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Cell proliferation indices, morphometry and DNA flow cytometry provide objective criteria for distinguishing low and high grade bladder carcinomas

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Abstract Argyrophilic nucleolar organizer region (AgNOR) analysis, proliferating cell nuclear antigen (PCNA/PC10) and MIB-1 immunohistochemistry, nuclear morphometry and DNA flow cytometry have been performed on formalin-fixed, paraffin-embedded biopsies from 50 patients with transitional cell carcinoma of the urinary bladder. The mean AgNOR count was 6.01 for the 17 grade 1 (G1), 7.59 for the 21 G2 and 13.33 for the 12 G3 carcinomas ($p<0.001$). The mean PCNA score was 15.03% for G1, 24.04% for G2 and 40.01% for G3 cases ($p<0.001$). The mean MIB-1 score was 11.31% for G1, 17.09% for G2 and 34.47% for G3 carcinomas ($p<0.001$). The mean nuclear area was 35.53 μm^2 for G1, 38.65 μm^2 for G2 and 83.62 μm^2 for G3 cases ($p<0.001$). Aneuploidy rates were significantly higher (91.7%) in G3 than in G2 (42.9%, $p<0.01$) or G1 cases (47.1%, $p<0.05$) but not different for G1 versus G2 cases ($p=0.94$). While many overlaps of values were seen between G1 and G2 tumours, no overlaps were found between G3 and G1/G2 tumours. Significant differences of values were also found between pTa and invasive tumours ($p<0.0001$ for AgNOR count and PCNA score; $p<0.001$ for MIB-1 score and mean nuclear area; $p<0.01$ for DNA ploidy); however many overlaps were seen. Our findings indicate that the quantitative parameters obtained with different methods are associated with histological grade of bladder urotheliomas and may improve the grading reproducibility. In addition, the absence of overlaps between G3 and G2/G1 carcinomas supports the tendency to classify bladder urotheliomas in only two categories of malignancy.

Key words Bladder cancer · Argyrophilic nucleolar organizer regions · Proliferating cell nuclear antigen MIB-1 · Nuclear morphometry · DNA flow cytometry

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Introduction

The histological grading of urothelial tumours is still the cause of some concern among pathologists. Several systems have been proposed; the most widely accepted is the WHO classification (Mostofi et al. 1973) that showed significant correlation with prognosis. Therefore, in clinical practice, grade 1 (G1) and 2 (low grade) tumours are treated conservatively, while grade 3 (high grade) tumours are treated more aggressively (Ooms et al. 1983b). All the grading systems are based on estimation of histological and cytonuclear atypia: this carries a risk of significant interobserver variation (Busch et al. 1975; Ooms et al. 1983a). Therefore there is a need for methods able to provide quantitative data, in order to improve the reproducibility of the histological grading and make a clear distinction between low and high grade malignant tumours.

The nuclear morphometry, based on random (Bjelkenkrantz et al. 1982; Ooms et al. 1983b; Helander et al. 1984) or selective sampling of nuclei (Blomjous et al. 1989a, c, 1990), was found to be significantly associated with histological grading.

Flow cytometry (FCM) methods for the analysis of the cellular DNA content in paraffin-embedded tissues (Hedley et al. 1983; Coon et al. 1986) showed significant association between ploidy and histological grade of bladder urotheliomas (Blomjous et al. 1988; Badalament et al. 1990; Lipponen et al. 1991a).

The evaluation of cell proliferative activity is an important adjunct to the histological classification of tumours (Hall and Levison 1990). The number of nucleolar organizer regions (NORs), loops of DNA which transcribe to ribosomal RNA (Gall and Pardue 1969) is related to cell proliferative activity (Derenzini et al. 1990). AgNOR counts are associated with histological grade in transitional cell bladder carcinomas (Rüschoff et al. 1989; Hufnagl et al. 1991; Lipponen et al. 1991b; Pich et al. 1992; Rüschoff et al. 1992; Skopelitou et al. 1992; Limas et al. 1993).

The proliferating cell nuclear antigen (PCNA/Cyclin), a nuclear protein involved in DNA synthesis (Mathews et al. 1984), can be detected in conventionally processed tissues using a monoclonal antibody against the PC10 clone (Hall et al. 1990). PCNA scores are associated with histological grade in bladder urotheliomas (Lipponen and Eskelinen 1992; Pich et al. 1992; Skopelitou et al. 1992; Waldman et al. 1993).

The reaction with the monoclonal antibody Ki-67, which detects a nuclear antigen expressed in all phases of the cell cycle, except G0 (Gerdes et al. 1983), has been employed in a large number of studies of cell proliferation and showed significant association with histological grade in bladder carcinomas (Mellon et al. 1990; Bush et al. 1991; Mulder et al. 1992). However, Ki-67 antibody is only effective on cryostat sections. The new monoclonal antibody MIB-1, prepared against recombinant parts of the Ki-67 antigen (Key et al. 1992) can react also in routinely-fixed, paraffin-embedded tissues (Cattoretti et al. 1992). To our knowledge, no studies have been performed so far on MIB-1 expression in bladder urotheliomas.

In this work we have performed nuclear morphometry, DNA-FCM, AgNOR analysis, PCNA and MIB-1 immunohistochemistry in 50 routinely fixed and embedded biopsies of transitional cell carcinomas of the bladder. The purpose was to assess if the quantitative and reproducible values obtained using different methods were associated with the tumour histological grade and could allow a more objective distinction between low and high grade tumours.

Materials and methods

Fifty patients (5 females and 45 males) who underwent transurethral biopsy or resection of a primary and untreated transitional cell carcinoma of the urinary bladder were included in this study. The mean age was 64.4 years (31–91). Tumour grade was established according to WHO (Mostofi et al. 1973): 17 were grade 1, 21 grade 2 and 12 grade 3 carcinoma. Tumours were staged according to TNM classification (Hermanek and Sobin 1987): 27 were stage pTa, 15 pT1 and 8 pT2–4. The samples were immediately fixed in 10% formol for 24 h and embedded in paraffin. Serial sections from the same tissue blocks were used for histology, morphometry, FCM, AgNOR staining, PCNA and MIB-1 immunostaining.

Haematoxylin-eosin stained sections (4 μm thick) were analysed with an automated image analyzer (Immagini & Computer, Rho, Milano, Italy), using the module IM5200 of the Image Measure software package (Microscience, Phoenix Technology, Seattle, USA), connected to a light microscope equipped with a 40× objective. The images were displayed on a monitor at the final optical magnification of 2000×. The nuclear areas of 150 random sampled nuclei were measured for each slide.

For DNA flow cytometry sections 100 μm thick from paraffin embedded material were treated with propidium iodide, according to methods previously described (Hedley et al. 1983; Coon et al. 1986). Ten thousand events were acquired using a FACScan flow cytometer with a 488 nm argon ion laser, equipped with doublet discrimination module and the Cell Fit program, Version 2.0 (Becton Dickinson, Immunocytometry Systems, San Jose, Calif., USA). Histograms were grouped as diploid and aneuploid according to the method of Joensuu and Klemi (1988).

For AgNOR staining and counting 3 μm thick sections taken to an aqueous medium were stained with the AgNOR method of Ploton et al. (1986), as previously described (Pich et al. 1991). Random fields, excluding areas of necrosis, were independently examined by two pathologists (AP and LC) using a 100× oil immersion lens; at least 100 tumour cells were counted in each case. Single AgNORs and individual AgNORs within clumps were counted by careful focusing through the whole thickness of the sections. When large polycyclic structures (overlapping NORs) were present, they were considered as a single AgNOR, if individual dots could not be identified. The internal control of the reaction was provided by the infiltrating lymphocytes that on average had one single silver stained dot. The mean number of AgNORs per nucleus was then calculated in each case. The interobserver variation was less than 5%.

Sections 4 μm thick on poly-L-lysine coated slides were immunostained with monoclonal antibody against PCNA (PC10) (Dakopatts, Glostrup, Denmark), as described elsewhere (Hall et al. 1990), using an immunoperoxidase method (ABC Complex, Dakopatts) with light haematoxylin counterstaining. Scoring of PCNA (PC10) was independently performed by two pathologists (AP and LC) using a standard light microscope equipped with an ocular reticle (magnification 15×) and a 40× objective. In each case, 1000 tumour cells were counted from 10 representative fields (areas in which the reaction was clearly positive): in these fields all the reactive nuclei were considered as positive, regardless of the intensity of the staining, and the fraction of positive cells was determined. The interobserver variation was between 10% and 15%.

MIB-1 staining and scoring was carried out on 4 μm thick sections on poly-L-lysine coated slides, taken to water, which were treated with 0.05% trypsin for 10 min, placed in glass box filled with 10 mM, pH 6.0 citrate buffer and processed in a microwave oven twice at 750 W and then rinsed in PBS. The sections were stained with MIB-1 monoclonal antibody (Immunotech, Marseille, France) at 1:100 dilution for 2 h at room temperature, using an immunoperoxidase method (ABC Complex, Dakopatts); they were counterstained with haematoxylin and mounted in resin. MIB-1 immunostaining was scored as the PCNA staining; the interobserver variation was less than 5%.

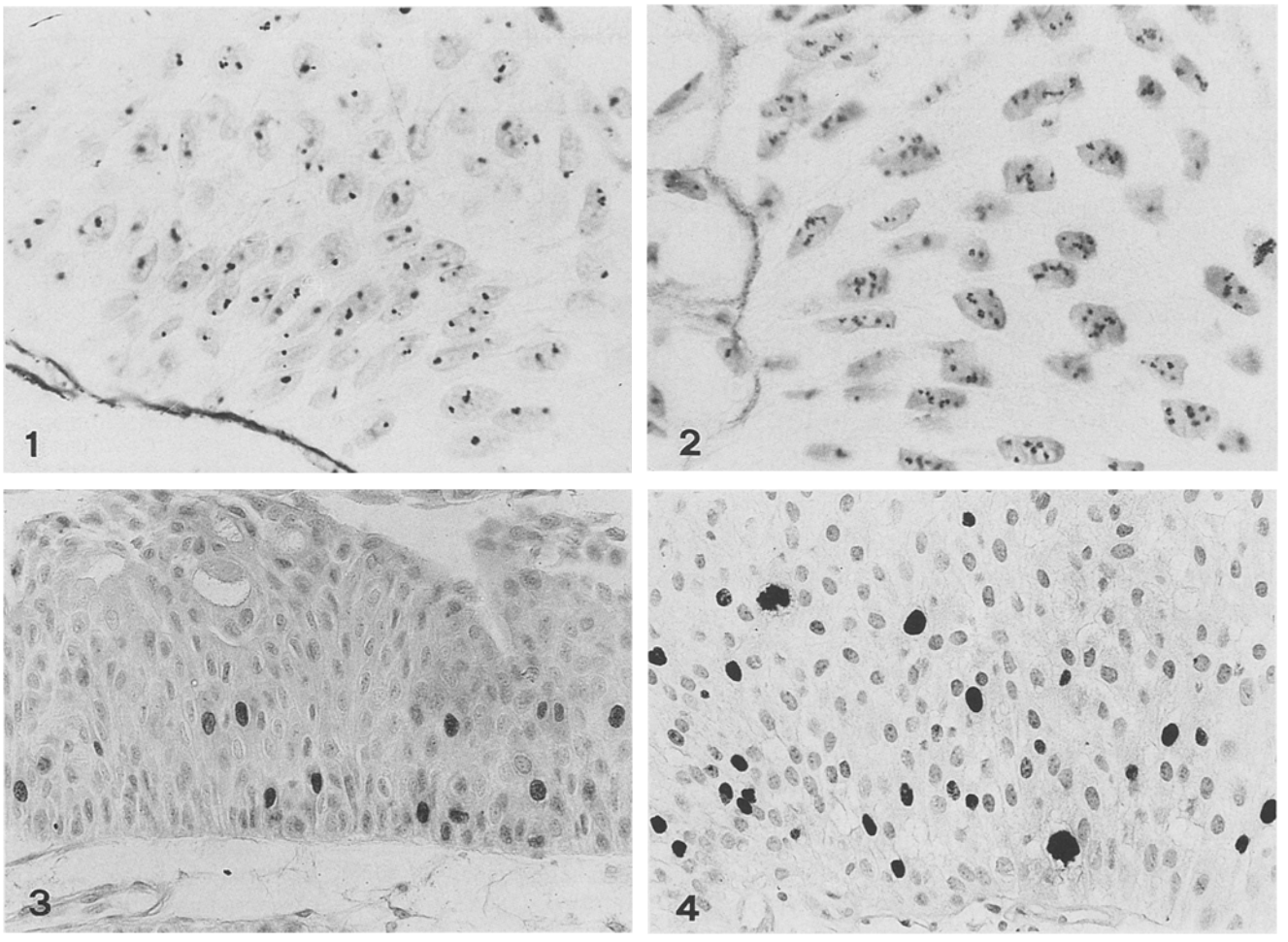
Associations between mean nuclear area, AgNOR counts, PCNA scores and MIB-1 scores with tumour histological grade and stage were assessed by ANOVA. Associations between ploidy/aneuploidy with tumour histological grade and stage were evaluated by chi-square or Fisher's exact test. As a level of significance, $p=0.05$ was taken.

Results

The mean nuclear area for the whole series was 48.38 μm^2 (SD: ± 23.12 μm^2; range: 19.21–129.04 μm^2). It was 35.53 μm^2 for G1, 38.65 μm^2 for G2 and 83.62 μm^2 for G3 carcinomas. The differences are significantly different for G1 versus G3 ($p<0.001$) and G2 versus G3 ($p<0.001$), not for G1 versus G2 ($p=0.26$). No overlaps were found between G3 and G1/G2 cases.

Twenty-two cases were diploid (44%) and 28 (56%) aneuploid; tetraploidy was detected in 6 cases (12%). Aneuploidy rates were significantly higher in G3 (91.7%) than in G2 (42.9%; $p=0.006$) or G1 cases (47.1%; $p=0.015$), not different for G1 versus G2 ($p=0.94$).

The mean AgNOR counts for the whole series was 8.43 (SD: ± 3.07; range: 5.05–15.6). It was 6.01 for G1, 7.59 for G2 and 13.33 for G3 carcinomas; the differences are significantly different for G1 versus G3, for G2 ver-



sus G3 and for G1 versus G2 ($p<0.001$). Whereas no overlaps of AgNOR counts were seen between G3 and G1 or G2 tumours, many overlaps could be found between G1 and G2 cases. The tumour cells of G1 and of part of G2 cases showed relatively large AgNORs, mostly grouped in two or three clusters (Fig. 1); some G2 and all G3 cases showed numerous small dots dispersed throughout the nucleus (Fig. 2).

The mean PCNA score for the whole series was 24.81% (SD: ±11.22%; range: 4.4–62.5%). It was 15.03% for G1, 24.04% for G2 and 40.01% for G3 cases; the differences are significantly different for G1 versus G3, for G2 versus G3 and for G1 versus G2 ($p<0.001$). Many overlaps were found between G1 and G2 carcinomas, while none was seen between G3 and G1/G2 cases. PCNA(PC10) staining was almost entirely confined to the nucleus; non-specific cytoplasmic staining was rarely seen; mitotic figures were always unstained. Variability was observed in the staining intensity, high in some areas, very poor in others. The PCNA positive nuclei were more numerous in the basal than in the superficial layers in well-differentiated papillary carcinomas (Fig. 3), but not in poorly differentiated and deeply invasive tumours.

The mean MIB-1 score for the whole series was 18.98% (SD: ±9.77%; range: 5–41.2%). It was 11.31% for G1, 17.09% for G2 and 34.47% for G3 cases. The differences are significant between G1 and G2, between G2 and G3 and between G1 and G3 cases ($p<0.001$). Many overlaps were found between G1 and G2 cases, whereas no overlaps were seen between G3 and G1/G2 cases. The positivity for MIB-1 monoclonal antibody was confined to the cell nucleus; the sections were homogeneously stained without background interference; some gradation in the intensity could be seen from nucleus to nucleus within the same section, but it was always easy to determine whether a nucleus was positive.

Fig. 1 Low grade bladder carcinoma stained by the argyrophilic nucleolar organizer region (AgNOR) technique. Small number and clustered distribution of AgNORs are visible. ×600

Fig. 2 High grade bladder carcinoma shows large number and scattered distribution of AgNORs. AgNOR staining, ×600

Fig. 3 Low grade papillary carcinoma of the bladder stained with proliferating cell nuclear antigen PCNA(PC10): the positive nuclei are more numerous in the deep layers. ABC immunoperoxidase, ×230

Fig. 4 High grade bladder carcinoma stained with the monoclonal antibody MIB-1: positive nuclei and mitotic figures are strongly stained, without background interference. ABC immunoperoxidase, ×230

Table 1 Morphometry, DNA-FCM and cell proliferation indices in grade 1, 2 and 3 bladder carcinomas

Variable		All series N=50	Grade 1 N=17	Grade 2 N=21	Grade 3 N=12	*p*
Nuclear area (μm^2)	Mean ± SD	48.38 ± 23.12	35.53 ± 8.28	38.65 ± 8.47	83.62 ± 18.98	G1 vs G2 = 0.26
	Range	19.21–129.04	19.21–49.8	21.58–54.51	64.93–129.04	G1 vs G3 = 0.0000
						G2 vs G3 = 0.0000
DNA ploidy	Diploidy (%)	22 (44%)	9 (52.9%)	12 (57.1%)	1 (8.3%)	G1 vs G2 = 0.94
	Aneuploidy (%)	28 (56%)	8 (47.1%)	9 (42.9%)	11 (91.7%)	G1 vs G3 = 0.015
						G2 vs G3 = 0.006
N° AgNOR/cell	Mean ± SD	8.43 ± 3.07	6.01 ± 0.71	7.59 ± 1.44	13.33 ± 1.57	G1 vs G2 = 0.0001
	Range	5.05–15.6	5.05–7.45	5.91–9.25	9.33–15.6	G1 vs G3 = 0.0000
						G2 vs G3 = 0.0000
PCNA score	Mean ± SD	24.81 ± 11.22	15.03 ± 4.73	24.04 ± 6.01	40.01 ± 7.88	G1 vs G2 = 0.0000
	Range	4.4–62.5	7.1–22.5	4.4–32.25	32.4–62.5	G1 vs G3 = 0.0000
						G2 vs G3 = 0.0000
MIB-1 score	Mean ± SD	18.98 ± 9.77	11.31 ± 4.77	17.09 ± 4.11	34.47 ± 4.11	G1 vs G2 = 0.0002
	Range	5–41.2	5–23.5	9–24.5	27.75–41.2	G1 vs G3 = 0.0000
						G2 vs G3 = 0.0000

Most mitotic figures were strongly stained in all cases (Fig. 4). The results are summarized in Table 1.

Significant differences were also found between pTa and invasive tumours. The mean nuclear area was 36.4 μm^2 (SD: ±8.42 μm^2; range: 19.21–54.51 μm^2) for pTa and 62.45 μm^2 (SD: ±26.91 μm^2; range: 21.58–129.04 μm^2) for invasive cases ($p < 0.001$); the aneuploidy rate was 40% for pTa and 74% for invasive cases ($p < 0.01$); the mean AgNOR counts was 6.44 (SD: ±1.05; range: 5.05–8.99) for pTa versus 10.78 (SD: ±3.02; range: 6.04–15.6) for invasive tumours ($p < 0.0001$); the mean PCNA score was 17.38% (SD: ±6.48%; range: 4.4–28.1%) for pTa versus 33.54% (SD: ±9.15%; range 22.5–62.5%) for invasive cases ($p < 0.0001$), and the mean MIB-1 score was 12.76% (SD: ±5.04%; range: 5–24.5%) for pTa versus 26.62% (SD: ±8.72%; range: 14.25–41.2%) for the invasive tumours ($p < 0.001$). However, there were numerous overlaps of values between pTa and invasive tumours.

Discussion

The first purpose of this work was to see if the morphometric values, DNA-FCM and proliferative activity were associated with histological grade in transitional cell carcinomas of the bladder. Progressive increase of nuclear area was seen with increase of histological grade but no significant difference was found between G1 and G2 tumours, in contrast to Blomjous et al. (1990), who also found larger values of the mean nuclear area. However, they measured selected large and abnormal nuclei, with values obviously greater than those we obtained by the evaluation of random nuclei. In fact, also in our series, when the 10 largest nuclei were selected in each case by the computer, the mean nuclear area resulted 57.03 μm^2 for G1, 76.23 μm^2 for G2 and 161.56 μm^2 for G3 cases, being significant the difference also between G1 and G2 tumours ($p < 0.001$).

DNA-FCM showed a significant association between ploidy/aneuploidy and histological grade. However, the rate of aneuploidy in our G1 tumours (47.1%) is higher than that reported in larger series, either in fresh (Norming et al. 1989) or paraffin-embedded specimens (Blomjous et al. 1988; Badalament et al. 1990; Lipponen et al. 1991a). The size of the specimens in which ploidy is evaluated may influence the final ratio of ploidy in a group of samples. Indeed, different DNA values are found in 15% of large tumours, when central and surface biopsies are compared (Heiden et al. 1990) and heterogeneity of DNA ploidy can be found in nearly 27% of bladder tumours (Lipponen et al. 1991a). It is obvious that at least in some diploid tumours, if only one sample is measured, there may be an undetected aneuploid cell population. Possibly our relatively high frequency of aneuploidy in G1 cases may be due to the size of the specimens and to the number of samples (3–4) evaluated in each case of surgically resected tumour.

AgNOR counts were significantly associated with histological grade. While many overlaps were seen between G1 and G2 cases, no overlaps were found between G3 and G1 or G2 cases, contrary to the findings of Cairns et al. (1989), Ooms and Veldhuizen (1989) and Hansen et al. (1992). Our data fit with the findings of Rüschoff et al. (1992) obtained by a morphometric evaluation of AgNORs, and indicate that AgNOR analysis is a valuable diagnostic tool for precise discrimination between high and low grade tumours.

Similar results were obtained using PCNA or MIB-1 immunostainings; however, the latter has some advantages over PCNA immunostaining. In fact, MIB-1 homogeneously stains the sections, with minimal variation in the intensity from nucleus to nucleus; such characteristic reduces the interobserver discrepancy in the scoring process and, together with the absence of background interference, makes MIB-1 staining particularly suitable for automated image analysis. MIB-1 also strongly stains mitotic figures, allowing an easy and quick count. It seems surprising that PCNA scores were higher than MIB-1 scores: however, PCNA is over-ex-

pressed or upregulated in some tumours (Hall et al. 1990; Yu et al. 1991) and the half life of PCNA protein is approximately 20 h, so that it may be immunologically detectable in more cells than would be expected from S phase fraction assay by FCM (Hall et al. 1990).

The second purpose of the work was to assess whether morphometry, DNA-FCM and the measurements of cell proliferation could provide objective criteria for distinguishing low and high grade bladder carcinomas. Grade 2 carcinomas of WHO classification represent a large and heterogeneous group, with very different survival rates (Bergkvist et al. 1965; Koss 1975). The current concept is gaining acceptance that the majority of transitional cell carcinomas exists as non-aggressive low grade or aggressive high grade lesions (Jordan et al. 1987). Indeed, selective nuclear morphometry and DNA-FCM can separate bladder carcinomas into only two subgroups with different survival rates (Blomjous et al. 1989b, 1990).

By using morphometry, AgNOR, PCNA and MIB-1 stainings, we found many overlaps of values between G1 and G2 tumours, but no overlap between G3 and G1/G2 cases. Therefore, our findings support the tendency to classify the bladder urotheliomas in only two categories: one, the low grade bladder carcinomas, which may include G1 and G2 categories of the WHO classification, the other, the high grade bladder carcinomas, which may include the G3 category of WHO classification. All the techniques used in the present study are reproducible and easy to be performed; they can also give prognostic information, especially DNA-FCM, of which the clinical relevance has been extensively documented (Wheeless et al. 1993). Contrary to DNA-FCM and morphometry, AgNOR, PCNA and MIB-1 stainings are inexpensive procedures that can be performed in all laboratories. They permit the simultaneous evaluation of the morphology and tumour cell kinetics in the same histological section and may allow the diagnosis of high or low grade malignancy even in the individual case.

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