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QUANTITATION OF AgNORs IN UROTHELIAL CANCER - EVALUATION OF DIAGNOSTIC PARAMETERS IN HISTOLOGY AND CYTOLOGY

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ABSTRACT

The value of morphometry of silver stained nucleolar organizer regions (AgNOR) was assessed in 80 consecutive patients which had a diagnostic bladder washout prior to subsequent transurethral biopsy or tumor resection. 47 cases had transitional cell cancer, 12 flat atypical bladder lesions currently free of tumor cystoscopically. 19 patients without history of bladder carcinoma and histologically no epithelial atypia served as normal controls. In tissue sections urothelium exhibiting no or mild dysplasia showed few but large AgNORs (mean number [MNN]$=3.4\pm0.5$, mean area [MNA]$=0.29\pm0.08\mu m^2$). Flat atypical epithelium with moderate or severe dysplasia (D2, Cis), as well as, carcinoma displayed numerous silver stained dots (MNN$=6.4\pm1.4$; MNA$=0.12\pm0.04\mu m^2$). Regression analysis revealed an inverse correlation between MNN and MNA ($r=0.67$, $p<0.001$). Using the quotient of both parameters (NQ=MNN/MNA) transitional cell bladder lesions could be subdivided into urothelium i.) with no or mild dysplasia (normal), ii.) exhibiting moderate dysplasia (D2) or low grade carcinoma (G1), and iii.) displaying severe dysplasia (Cis) or high grade carcinoma (G2,G3). Identical diagnostic groups could be separated in the cytological specimens by determination of the mean total AgNOR area (TNA) in 30 most atypical cells. Thereby, the quotient of TNA in atypical and TNA in normal urothelial cells (AgNOR-index) proved to be the most sensitive parameter in detecting malignancy in urinary cytology.

Key words: AgNOR, cytology, histology, image analysis, morphometry, transitional cell bladder cancer.

INTRODUCTION

During the last five years, the diagnostic and prognostic value of the silver staining technique of nucleolar organizer regions (AgNOR) has been demonstrated in several human organ tumors (Crocker 1990, Rüschoff 1992). This new method is a valuable and inexpensive tool of evaluating the proliferative activity in routine histopathology. Application to urinary transitional epithelium revealed, however, inconsistent results. Although a step-wise increase of AgNOR number per cell from normal to dysplastic and neoplastic urothelium was found, a disappointing overlap between these diagnostic groups as well as between the various malignancy grades was evident (Ooms and Veldhuizen 1989, Cairns et al. 1989). In addition, Mansour et al. (1990) found no correlation between clinical outcome and mean AgNOR number; whereas, Lipponen and Eskelinen (1991) demonstrated a close relationship to clinical stage.

Since these studies have been performed by enumeration of AgNORs by eye, the current study demonstrates the diagnostic value of quantitative AgNOR analysis using digital image analysis. In addition, this is the first study demonstrating the applicability of the AgNOR technique to urinary cytology.

MATERIAL AND METHODS

The study comprised 80 consecutive patients, 47 with a histologically confirmed papillary and/or infiltrating bladder carcinoma, and 12 with flat atypical mucosal field alterations (moderate to severe dysplasia, D2/Cis) currently free of tumor cystoscopically. 19 patients had no history of bladder cancer and histology revealed normal urothelium or a mild dysplasia due to some degree of inflammation. In all cases, a diagnostic bladder washout was performed prior to subsequent transurethral biopsy or tumor resection.

Aliquots of the urinary washouts were freshly processed onto poly-l-lysin covered glass slides. After air drying overnight two slides were routinely stained with the May-Grünwald-Giemsa technique; another two slides were fixed in buffered 4 per cent formaldehyde (pH=7.0) prior to AgNOR staining.

The corresponding histological material was routinely fixed and paraffin embedded. Each block was serially sectioned at 3-5μm thickness. One was stained with hematoxylin and eosin (H&E), the other with colloidal silver. Typing and grading was done following the recommendations of the WHO (Mostofi et al. 1973). In flat non-infiltrating lesions definition of moderate (D2) and severe (D3) dysplasia corresponded to atypical hyperplasia and carcinoma in situ (Hofstädter et al. 1986, Koss 1985). TNM classification of tumors was done according to UICC (UICC 1987).

Silver staining for AgNOR followed the description given by Ploton et al. (1986). Silver incubation of 27 minutes gave the best staining result in tissue sections and incubation of 32 minutes in cytological specimens. This was determined by qualitative criteria (Crocker et al. 1989) and by staining time series as described previously (Rüschoff et al. 1990b).

Morphometry was done by single cell measurements using a digital image analysis system (Cue2, Olympus Optical Corp., Hamburg, FRG). Since AgNORs are in the region of 0.1-2μm^2 in area (Derenzini et al. 1986), a relatively high final magnification of 4000x was used on the monitor of the image analyser; the microscope magnification was 1000x. The

same set of AgNOR and nuclear parameters was determined both in histological and cytological specimens (Table 1). In tissue sections, 50 individual nuclei were selected at random and measured in the center of 3 different microscopic fields. Each nucleus was evaluated at that focal plane which yielded the best resolution of AgNORs on the image analyser screen. In cytological specimens nuclei were chosen in two different ways. At first, 50 urothelial cells were evaluated at random. Thereafter measurements were directed towards the 30 most atypical cells exhibiting large nuclei with high AgNOR content. For comparison of staining variabilities, in every slide TNA of 10 normal urothelial cells (TNAn) was determined. An AgNOR index was derived by forming a quotient of TNA in presumably atypical cells (TNAa) and TNAn. Thereby, only those nuclei showing undoubtably silver stained AgNORs were measured; i.e. only clearly visible cells not covered by other nuclei were taken into account.

Table 1. Morphometry of silver stained nuclei in urothelial bladder lesions

Parameters	Abbreviation
AgNOR values	
mean number/cell	MNN
mean area/AgNOR dot	MNA
total area/cell	TNA
Nuclear values	
mean area/cell	MNCA
mean nucleolar number/cell	MNCLN

For statistical analyses the Wilcoxon rank test for unpaired samples was used to compare between-group values. Regression analysis was performed to calculate the correlation coefficient between MNN and MNA. Values of $p < 0.05$ were regarded as significant.

RESULTS

Silver staining of biopsies yielded good and reproducible results with clearly discernible black AgNOR dots mainly within the nucleoli (Fig. 1). Similarly, in cytological specimens nuclei showed dispersed and clustered AgNOR dots (Fig. 2). The specific AgNOR staining reaction was, however, markedly reduced in case of background staining due to bacterial overgrowth and/or admixture of urea crystalls.

In comparison to tissue sections, cytological specimens showed a two to sevenfold increase of MNN being most elevated in high grade carcinoma (G3) and carcinoma in situ (Cis). A clear cut difference between normal and carcinomatous and/or atypical flat bladder lesions was found by determination of MNN and MNA in histology and by use of MNN and TNA in cytology. With respect to the various malignancy grades, an overlap of AgNOR values was, however, evident (Table 2). In addition, infiltrating carcinomas (> pTa) showed higher AgNOR values than non-infiltrating papillary lesions both in histology and cytology (data not shown).

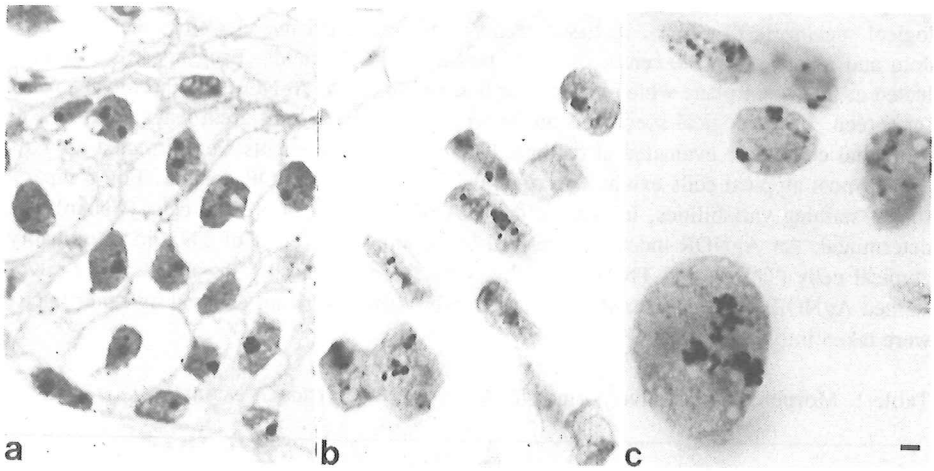

Fig. 1. AgNORs in histological sections of a) normal urothelium, b) low grade papillary carcinoma (G1,pTa), and c) high grade infiltrating carcinoma (G3,pT2). AgNOR stain, Barr: 1μm.

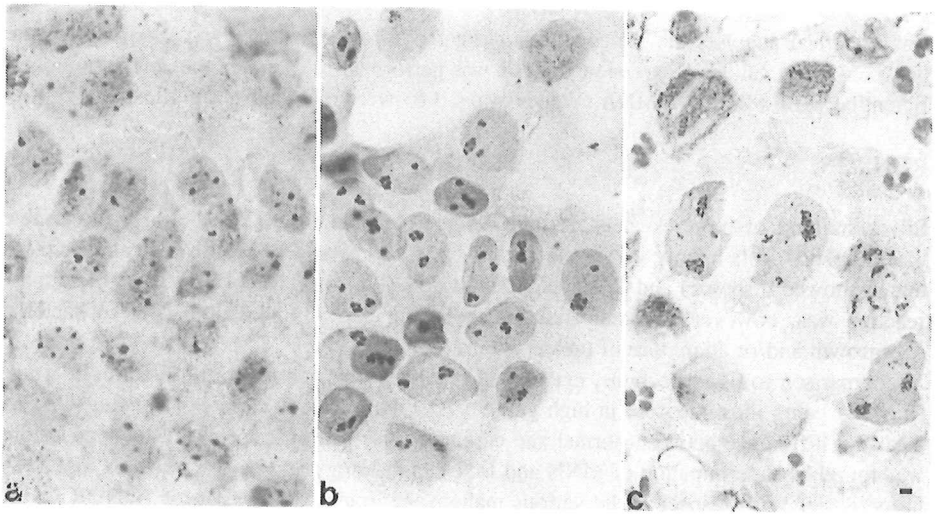

Fig. 2. AgNORs in cytologies of a) normal urothelium, b) low grade papillary cancer (G1,pTa), and c) high grade infiltrating carcinoma (G3,pT2). AgNOR stain, Barr: 1μm.

Table 2. Results of AgNOR and nuclear morphometry in histology and cytology (n=80)

	MNN		MNA	TNA	MNCA		MNCLN	
	(hi)	(cy)	(hi)	(cy)	(hi)	(cy)	(hi)	(cy)
No	3.2	7.5	0.30	1.9	26.3	89.2	1.3	2.2
(n=11)	±0.7	±1.2	±0.10	±0.4	±2.5	±21.5	±0.2	±0.4
D1	3.8	12.7	0.25	2.3	31.7	98.1	1.4	2.9
(n=8)	±0.4	±3.5	±0.05	±0.9	±3.1	±13.1	±0.4	±0.7
D2	6.3	29.7	0.16	6.0	39.8	126.2	1.9	3.9
(n=6)	±1.5	±7.7	0.04	±2.0	±9.1	±19.8	±0.6	±0.4
D3/Cis	9.4	53.8	0.10	12.5	103.7	210.9	2.3	6.1
(n=6)	±1.9	±19.5	±0.02	±7.1	±32.5	±81.2	±0.9	±1.8
G1	5.4	24.5	0.14	4.6	43.2	128.4	2.3	3.7
(n=21)	±1.2	±7.9	±0.06	±1.6	±7.5	±38.4	±0.7	±0.8
G2	6.2	38.1	0.12	8.5	51.2	154.1	2.7	4.9
(n=20)	±1.4	±14.2	±0.04	±4.3	±12.3	±48.3	±0.8	±1.1
G3	7.3	44.3	0.11	11.3	70.8	212.6	3.2	5.5
(n=6)	±1.6	±15.9	±0.05	±7.4	±21.4	±85.1	±0.9	±0.7

Abbreviations: hi: histology, cy: cytology; MNN, MNA, TNA,MNCA, MNCLN: see table 1; no: normal urothelium, D1-D3: various grades of dysplasia, G1-G3: malignancy grades

In tissue sections, regression analysis revealed a close relationship between MNN and MNA ($r=-0.67$, $p<0.001$). Thus, the quotient of both parameters proved to be advantageous over counting of AgNOR dots as well as determination of TNA. In cytological specimens, however, TNA in the 30 most AgNOR rich (atypical) cells gave a high diagnostic yield and was superior to measurements in 50 randomly selected cells (data not shown). In addition, the AgNOR index derived from the quotient of TNA in presumably atypical cells (TNAa) and TNA in normal urothelial cells (TNAn) proved to be a very sensitive tool in detecting malignancy in urinary cytology (Fig. 3).

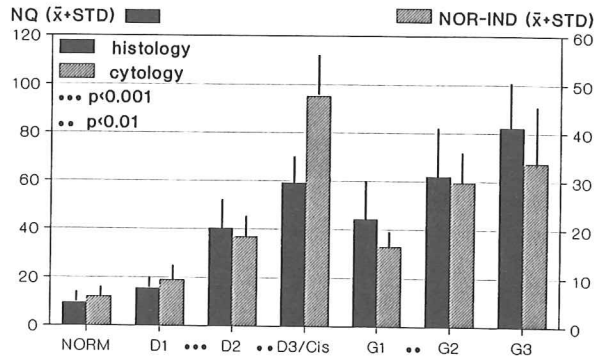

Fig. 3. Diagnostic value of AgNOR analysis in histology and cytology of bladder cancer determining NOR quotient (NQ), resp. NOR-index. D1-D3: grades of dysplasia in flat lesions. G1-G3: grades of malignancy in papillary and/or infiltrating cancer.

DISCUSSION

The presented study demonstrates the feasibility and diagnostic value of morphometry of AgNORs in neoplastic bladder lesions. This technique helps to discriminate between benign and atypical flat mucosal field alterations, as well as, between low grade and high grade carcinomas both in histology and cytology. The quotient of MNN and MNA is the most important diagnostic parameter in tissue sections. In cytology, TNA is closely related to the degree of dysplasia and grade of malignancy.

Evaluation of AgNORs has been shown to be a reliable marker of malignancy in various organ tumors (Crocker 1990). In tissue sections of urothelial neoplasms, however, this technique revealed conflicting results. Initially, Ooms and Veldhuizen (1989) found determination of AgNORs being of limited value in grading bladder carcinomas. In our view, their so-called argyrophilic bodies are mainly nucleoli. With respect to our data, enumeration of nucleoli, however, hampers the differentiation between various malignancy grades in bladder cancer (see table 2). This clearly shows that the staining result of the colloid silver technique cannot be evaluated without consideration of staining standards. Since AgNORs correspond mainly to the fibrillary centers at the ultrastructural level (Derenzini et al. 1986), only those silver stained slides should be evaluated that exhibit clearly discernible black dots primarily within the nucleoli (Crocker et al. 1989). Thus, staining time has to be adapted to the individual argyrophilia of a given tissue section. In this respect, determination of MNA in small resting lymphocytes has been shown to be a helpful parameter for quantitative assessment of the stainability of a given tissue section (Rüschoff et al. 1990). Similarly, in cytological specimens the quotient of TNA in presumable atypical and TNA in normal urothelial cells ("AgNOR index") compensates inevitable staining variabilities and thus, contributes to a standardized AgNOR morphometry in cytology (Rüschoff et al. 1992).

Moreover, morphometry of AgNORs has additional advantages over counting of AgNORs simply by eye. Cairns et al. (1989) found a step-wise increase of AgNOR number from normal to dysplastic and neoplastic urothelium. There was, however, a disappointing averlap between these diagnostic groups. In contrast, we observed a strong correlation between malignancy grade and the degree of dysplasia if number *and* size of silver stained dots were taken into consideration. In accordance with Crocker and Egan's values (1988) in non-Hodgkin's lymphomas, an inverse correlation bewteen MNN and MNA was found ($r=-0.67$, $p<0.001$). Thus, the quotient of both parameters (NQ) highly significantly discriminated between mild versus moderate to severe urothelial dysplasia (D2, Cis) in tissue sections. A prerequisite is an appropriate magnification of the light microscope. Since AgNORs are in the range of $0.1\text{-}2\mu m^2$, use of high primary magnification (x1000) is mandatory due to optical laws (Böck 1989). The AgNOR content of moderate dysplasia (D2) assessed by NQ was thereby in the range of well-differentiated papillary carcinoma (G1). The NQ of severe dysplasia (D3/Cis) was in the range of high grade carcinoma (G2/G3) indicating an almost identical proliferative potiential of these lesions.

To a certain degree, these data are similar to those obtained by DNA cytometry where aneuploidy is related to G2 and G3 carcinomas (Gustavson and Tribukait 1985), as well as, high grade flat bladder lesions (Hofstädter et al. 1986). Since evaluation of AgNORs can be regarded as a proliferation marker in tumor pathology (Trerè et al. 1991), comparable results have also been obtained by using Ki67 and BrdU-anti-BrdU immunostaining (Bush

et al. 1991, Mellon et al. 1990, Tsujihashi et al. 1991). In contrast to these techniques, however, AgNOR staining can be applied to routinely processsed paraffin embedded tissues and is economical with respect to time and expenses.

Most interestingly, we have shown that the AgNOR technique is of diagnostic value in urinary cytology. However, well preserved cell suspensions are of importance. Immediate fresh processing of bladder washouts onto glass slides and complete emptying of the bladder prior to irrigation are preconditions for reproducible staining results. Bacterial overgrowth, admixture of urinary crystalls and marked inflammation with granulocytes covering the transitional cells interferes with the specific AgNOR staining. It is, therefore, advisable to prepare several slides from different aliquots of varying cell density. In addition, alcohol based fixatives, such as Merckofix^R, result in more intense silver reactions than formalin. For diagnostic use, we found the measurement of TNA in the 30 most atypical cells (large nuclei with multiple AgNOR dots) to be the most sensitive method to discriminate between normal, low grade or high grade atypical urothelial cells. In contrast to tissue sections, cytological specimens contain the total AgNOR material within one focal plane. Thus, TNA proved to be a very sensitive parameter for the assessment of the AgNOR content in cytology. This parameter is far less sensitive in tissue sections. In breast cancer, for example, we found a good separation between benign and malignant, as well as, between the different malignancy grades determining NQ (Rüschoff et al. 1990a). In contrast, Derenzini et al. (1990) found a broad non-diagnostic overlap between TNA values in histological sections of breast tumors using a relatively low magnification (x40 objective lens) of the light microscope.

For diagnostic purposes, the method of cell selection seems to be of additional importance. We found measurements of the 30 most AgNOR rich nuclei being a reproducible and valuable tool for the evaluation of AgNORs in cytology (Rüschoff et al. 1992). Generally, this is in accordance with other morphometric studies in bladder cancer in which measurements favoring the most atypical cells gave a high diagnostic and prognostic yield (Blomjous et al. 1989, Lipponen et al. 1990). Accordingly, Lipponen et al. (1991) found similar results by AgNORs counts in the most atypical cells in tissue sections. AgNOR counting at random, however, seems to be of no predictive significance (Mansour 1990). Finally, studies are in progress to determine the value of AgNOR staining in urinary cytology for monitoring of patients with bladder cancer.

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