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# Studies on urinary bladder carcinoma by morphometry, flow cytometry, and light microscopic malignancy grading with special reference to grade II tumours

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**Summary.** Biopsies from 28 patients with urinary bladder carcinoma were investigated by flow cytometry and morphometry. Histopathological grading on 1.5 μm thick glycol methacrylate sections was also performed.

Nuclear profile areas, nuclear volume densities and mitotic indices were usually larger in the higher grades of malignancy. All grade I tumours were diploid and all grade III tumours were aneuploid. Out of 13 grade II tumours 8 were diploid and 5 aneuploid. In these latter five cases nuclear profile areas were at the high end of the spectrum.

The data show that flow cytometry and morphometry could be a valuable tool in the diagnosis of urinary bladder carcinoma. Our data also suggest that a subdivision of the grade II tumours might be possible and meaningful in the assessment of prognosis.

**Key words:** Urinary bladder carcinoma – Morphometry – Karyometry – Flow cytometry

## Introduction

Most neoplasms arising in the urinary bladder are of epithelial origin, and approximately 90% reveal different degrees of urothelial maturation, varying from regular, urothelial cell papillomas to high grade carcinomas. Squamous cell-carcinomas comprise 5–10% and adeno-carcinomas approximately 1% of bladder tumours. Histological grading of urothelial tumours is based on the degree of structural, cellular and nuclear atypia and it has been demonstrated that grading is correlated to the survival of the patients (Bergkvist et al. 1965). Malignancy grading, however, is not always an easy task, and repeated investigations of the same section sometimes give different results (Busch et al. 1977). This is of course related to the fact that

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the method is partly subjective and hence inter- and intra-observer variations occur (Sherman et al. 1984).

Several grading systems are in use. The WHO system (1973) is widely accepted, and comprises three grades. In addition, it recognizes completely undifferentiated tumours, where it is impossible to state whether the cancer is of urothelial origin or not.

Grade II urothelial carcinoma is a large, heterogeneous group with around 50% 8-year mortality (Bergkvist et al. 1965). The recurrence rate is relatively high. Some of the cases recur as Grade II tumours, whereas others "progress" into Grade III, or become completely undifferentiated.

It would be of value to establish objective criteria to classify Grade II into well defined sub-grades, with the hope that these would turn out to be related to prognosis (Farsund et al. 1984). Treatment may then become different in the two sub-grades.

In order to facilitate such subdivision, we decided to investigate a series of human bladder carcinomas with morphometry and flow cytometry, in order to see whether these methods might be used to divide Grade II tumours into sub-grades. These two methods were also compared, and correlated to conventional histological grading.

## Material and methods

The investigation was carried out on TUR biopsies from 28 patients with clinically suspected or verified urinary bladder carcinoma. Of the patients 22 were male, and 6 female; the median age for the males was 69 years (range 49 to 80 years), and for the females 76 years (range 41 to 84 years). None of the patients had previously been treated with radiotherapy or with cytostatic drugs. One fairly large biopsy specimen was obtained from each patient using isotonic glycine as irrigating medium.

Each biopsy was divided perpendicularly to the subjacent muscularis into two equal parts: one was processed for light microscopy and the other for flow cytometry. The specimens for light microscopy were fixed without delay at room temperature for 24 h in a solution containing 2% formaldehyde and 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Following a rinse in the same buffer the biopsies were dehydrated in rising concentrations of ethanol, infiltrated and embedded in glycol methacrylate.

Sectioning took place in a JB-4 microtome (Sorwall) with the thickness feed set at $1^1/_2$ µm. The sections were transferred to a 22° C water bath for brief stretching and were then mounted on glass slides. Haematoxylin and eosin were used for staining.

The grading of the tumours and the morphometric analyses were performed independently by separate investigators on the same section. The tumours were graded according to the WHO system (1973).

*Nuclear profile areas* were measured by light microscopy using a ×100 oil immersion objectives lens and a projection tube. The tracing device was operated on a Hipad digitizing tablet (Bausch & Lomb) connected to a Luxor ABC 80 microcomputer. Nuclear profile areas were measured both on the sectioned material and on the smears (vide infra).

*Nuclear volume densities* (i.e. the proportion of tissue volume – except for vessels – taken up by nuclei) were estimated in the sections, using the point counting method. For this purpose the microscope was equipped with a ×40 objective lens and a square grid inserted into the eye-piece. About 600 test points were counted in each tumour corresponding to an area of about 0.08 $mm^2$.

*Mitotic index* was calculated using the same equipment. One thousand nuclei were counted in each section.

All morphometric analyses were carried out by systematic sampling with a random start, in all layers of the tumour (except for the most superficial layer).

The other portion of each biopsy was used for *flow cytometry* and was immediately immersed in phosphate buffered saline (PBS). The tissue was minced and pipetted so as to produce a single cell suspension, which was then filtered through a 75 μm mesh nylon filter. The filtrate was diluted with PBS and centrifuged (1,000 rpm for 5 min). The pellet was resuspended in fresh PBS and fixed with ice cold ethanol (final concentration 70%). A few drops of the cell suspension were spread onto gelatin coated glass slides, dried, and stained with Papanicolaou. These smears were used for measuring nuclear profile areas as described above. The rest of the cells were stained with propidium iodide (PI, Calbiochem) and fluorescein-isothiocyanate (FITC, Isomer I, Becton Dickinson) as described by Crissman et al. (1981) for simultaneous analysis of cellular DNA and protein. The cells were washed once in PBS, resuspended in 2 ml PBS containing 190 ug/ml RNase and 0.075 ug/ml FITC, and left in the dark for 30 min at room temperature. They were stored at 4° C overnight and 1 ml of PBS containing 51 ug PI was added 1 h before analysis the next day. The samples were kept on ice and analysed in an Ortho Cytofluorograph 50H (Ortho Instruments, Westwood, USA) using 200 mW of excitation light at 488 nm. The intensity of the red fluorescence (DNA content), green fluorescence (protein content) and the red fluorescence pulse width (indicating nuclear diameter) were recorded. Cell doublets and larger aggregates could be excluded by their red fluorescence pulse width.

Fluorescent microspheres (Polysciences, Inc.) and two samples with known DNA and protein contents were always measured for control purposes.

The degree of ploidy was expressed as the DNA index (DI). The DI was obtained from each DNA histogram by calculating the ratio between the channels representing the DNA content of the aneuploid cells and that of those with diploid DNA content.

A sample was considered aneuploid when a distinct population of aneuploid cells was seen or when the number of tetraploid cells was higher than in controls (Devonec et al. 1982; Farsund and Hoestmark 1982) and was linked to corresponding S and $G_2$-phase fractions.

For each sample the channels representing the mean cellular protein content and the mean DI were obtained from the respective histograms. The ratio between the mean DI and the mean protein content (DPI) was calculated to express the nucleus/cytoplasm ratio.

## Results

### *Grading*

The majority of tumour biopsies were classified as urothelial carcinomas. Nine were of grade I, 13 of grade II, 4 of grade III, the remaining 2 being undifferentiated carcinomas.

### *Morphometric variables correlated to grade*

*Mean values* for *nuclear profile areas* were usually higher in tumours of higher malignancy grade, but there was some overlapping between the grades (Fig. 1 A and B). However, the 2 undifferentiated tumours displayed relatively small nuclei. Nuclear profiles *larger than* 90 $\mu m^2$ in sections were not present in any of the grade I tumours. Nine out of 13 grade II tumours had nuclear profiles larger than 90 $\mu m^2$. In the smears nuclear profiles larger than 110 $\mu m^2$ were absent in all grade I tumours and in 7 out of the 13 grade II tumours (Table 1).

There was a positive correlation ($r = +0.78$, $p < 0.01$) between nuclear profile areas in the sections and in smears (see Fig. 2).

*Nuclear volume densities* were (generally) larger in the tumours of higher grade than in those of lower grade (Fig. 3).

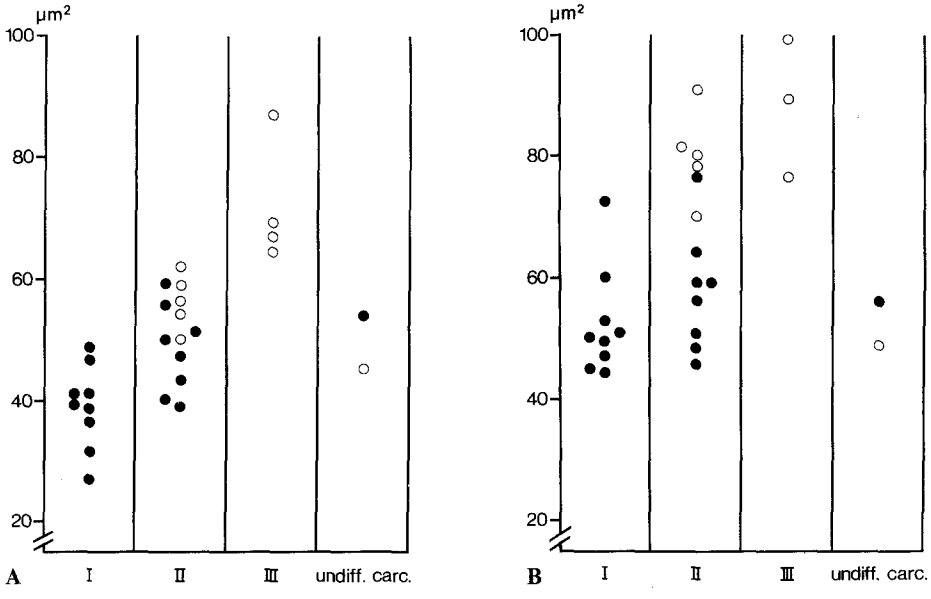



**Fig. 1 A, B.** The *mean values* of the nuclear profile areas in each of the 28 biopsies. **A** in sections. **B** in smears. I, II, III = malignancy grades and undifferentiated carcinoma. ● = diploid cases; ○ = aneuploid cases

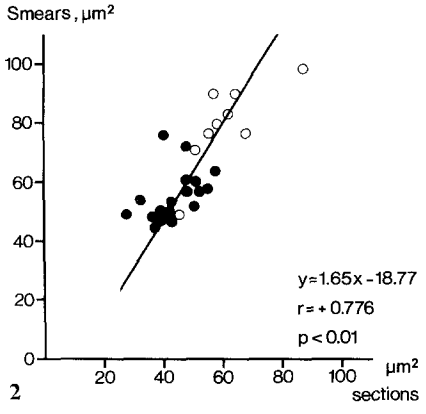
**Table 1.** Nuclear profiles exceeding 90 μm² in sections and 110 μm² in smears in grades I, II, III, and in undifferentiated urinary bladder carcinoma. Mean values given in per cent of the total number of nuclei (range in parenthesis)

| | Sections | | Smears | |
|---|---|---|---|---|
| | >90 μm² | No. of patients | >110 μm² | No. of patients |
| I | 0 | 9 | 0 | 9 |
| II | 2 (0–7) | 13 | 6 (0–22) | 13 |
| III | 20 (13–35) | 4 | 19 (9–26) | 3 |
| Undiff. carc. | 2 (1–3) | 2 | 1 | 2 |

*Mitotic index* in grade I tumours was 0–0.3%, and in grade II tumours 0–1.7% (median value 0.6%). Among the grade III tumours no mitoses were found in one case; in the remaining three cases the mitotic index ranged between 1.2–2.5%. In the two undifferentiated cases the mitotic index reached 1.5 and 2.5%, respectively.

*Flow cytometric variables correlated to grade*

As shown in Fig. 4 all biopsies of grade I tumours were diploid, and all of the grade III tumours were aneuploid. Among the 13 grade II tumours



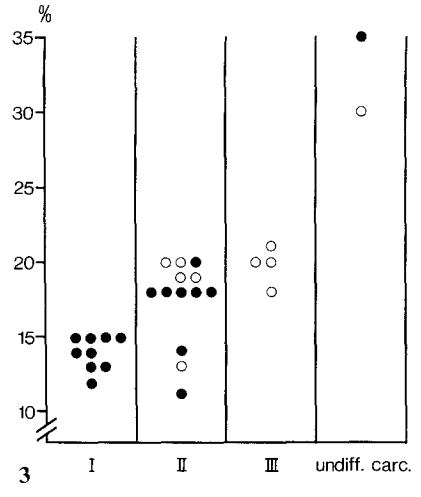
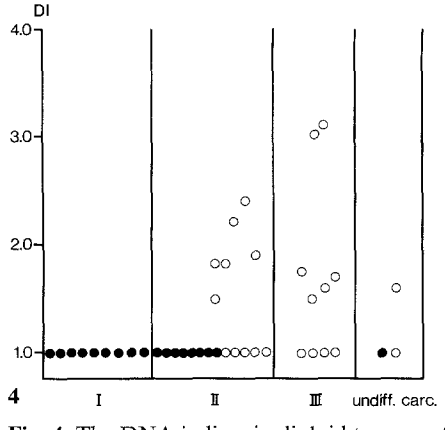




**Fig. 2.** Correlation between mean nuclear sizes in sections and in smears. Ploidy *symbols* as in Fig. 1

**Fig. 3.** The mean values of nuclear volume densities in each of the 28 biopsies. I, II, III = malignancy grades and undifferentiated carcinoma. Ploidy *symbols* as in Fig. 1



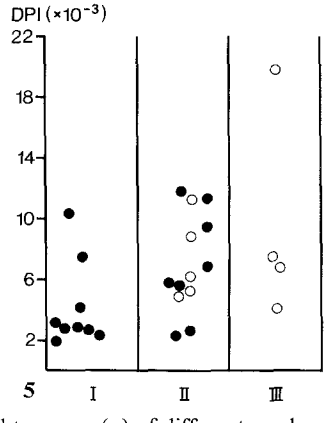




**Fig. 4.** The DNA indices in diploid tumours (●) and aneuploid tumours (○) of different grades. Aneuploid tumours have two or more peaks in their DNA patterns, hence the number of *open circles* is higher than the number of tumours investigated

**Fig. 5.** The ratio (DPI, arbitrary units) between the mean DNA index and the mean protein content calculated for diploid (●) and aneuploid (○) tumours of different grades

8 were diploid and 5 aneuploid. The highest DNA indices were found in grade III tumours. The DPI values tended to be higher in grade II than in grade I tumours (Fig. 5). Examples of FCM results on tumours with a diploid and an aneuploid DNA pattern respectively are shown in Fig. 6.

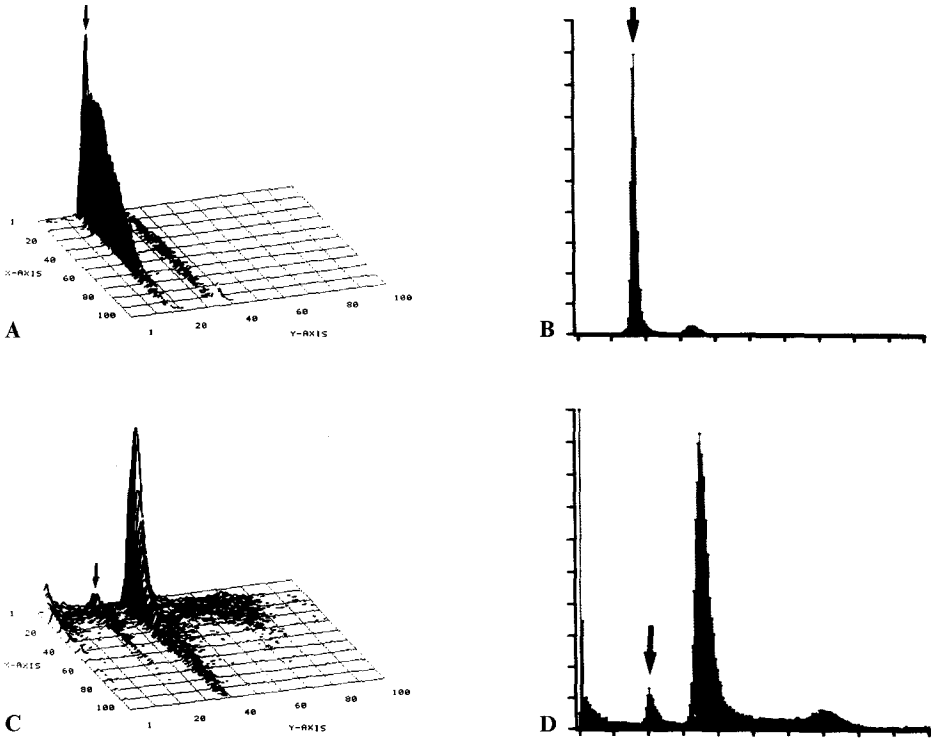



**Fig. 6.** Flow cytometric analyses of a diploid grade I tumour (**A** and **B**) and of an aneuploid grade III tumour (**C** and **D**). In **A** and **C** cellular DNA (y-axis) is *plotted* versus cellular protein content (x-axis). In **B** and **D** the various DNA peaks are shown. The height of the curves reflect the number of cells. *Arrows* point to the position of the DNA content of diploid cell nuclei

*Correlation between variables from flow cytometry and morphometry*

There was a significantly positive correlation ($r = +0.63$, $p < 0.01$) between the DNA (red) fluorescence pulse width (nuclear size) measured by FCM and nuclear areas in smears measured by morphometry (Fig. 7).

The eight aneuploid tumours of grade II and III displayed larger nuclei in smears than in all but two of the diploid cases (Fig. 1 B).

*Combination of selected variables from morphometry*

By analogy with what has been proposed for premalignant conditions in the nasal epithelia (Boysen and Reith 1982) we also combined the weighted results from various morphometric varibles to investigate the correlation with the malignancy grading. The procedure resulted in a complete separation of the grade II tumours into two groups: one resembling the grade I tumours and the other approaching grade III tumours (Fig. 8).

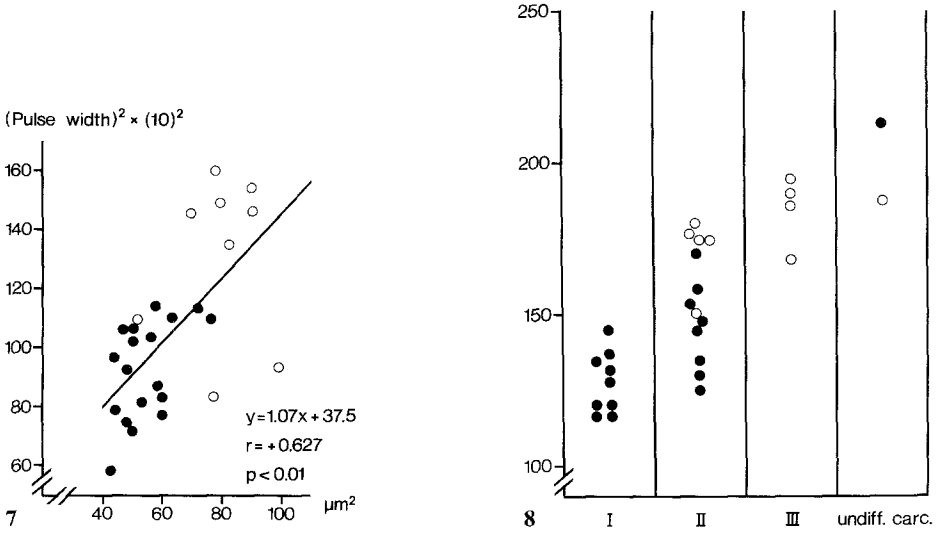



**Fig. 7.** The correlation between mean nuclear size as measured by FCM (red fluorescent pulse width, arbitrary units) and mean nuclear area obtained by morphometry in smears (abscissa)

**Fig. 8.** The correlation between the malignancy grades and the values obtained by combining three variables, vis. mean nuclear profile areas in sections + 3 × nuclear volume densities + 0.5 × pulse width as measured by FCM (ordinate)

## Discussion

Tumour type, stage, and grade are important prognostic factors in bladder tumours. The choice of therapy is based upon these variables. Staging is based on clinical signs, x-ray findings and macroscopical peroperative findings. The reliability of the histological typing can be facilitated by the use of special staining methods such as van Gieson. The malignancy *grading*, however, presents greater problems. Several investigators have reported considerable inter- and intraobserver differences in the grading of these tumours (Busch et al. 1977; Sherman et al. 1984). The difficulties have led to the introduction of different classification systems and – more recently – to the proposal of introducing more objective criteria in the diagnostic procedure (Sherman et al. 1984).

In the present work the correlation between two such more objective – quantitative – methods, viz. flow cytometry and morphometry has been investigated. Flow cytometry has been extensively used for the evaluation of bladder carcinoma (Tribukait and Esposti 1979; Gustafson et al. 1982; Tribukait et al. 1979). By this method a very large number of cells are measured with respect to various cell components like DNA, RNA, and protein as well as to nuclear size (Klein et al. 1982; Collste et al. 1980). All cells within the tissue samples are analyzed in our system and inflammatory cells can not be excluded. The equipment is expensive, but the method is relatively fast.

In contrast, morphometry requires only an inexpensive measuring scale inserted into the eyepiece of any light microscope. Paraffin sections can be used, but morphometry is difficult to carry out with high precision on such sections because of their thickness and uneveness (Helander 1983). Therefore we have preferred to analyze semi-thin (1.5 μm) sections of methacrylate embedded tissues. Even though only a small number of the nuclei are evaluated in the morphometric part of the study, this method is rather time consuming.

In this context it should be emphasized that urothelial tumours may vary in grade in different parts of the biopsy. This might lead to discrepancies between the morphometric results and those from flow cytometry, and can explain why one of our undifferentiated cases was diagnosed as diploid. This stresses the importance of always combining histological grading and the discriminatory capacity of the other methods.

The positive correlation between the nuclear areas in sections and smears demonstrate that both these methods could be used. As expected the nuclear areas in sections were smaller than those in smears (in the latter case only equatorial planes are measured, whereas in the sections planes from all levels are present). Theoretically the areas of the sectioned nuclei should be 67% of those in smears. In our study a figure of 79% was obtained. This difference could be explained by an 8% shrinkage of the radii of the nuclei in the smears, which might be expected since these nuclei were fixed in ethanol and dried on to the glass slides.

A correlation between the *mean nuclear size* and the subjective malignancy grading was present, but there was some overlapping between grades I and II. Further information could be gained from studying the correlation between the proportion of large nuclei ($>90$ μm$^2$ in the sections, $>110$ μm$^2$ in smears) and the grade of malignancy (Table 1).

In this context it should be kept in mind that nuclear enlargement can be seen also in non-malignant conditions, such as in inflammatory reactions. However, in such cases the nucleo-cytoplasmic ratio generally remains normal – thus the nuclear enlargement is paralleled by a cellular enlargement (Boon 1980).

Measurement of cell size is a difficult and time-consuming task, both in the sections and in the cell suspensions, since the cell borders are often difficult to discern. In contrast, the determination of *nuclear volume density* is rapid and precise. This variable provides relative information on cell size and in the present study it discriminated fairly well between grade I and grade II tumours. Boysen and Reith (1982) studied premalignant lesions of the nasal mucosa by stereological methods. In order to obtain a reliable discrimination between the different lesions, they suggested weighting of several morphometric variables. This approach proved to be of value also in the present study. Weighting of the morphometric data in this way resulted in a separation of the grade II tumours into two groups (Fig. 8).

The flow cytometric data show that the grade I tumours were diploid, while 38% of the grade II tumours and all grade III tumours were aneuploid. This is in agreement with previous studies (Tribukait and Esposti 1978; Tribukait et al. 1979; Farsund et al. 1984).

The DPI seems to be higher in grade II tumours than in grade I tumours (Fig. 5), indicating that the nucleo-cytoplasmic ratio is higher in grade II tumours. This is in agreement with the morphometric data which show the nuclear volume density to be higher in the more malignant grades (Fig. 3).

There is also a positive correlation between nuclear size as measured by FCM (red fluorescence pulse width) and nuclear area as measured by morphometry (Fig. 7).

The fact that nuclear profiles larger than 90 $\mu m^2$ (110 $\mu m^2$) appear mainly in tumours with high DNA indices indicates that the nuclear enlargement is due to increased DNA content (Table 1).

In the present investigation we have also correlated the results from flow cytometry and morphometry (both of them objective methods for tissue examination) with the traditional, more subjective malignancy grading. In the absence of survival data for these patients it is too early to determine which variables are the most reliable for predicting the prognosis of the disease. The histological grading of the tumour biopsies – objective or subjective – will always be of fundamental importance for the planning of treatment for a patient with bladder cancer. It is also obvious that the general health condition of the patient plays an important role.

Grade II tumours appear to be a rather heterogeneous group requiring more precise histological evaluation. Our data show that this tumour grade can be subdivided into two groups: one with diploid, relatively small nuclei and another group with larger, aneuploid nuclei. Farsund et al. (1984) reported similar results with flow cytometry and found that the aneuploid fraction of grade II tumours was correlated with "involvement of the surrounding mucosa". In an extended prospective study during the next couple of years we hope to be able to evaluate whether or not the prognosis differs between these two groups.

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