tumour grade, which makes selection inevitable (van der Poel et al. 1988).

The problem with selection is the risk of subjectivity decreasing reproducibility. For this reason selective morphometry can only be used if the criteria for the selection of nuclei are clear and if there is little inter-individual and intra-individual inconsistency (Ooms et al. 1983). Selective morphometry was compared with non-selective morphometry to answer the questions: how reproducible are the cytomorphometrical measurements of bladder tumours, can selection of nuclei increase reproducibility and how does selection affect the correlation of cytomorphometry with the histomorphometrical grading?

Materials and methods

The material consisted of 39 consecutive cases with the diagnosis of bladder tumour, graded histologically according to the WHO system. The histological material obtained by transurethral resection was graded histologically and histomorphometrically according to the method described by Ooms et al. (1983). The histomorphometrical grading served as the basis for the grading of the cytological material. Eleven were graded as grade I, 16 as grade II and 12 as grade III. The cytological material was obtained by means of bladder washing with physiological saline solution (Zein et al. 1984). The material was obtained in the Westeinde Ziekenhuis in The Hague. The earlier described fixation process was used (van der Poel et al. 1988). The sediment obtained after centrifuging was stained using the Papanicolaou method.

Cytomorphometry. Cytomorphometrical measurements were made using a microscope connected with a computer MOP-video plan of KONTRON. Fifty nuclei were measured in each slide, and each slide was measured twice by two technicians. Both technicians did one measurement at random and one after selecting the nuclei. The cytomorphometrical measurements took about 15 min per slide.

In the selection process the slide is first screened for single, highly malignant cells. These cells are always found in the exfoliat-

Offprint requests to: H.G. van der Poel, Leiden Cytology and Pathology Laboratory, P.O. Box 16084, 2301 GB Leiden, The Netherlands

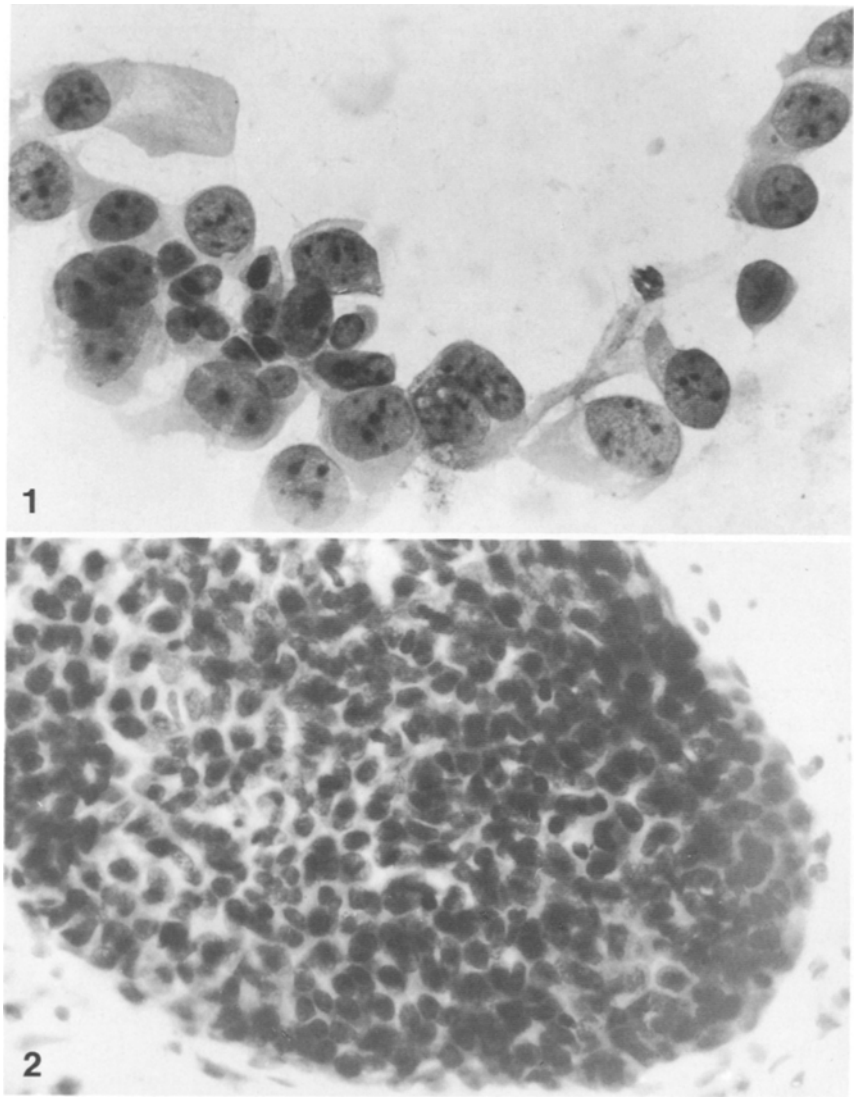

Fig. 1. High-grade malignant cells of grade III tumour material. Increased N/C ratio, eccentric position of the nuclei and multiple nucleoli are present. ×400

Fig. 2. Large papillary cell group as present in grade I tumour material. ×150

ed material of high-grade carcinomas. Not all loose-lying cells are malignant; squamous cells and normal urothelial cells do occur singly but can easily be recognized as benign (Crabbe 1971). The cellular features of the single, high-grade malignant cells are as follows: the cytoplasm is variably dense, with vacuoles and irregular cell shapes; the nucleus is enlarged with an increased N/C ratio (approx. >0.60, Boon et al. 1981), is eccentrically situated in the cytoplasm, of irregular shape, with a prominent nuclear border, coarse chromatin pattern, and multiple, irregularly shaped, prominent nucleoli with an unfavourable low nucleus/nucleolus ratio (Fig. 1).

When single cells are present with the above-mentioned features these are used for morphometry.

When no loose-lying cells with the described malignant features are present, the slide is screened for small papillary cell groups.

Two types of small papillary cell groups are selected, the first consisting of loose clusters of only 5–15 clearly malignant cells and the second of small groups of less than 50 cells with round nuclei with little nuclear overlapping.

If neither of these two types of cell groups are found, the slide is screened for large papillary groups. These consist of more than 50 cells, have smooth outlines, consist of many nuclei and are often very dense, making measurement possible only at the edges of the cell groups (Fig. 2).

Statistical analysis. As in our earlier studies (Boon et al. 1981; van der Poel et al. 1988) we found a strong linear dependency between standard deviation and mean nuclear area (mean of correlation in the four groups: 0.83). The statistical analysis of the data is complicated by the fact that a systematic correlation exists between standard deviation and mean value. In an earlier histomorphometrical study Ooms et al. (1983) also found this correlation. We attempt to eliminate this correlation by applying a variance-stabilizing transformation on the original data. We tested this method and found it acceptable. As variance-stabilizing transformation we used the natural logarithm.

In order to investigate the inter-individual reproducibility of a certain factor when measured by two different technicians using the same protocol one should, at least in our opinion, pay attention to the following guidelines:

1. The measurements should be highly correlated. How large the correlation coefficients should be is a somewhat subjective affair. We propose that $r>0.90$ or $r>0.95$. An interpretation of r is that the bivariate scatterplot is shaped as an ellipse for which the length of minor axis l_1 is $\sqrt{(1-r)}/\sqrt{(1+r)}$ times the length of the major axis l_2 (for $r=0.90$ $l_1:l_2=1:\sqrt{19}$, for $r=0.95$ is $l_1:l_2=1:\sqrt{39}$).
2. The measurements should be exchangeable. This implies, among other things, that the measurements performed by one technician might have been performed by any other technician just as well. This is tested by assuming joint normality of (X, Y), where X

Table 1. The log mean variables and standard deviation of nuclear area for the two technicians

	Grade 1			Grade 2			Grade 3		
	n	mean	SD	*n*	mean	SD	*n*	mean	SD
Technician A									
selective	11	3.58	0.14	16	4.17	0.16	11	4.67	0.25
at random	11	3.66	0.19	15	3.95	0.20	11	4.12	0.17
Technician B									
selective	5	3.34	0.12	16	4.20	0.29	11	4.74	0.27
at random	5	3.61	0.17	16	3.86	0.20	11	4.21	0.21

Table 2. Results regarding the reproducibility of selective and non-selective morphometry (two technicians)

Selective	*r*	0.90
	P-value	0.015
Non-selective	*r*	0.55
	P-value	0.5*E-3

Table 3. Correlation between histological grade and the log mean nuclear area

	Selective	Non-selective	Significance of increase
Technician A	($n=38$)	($n=38$)	
	0.93	0.66	*
Technician B	($n=33$)	($n=34$)	
	0.86	0.50	**

is the random variable describing the measurement of technician A and Y that of technician B and using the likelihood-ratio statistic for testing whether X and Y have the same distribution.

In order to test whether the inter-individual sample correlation coefficient increases significantly due to the selection process we applied a divided sample approach (to obtain independent samples) and used Fisher's z-transformation to obtain a test statistic.

To test the increase in consensus between cytomorphometry and histomorphometry when using selection, let X denote a certain factor measured without selection and let Y denote the same factor with selection of nuclei to measure. In order to test whether X and Y are equally correlated with G (=grade) we postulate (to avoid statistical complexity) the ratio of variance of X to that of Y to be known and equalling, τ^2, say. Now testing $\mathcal{H}$: $\rho(G, Y)=\rho(G, X)$ is equivalent with testing $\mathcal{H}'$: $\rho(G, Z_\tau)=0$, where $Z_\tau=X-\tau Y$. Under rather general conditions we have that under $\mathcal{H}'$ (Muirhead 1982)

$$T=\sqrt{(n-2)}\,\frac{r_{G,Z_Y}}{(1-r^2_{G,Z_Y})^{1/2}}\sim t_{n-2}$$

Now we replace the nuisance parameter τ^2 by its obvious sample estimate to obtain an approximate test.

Results

For reasons indicated above we used the mean log nuclear area as a potential factor. As shape factors are well known to be highly irreducible and introducing other size factors seems redundant no other factors have been entered into the analysis. Note that we have essentially four data-sets, namely the measurements of two technicians (A,B) each of which measured once with and once without selecting the nuclei (Table 1).

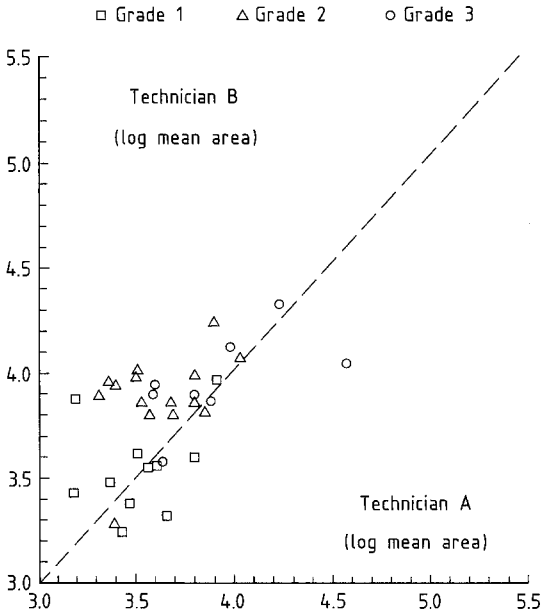

Fig. 3. Reproducibility and separability of the mean nuclear area without selection (non-selective morphometry)

The measurements based on the selection process gave rise to a significantly larger sample correlation ($P<0.001$, based on the divided sample test statistic) than the random measurements. However, the hypothesis of exchangeability is still somewhat contradicted, although to a lesser extent than the exchangeability of the random measurements (Table 2).

Not only does the selection improve reproducibility, it also increases the consensus between the technician (cytomorphometry) and pathologist (histomorphometry), in the sense that the sample correlation coefficient between the grade and the mean log area increases significantly (Table 3).

To illustrate the above-discussed reproducibility and consensus of the pathologist and technician the outcomes of the random measurements of both technicians are displayed in Fig. 3, whereas those based on the selection process are displayed in Fig. 4.

Discussion

The cytology of the urine of patients with bladder tumours is a valuable method in the diagnosis of new cases and is especially useful in the follow-up of patients known to have bladder neoplasms (Flanagan et al. 1978; Morrison et al. 1984).

Cytomorphometry of cells in the urine is used as a method to achieve a more reproducible method of cytological grading (Montironi et al. 1985; van der Poel et al. 1988). In an earlier study we found a correlation

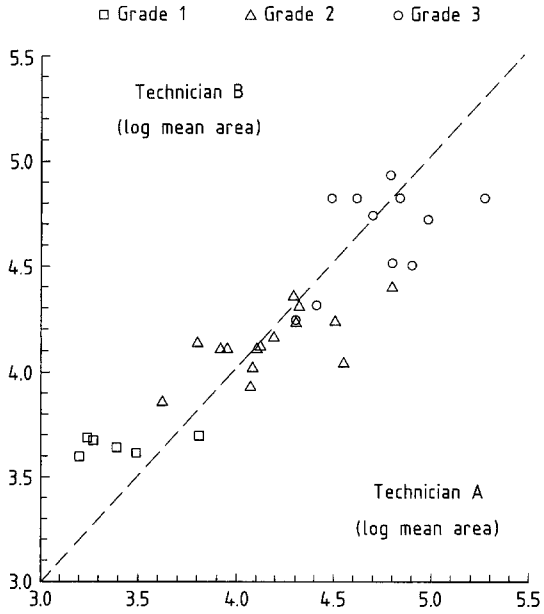

Fig. 4. Reproducibility and separability of the mean nuclear area with selection (selective morphometry)

value of 0.743 for cytomorphometrically determined nuclear area with histological tumour grade (van der Poel et al. 1988). The reason for an increase in nuclear area with tumour grade is the increase in amount of DNA in the tumour nucleus. Helander et al. (1984) found that grade I tumours were always diploid and grade III tumours always consisted of aneuploid nuclei. The amount of DNA measured by flow cytometry shows a positive correlation with nuclear size (Farsund et al. 1984; Helander et al. 1985).

In the current study urothelial cell nuclei selected at random were first measured. The correlation between the mean nuclear area and the tumour grade was low. Colpaert et al. (1987) showed that subjective grading is influenced by a small number of larger nuclei, representing tumour grade. They found that 50% of the nuclei in grade III tumours were within normal values (Colpaert et al. 1987). If nuclei chosen at random are measured, then low-grade cells are measured in addition to the true tumour grade cells. To avoid the influence of these less specific nuclei, we then repeated the measurements, selecting the most malignant nuclei.

When screening the slide at low magnification three different types of cell grouping can be recognized: single malignant cells, small papillary groups and large papillary cell groups. This division forms the basis of the selection process. When single malignant cells were present, this indicated loss of inter-cellular cohesiveness – a feature of malignancy and always present in grade III carcinomas (Boon et al. 1986; Collste et al. 1980; Rife et al. 1979). Small papillary cell groups were present in grade II as well as grade III tumour material. Exclusively large papillary cell groups occurred mainly in grade I tumours. This kind of cell group frequently was found in tumours of higher grade but then always next to small cell groups or single cells. The number of papillary groups in the material depends on both the size and the location of the tumour (Rife et al. 1979). Low-grade tumour cells, characterized mainly by cell groups and less malignant cellular features are selected in the last phase of the selection procedure.

A repeatable and reliable selection of nuclei of be achieved by analysing carefully the cellular features accompanying malignancy. Many studies have discussed cellular features used in the grading of bladder tumours (Bergkvist et al. 1965; Beyer-Boon 1977; Colpaert et al. 1987; Kalnins et al. 1970; Murphy et al. 1984). Ooms et al. (1983) described a histomorphometrical grading system in which selection is made on the basis of deep, superficial and large nuclei in bladder tumours. They found a significant correlation with histological tumour grade. In a subsequent article they stressed the importance of a clear selection of nuclei and showed that the histomorphometrical grading had better inter-individual consistency than histological (Ooms et al. 1985). The process tested in this study uses the different features of malignancy and makes a step-wise analysis of the material possible. The difference between the cytological criteria (Beyer-Boon 1977; Murphy et al. 1984; Rife et al. 1979) and the method used in the present study is the fact that in the current study, attention is paid to the cell groups first. As the cell grouping is a feature that can be appreciated at low magnification this was used as the first step in the present analysis. The reason for this attention to the cell groups is that it simplifies screening and makes a graded analysis of the material possible. Individual cellular features become important in the second phase.

This study shows that: cytomorphometry of bladder washing material is a useful method for grading of bladder carcinoma but that it is important how nuclei for measurement are chosen, since in cytological material less atypical and some normal cells may be found. A selection process, based on the way cells are situated, described in our study, increases reproducibility and gives a better correlation with histological tumour grade. Cytomorphometrical studies ought to contain a description of the way nuclei are selected for measurement.

Acknowledgements. The authors thank Dr. Robin Kirk for his critical review of the text and some refreshing comments.

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