Virchows Archiv A Pathol Anat (1989) 414:365–369

Virchows Archiv A
Pathological Anatomy and Histopathology

Argyrophilic proteins of the nucleolar organizer region in bladder-tumours

E.C.M. Ooms and R.W. Veldhuizen
Department of Pathology, Westeinde Ziekenhuis, Lijnbaan 32, Den Haag, The Netherlands

Summary. The introduction of a simple one-stage silver nitrate stain as a method of displaying nucleolar organizer regions (NOR's) in formalin-fixed tissue has made it possible to examine the significance of these structures in tumour pathology. In the current study argyrophilic proteins of the NOR's were studied in 39 consecutive patients with bladder tumours. The results indicate that the counting of silver staining particles is only of limited value in grading bladder tumours and that this method is not superior to other additional grading techniques such as histo-morphometry and flow cytometry.

Key words: Bladder carcinoma – Transitional cell carcinoma – Nuclear organizer region – DNA – Nucleolus

Introduction

Nucleolar organizer regions (NOR's) are loops of D.N.A. that transcribe ribosomal R.N.A. The number of this regions (NOR's) per nucleus correlates with cellular activity and may be an indicator of the degree of malignancy in tumours. The possible significance in tumour pathology has been studied in melanoma (Crocker and Skilbeck 1987), lymphoma (Crocker and Paramjit Nar 1987) and breast carcinoma (Smith and Crocker 1988). The introduction of a simple one-stage silver nitrate stain as a method of displaying nucleolar organizer regions in formalin-fixed tissue made it possible to examine the significance of these structures in routine pathology. In the current study argyrophilic proteins of the NOR's are described in 39 consecutive patients with bladder tumours.

Offprint requests to: E.C.M. Ooms

Materials and methods

Thirtynine consecutive patients with bladder tumours of different grades have been studied. The tumours were graded according to the WHO grading system (Mostofi 1973) and the Morphometrical Grading System developed by Ooms et al. (Ooms et al. 1981) and staged according to the TNM staging system of 1987. The patients caracteristics are described in Table 1.

The staining procedure used by Smith and Crocker described in (Smith et al. 1988) has been used by us: Sections of 3 μm thickness were cut from the routinely processed paraffin blocks. These were dewaxed in xylene (3–5 min), and then rehydrated through ethanols to distilled, deionized water. The Ag-NOR solution was prepared by dissolving gelatine in 1 g/dl aqueous (distilled, deionized) formic acid at a concentration of 2 g/dl. This solution was mixed (1:2 volumes) with 50 g/dl aqueous (distilled, deionized) silver nitrate solution, to give the final working solution. This was immediately poured over the tissue sections and left for 30 min at room temperature in a dark place. The silver colloid was then washed off with distilled, deionized water and the sections dehydrated through graded ethanols to xylene and mounted in synthetic medium, DPX.

The staining reaction time was found to be quite critical. By allowing it to continue beyond 35 min, the distinction between the resulting Ag-NOR dots within clusters became progressively blurred, whereas reaction times of less than 25 min resulted in no appreciable Ag-NOR staining. Counterstaining was also found to be detrimental to the definition of Ag-NOR dots and was therefore not used.

For counting sections were examined under an oil immersion lens at a magnification of 1000. Per case 50 nuclei were studied. Cells were selected for the counting procedure in the following way: in each case 10 different microscopic fields were selected randomly. In each field 5 neighbouring cells were then selected for the counting procedure, the nucleus in the center of the field being the first selected item. Because of the difficulty in the interpretation of argyrophilic proteins in the nucleus we separated different elements for counting: small fragments (satellites), medium sized particles (nucleoli?), large round particles (nucleoli) and large non-round (rodshaped) elements (nucleoli) (Fig. 1).

For each grade the mean values and their standard deviations were calculated for the absolute counts of each type of silver staining fragment. The results of the absolute counts are shown in Fig. 2A. The relative amount (%) of each fragment in the different grades has been calculated histiograms have been constructed from these percentages (Fig. 2B). The data were analysed by means of student's unpaired t-test.

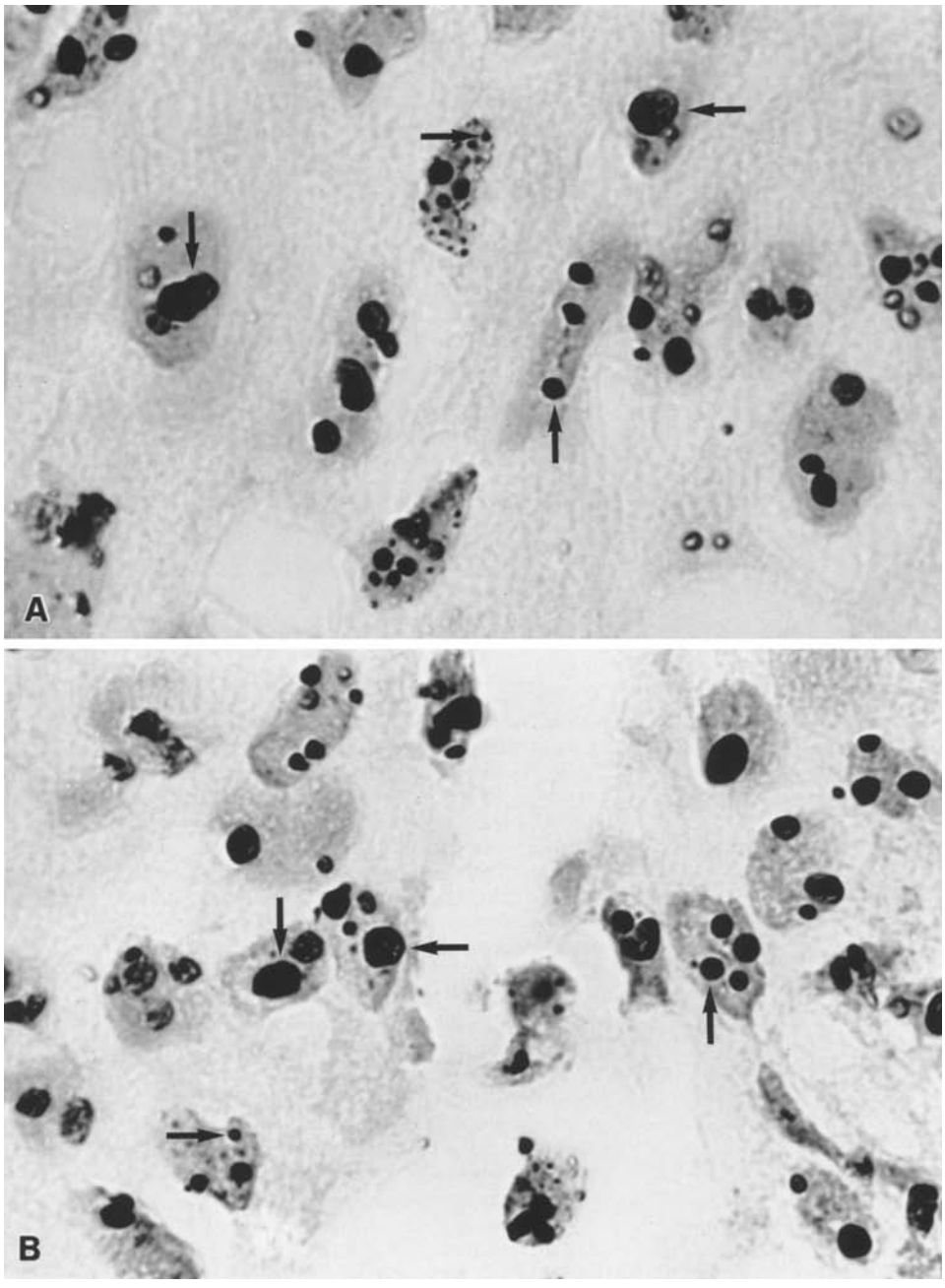

Fig. 1. Argyrophilic bodies (Ag-NOR's) in nuclei of bladder carcinoma cells. **(A)** and **(B)** show the different types of argyrophilic bodies as used in our scoring systems: small, intermediate, large round, large non round

Results

The results of the counting of the different silver staining particles are listed in Table 2. This large table gives information on the considerable overlap of the counts in the different grades.

This overlap is nicely illustrated by the standard deviation of the mean values per grade (group) which can be found in Table 3 A (absolute counts) and 3 B (relative counts). The mean values of the absolute counts are listed in Table 3 A. In higher grades there is a greater number of small, large round and large rodshaped (non round) particles. For these values the differences are only significant for the distinction between grade I and grade III tumours. The differences are not significant for the grade I and grade II tumours or for grade II and grade III tumours. The figures show that high counts are an indication of high grade malignancy. Low absolute counts, however, can be found in all different grades. Obviously the same holds true for the relative values (Table 3 B).

Table 1. Characteristics of the 39 consecutive patients with bladder tumours

Rang number	grade WHO '73	Morphometrical grade (Ooms '81)	PT Stage TNM '87	Sex M/F	Age
A I	I	I	T1	M	70
B I	I	I	T1	V	61
C I	I	I	T1	V	54
D I	I	I	T1	M	69
E I	I	I	T1A	V	69
F I	I	I	T1A	M	76
G I	I	I	T1	M	73
H I	I	I	T1	M	54
I I	I	I	T1	V	50
J I	I	I	inv. pap	M	58
K I	I	I	T1	M	72
L I	I	I	T1	M	76
A II	II	II	T1A	M	59
B II	II	II	T1A	M	70
C II	II	II	T1A	M	84
D II	II	II	T1	M	53
E II	II	II	T2	M	75
F II	II	II	T2	V	84
G II	II	II	T2	V	93
H II	II	II	T1	M	64
I II	II	II	T3	M	80
J II	II	II	T1	M	76
A III	III	III	T1	M	59
B III	III	III	T3	V	74
C III	III	III	T2	M	79
D III	III	III	T3	M	91
E III	III	III	T3	M	43
F III	III	III	T3	M	80
G III	III	III	T3	M	69
H III	III	III	T2	M	58
I III	III	III	T2	M	53
J III	III	III	T2	V	69
K III	III	III	T2	M	77
L III	III	III	T1	V	76
M III	III	III	T3	M	60
N III	III	III	T3	M	77
O III	III	III	T2	M	89
P III	III	III	T3	M	69
Q III	III	III	T3/4	M	82

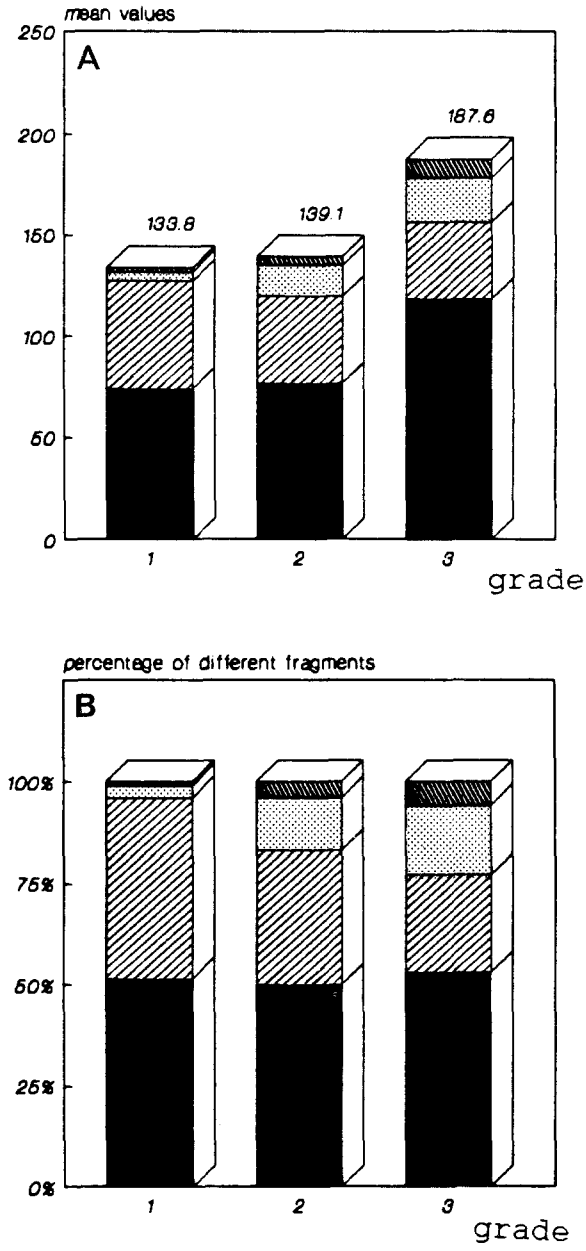

Fig. 2. Correlation of different patient groups by silver staining fragments. The mean values of the absolute counts (**A**) and the relative values (**B**) in stacked bars. ■ small; ▨ medium; ▩ 1 = large round; ▧ 1 = large rod. In each case Agnors have been counted in 50 nuclei

The mean values of the absolute counts (Table 3A) and of the relative values (Table 3B) have been visualized in histiograms (Figs. 2A and 2B).

Discussion

Grading of bladder tumours has been a subject of continuous discussion in the literature (Bergkvist et al. 1965; Mostofi et al. 1973; Ooms et al. 1981; Ooms et al. 1985). Methods have been developed to come to a more objective and reproducable grading system since subjective grading leads to a great inter- and intraobserver variation (Ooms et al. 1981, 1985). Recent reports suggest that in some situations silver-binding nucleolar organizer region counts on tissue sections may facilitate the prediction of biological behaviour of tumours. This has been described in melanoma (Crocker et al. 1987), lymphoma (Crocker et al. 1987) and breast cancer (Smith et al. 1988).

In the current study we examined 39 consecutive patients with bladder tumours. The staining method described by Smith and Crocker (Smith et al. 1988) appeared to be reproducable in our

Table 2. Counting results of each case. In each case 50 nuclei were randomly selected for the counting procedure

Rang number	small fragments	medium	large round	large non-round	total
A I	91	36	24	11	162
B I	72	66	4	0	142
C I	47	71	3	6	127
D I	80	52	6	3	141
E I	122	43	0	1	166
F I	43	59	0	1	103
G I	13	71	6	0	90
H I	156	27	2	0	185
I I	102	52	2	0	156
J I	17	59	0	0	176
K I	52	53	9	1	125
L I	90	50	1	0	141
A II	87	43	3	1	134
B II	79	45	4	1	129
C II	23	34	14	13	84
D II	54	39	46	1	140
E II	47	73	12	0	132
F II	44	20	38	10	112
G II	138	59	2	0	197
H II	202	22	3	3	230
I II	47	25	23	5	100
J II	43	73	6	9	131
A III	215	37	0	1	153
B III	168	33	7	0	208
C III	77	51	39	1	168
D III	142	75	21	32	170
E III	48	26	34	20	128
F III	75	28	42	13	158
G III	125	46	13	6	190
H III	123	31	19	4	177
I III	74	14	38	14	140
J III	199	39	0	0	238
K III	248	22	1	0	271
L III	16	54	1	2	73
M III	105	75	29	22	231
N III	316	19	0	0	335
O III	25	34	45	11	115
P III	32	19	43	11	105
Q III	25	42	43	20	130

laboratory and therefore we followed this procedure strictly. Staining reveals numerous silver staining structures, varying greatly in size and shape. The larger stained structures probably correspond to nucleoli. The significance of the smaller particles remains obscure since they probably are strongly influenced by fixation artefacts. We therefore divided the different staining particles into 4 groups: – small fragments (probably satellites, r-DNA?, nuclear fragments from previous mitosis) – medium sized particles (possibly small nucleoli) – large round particles (nucleoli) and large non-round particles (abnormal nucleoli). The results indicate that the counting of silver staining particles are of limitted value in grading bladder tumours. Low counts of the different particles are found in all tumour grades, high counts are an indication of high grade malignancy. The high standard deviations show that, at least in our hands, this technique is not superior to other methods of grading such as morphometry and flow cytometry.

The study of argyrophilic proteins in the nucleolar organizer region has been studied extensively in tumour pathology. The argyrophilia of the Ag-NOR proteins appears as a preferential cytochemical marker for both the R-DNA and of the level of the actual or potential transcribtion of the R-DNA. Consequently, silver staining is a useful tool for the study both of the structure of the nucleolus and for variation of nucleolar activity. Ochs demonstrated that nucleolar silver staining protein is the major silver staining protein of the nucleolus and that it is directly or indirectly associated with R-DNA (Ochs et al. 1984). Other evidence indicates that protein C23, or its degradation products, is associated with preribosomes in the nucleus and with ribosome maturation (Escande et al. 1985;

Table 3. **(a)** mean values and SD of the counts per grade (counts in 50 randomly selected nuclei)

Type:	small			medium			large round			large rod		
	mean	%	sd	mean	%	sd	mean	%	sd	mean	%	sd
Grade I	73.8	51	40.4	53.3	44	12.8	4.8	3	6.4	1.9	1	3.2
Grade II	76.4	50	51.9	43.3	33	18.6	15.1	13	14.9	4.3	4	4.5
Grade III	118.4	53	84.3	37.9	24	17.4	22.1	17	17.5	9.2	6	9.5

(b) relative amount of different silver staining particles in each grade (counts in 50 randomly selected nuclei)

Type:	small		medium		large round		large rod	
	mean	sd	mean	sd	mean	sd	mean	sd
Grade I	51%	19%	44%	20%	3%	4%	1%	2%
Grade II	50%	18%	33%	14%	13%	12%	4%	5%
Grade III	53%	25%	24%	15%	17%	14%	6%	6%

Gas et al. 1985). Following the demonstration of the selectivity of the silver techniques for C23 en 100 K proteins, it has been postulated that staining sites in the intact cell represent the in situ localisation of these proteins. However, the true function of the nucleolar silver staining protein remains a mystery. Moreover, pathologists are not yet familiar with the possible artefacts caused by fixation and further processing of the material. Silver staining is not yet an entirely reliable method for the examination of the NOR's.

The current study indicates that the examination of argyrophilic proteins of the nucleolar organizer region may, in a modest way, contribute to a more accurate grading of bladder tumours, but there may be pitfalls in this new diagnostic method. Further investigation (on fixation artefacts and the reproducibility of counting procedures) is needed before this technique can be implemented in routine diagnostic tumour pathology.

Acknowledgments. The authors are indepted to: Eric de Graaf (statistical analysis), Marianne van Huffelen (word-processing), Frans van Leeuwen (graphics), George Patho (photographic assistance).

References

Bergkvist A, Ljungqvist A, Moberger G (1965) Classification of bladder tumors based on the cellular pattern. Acta Chirurg Scand 130–371

Crocker J, Skilbeck N (1987) Nucleolar organiser region associated proteins in cutaneous melanotic lesions: a quantitative study. J Clin Pathol 40:885–889

Crocker J, Paramjit N (1987) Nucleolar organizer regions in lymphomas. J Pathol 151:111–118

Escande ML, Gas N, Stevens BJ (1985) Immunolocalization of the 100 K nucleolar protein in CHO cells. Biol Cell 53:99–110

Gas N, Escande ML, Stevens BJ (1985) Immunolocalization of the 100 kDa nucleolar protein during the mitotic cycle in CHO cells. Biol Cell 53:209–218

Mostofi FK, Sobin LH, Torloni H (1973) Histological typing of urinary bladder tumours. Geneva, Wolth Health Organization no. 10

Ochs RL, Busch H (1984) Further evidence that phosphoprotein C23 (110 kD/p I5.1) is the nucleolar silver staining protein. Exp Cell Res 152:260–265

Ooms ECM, Essed E, Veldhuizen RW, Alons CL, Kurver PHJ, Boon ME (1981) The prognostic significance of morphometry in T1 bladder tumours. Histopathology 5:311–318

Ooms ECM, Anderson WAD, Alons CL, Veldhuizen RW, Boon ME (1983) An analysis of the performance of pathologists in grading bladder tumors. Hum Pathol 14:140–143

Ooms ECM, Kurver PHJ, Veldhuizen RW, Alons CL, Boon ME (1983) Morphometric grading of bladder tumors in comparison with histologic grading by pathologists. Hum Pathol 14:144–150

Ooms ECM, Blok APR, Veldhuizen RW (1984) The reproducibility of a quantitative grading system of bladder tumours. Histopathology 9:501–509

Smith R, Crocker J (1988) Evaluation of nucleolar oragnizer region – associated proteins in breast malignancy. Histopathology 12:113–125

Received August 10, 1988 / Accepted December 29, 1988