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Nucleolar and argyrophilic nucleolar organizer region counts in urothelial carcinomas with special emphasis on grade II tumors

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Abstract Prognostic assessment of bladder carcinomas of intermediate differentiation is difficult. This study therefore investigated the prognostic values of nucleolar status and silver staining of argyrophilic nucleolar organizer regions (AgNORs) in grade II bladder carcinomas. In biopsies from 34 grade II transitional cell carcinomas of the urinary bladder the number of nuclei with nucleoli, the location of nucleoli within the nucleus and the number of AgNORs were determined in 1000 or 200 nuclei per section respectively. Ten biopsies showing normal urothelium, 18 cases with mild to severe atypia, 27 grade I, 34 grade II and 12 grade III transitional cell carcinomas were also studied. Significantly differing nucleolar and AgNOR values were found comparing normal urothelium/grade I carcinomas with severe urothelial atypia/grade III carcinomas. Grade II carcinomas, however, were inhomogeneous. One subgroup had nucleolar and AgNOR values resembling grade I carcinomas while the second group had values similar to those of grade III carcinomas. This subdivision of grade II carcinomas correlates with results reported for DNA-cytometry. The results suggest a subdivision of patients with grade II transitional cell carcinomas into a low risk and high risk group.

Key words Transitional cell carcinoma · Nucleoli Argyrophilic nucleolar organizer regions

Introduction

Strategies for the treatment of transitional cell carcinomas of the urinary tract, especially of the urinary bladder, are based either on the extent of stromal invasion and/or on the estimated proliferative potential of the tumour. Using these criteria, it is possible to classify nearly 90% of bladder carcinomas as low or high risk malignancies. However, the prognosis of carcinomas with limited invasion and intermediate differentiation showing mixed papillary/solid growth pattern is difficult to predict. Various methods assessing cell proliferation such as thymidine autoradiography, DNA-cytometry and immunohistochemical proliferation assays have been used to subgrade this group of bladder tumours [1, 2, 3, 12, 26]. The results of these studies were promising, but there is still a need for simple measures to predict the prognosis of the intermediate group of bladder carcinomas. It was therefore the purpose of this investigation to test the count of silver stained nucleolar organizer regions (AgNORs) in combination with the occurrence and location of nucleoli to subgrade these tumours.

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Materials and methods

One hundred and three urinary bladder biopsies were fixed in 4% formalin, embedded in paraffin blocks and cut into 3 µm thick slices, transferred onto slides and dewaxed. For standard histopathological diagnosis, the slides were haematoxylin and eosin stained. The tumours were graded and staged according to the criteria of the World Health Organisation and TNM-classifications [13, 20].

The number of nuclei containing nucleoli and the number of nucleoli inside each nucleus were determined. The nucleolar size was related to the nuclear size and the relationship expressed as the ratio of nucleolar and nuclear diameter. Two groups (cut off point 0.125) were distinguished. Furthermore, we evaluated the intranuclear location of the nucleoli (central or eccentric position). Nucleoli in intermediate location were not considered. On average, 1000 nuclei on haematoxylin and eosion stained slides were evaluated per section [8, 9, 11, 15, 19] (Fig. 1).

For AgNOR staining two solutions were mixed (2:1):500 g/l aqueous silver nitrate solution and 20 g/l gelatin in 10 g/l aqueous formic acid. After staining the slides were incubated at 37° C for 30 min [22]. Staining was considered successful when silver stained dots (AgNORs) were clearly visible within the nucleoli.

The AgNORs whithin and also outside the nucleoli were counted following the recommendation of Crocker [5]. AgNOR counting was performed after focussing on the nuclear membrane and the fine granular matrix. Overlapping dots were not counted. Two hundred nuclei were evaluated per section. The AgNOR and nucleolar analyses were done at a 1000 fold magnification using oil immersion and an eyepiece graticule (Leitz/Orthoplan). The re-

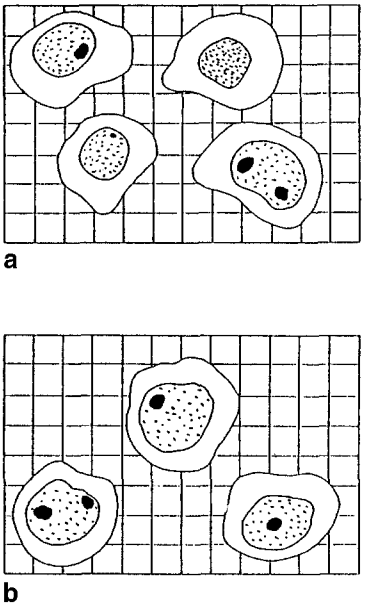

Fig. 1. **a** Distribution of number and size of nucleoli within nuclei (solitary or multiple nucleoli). **b** Location of nucleoli within nuclei (central or eccentric)

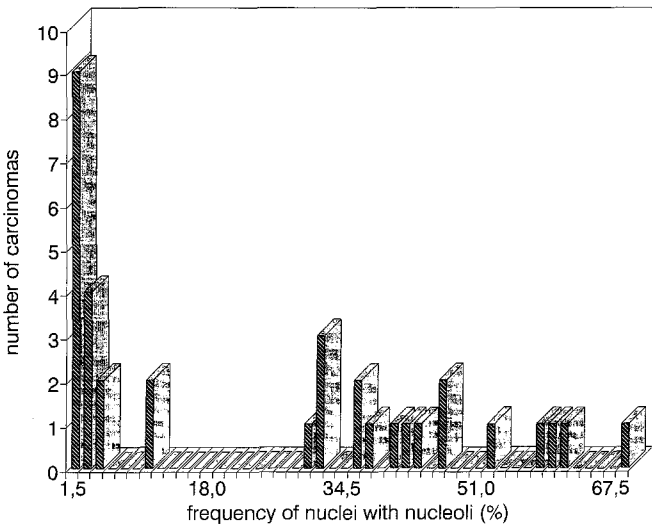

Fig. 2. Distribution of the frequency of nuclei with nucleoli in grade II urothelial carcinomas

sults were analysed statistically by applying the Student's *t*-test and the Shapiro-Wilk normality test. Data are expressed as mean ±standard deviation. The cut off point was determined according to the Gauss distribution curves of both subgroups. The cut off point was then defined as the value which has an equal probability to belong to either subgroup.

Results

The histological diagnoses in 103 bladder biopsies were as follows: 10 biopsies showed normal transitional epithelium and 75 transitional cell carcinoma. Twenty seven of these carcinomas were grade I, 36 grade II and 12 grade III. Mild to severe urothelial atypia (D1, D2, D3) was diagnosed in 18 cases. The stage of the grade I carcinoma was mostly pTa (13 cases). Fourteen cases of grade I and all 34 cases of grade II carcinomas had superficial stromal invasion (stage pT1), whereas the grade III carcinomas showed deep invasion (pT2-pT4b).

Nucleolar Status

The frequency of nuclei with nucleoli (nucleolar frequency) in transitional cell carcinomas with grade I malignancy ranged from 1.1%–1.6%. In contrast, the nucleolar frequency in grade III carcinomas increased to mean values of 63.1±30.2. The nucleolar frequency of grade II/pT1 carcinomas did not follow a Gaussian distribution, as demonstrated by the Shapiro-Wilk test ($P<0.0093$). The distribution of nucleolar frequencies was characterized by low values of nucleolar frequency and a second group of high values with an obvious gap in between (Fig. 2). Therefore we subdivided the grade II carcinomas in two groups showing normal distribution (cut off point 12.12). The first group consisted of 17 cases with low values and an average value of 3.6±2.4% (all values smaller than the cut off point). The other 17 cases with high values had an average of 41.1±11.2%. This difference was highly significant ($P<0.001$).

Grade I papillary carcinomas and the 17 cases of grade II carcinomas with low nucleolar frequency taken together had nuclei with one singular nucleolus, whereas grade III carcinomas and the other 17 cases of grade II carcinomas with increased number of nuclei with nucleoli showed an average of 1.8±0.8 and 1.5±0.5 respectively. The grade I carcinomas had small to medium sized and centrally located nucleoli, whereas grade III carcinomas had large and peripherally located nuclei. Similarly to the frequency of nuclei containing nucleoli and the number of nucleoli per nucleus, the first group of grade II carcinomas (GIIa) had small to medium sized centrally located nucleoli, whereas the other 17 cases (GIIb) showed values similar to grade III carcinomas (Table 1; Figs. 3, 4).

AgNOR analysis

The mean number of AgNORs per nucleus in normal transitional cell epithelium was 2.5±0.6. D1 and D2 showed a mean value of 4.5±0.3. The mean AgNOR value of D3 or situ carcinoma was 9.2±2.4. The differences were highly significant ($P<0.001$).

Well differentiated papillary transitional cell carcinomas with mild cellular atypia had a mean value of 5.2±2.1. The solid grade III transitional cell carcinomas had an average value of 15.2±3.6.

Table 1 Nucleolar status of urothelial carcinomas

Histology	Grade	Number of cases	Frequency of nuclei with nucleoli (%)	Number of nucleoli/ nucleus	Nucleolar/ nuclear diameter		Location of nucleoli in nuclei	
					<0.125 (%)	>0.125 (%)	central (%)	eccentric (%)
Papillary	I	27	1.6+0.4	0.5+0.4	87.1	12.9	100.0	–
Papillary	IIa	17	2.5+3.3	0.9+0.5	76.3	23.7	77.2	22.8
Papillary	IIb	17	43.5+12.0	1.5+0.5	18.9	81.1	10.5	89.5
Solid	III	12	63.1+30.2	1.8+0.8	1.9	98.1	–	100.0

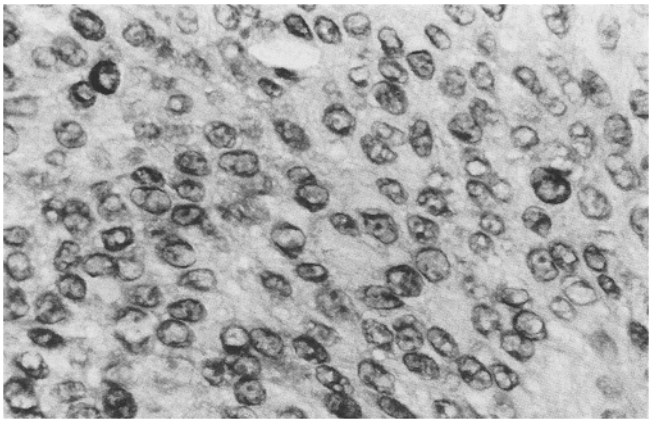

Fig. 3. Urothelial carcinoma (grade IIa) with few singular nucleoli in central position within the nuclei. Haematoxylin and eosin ×625

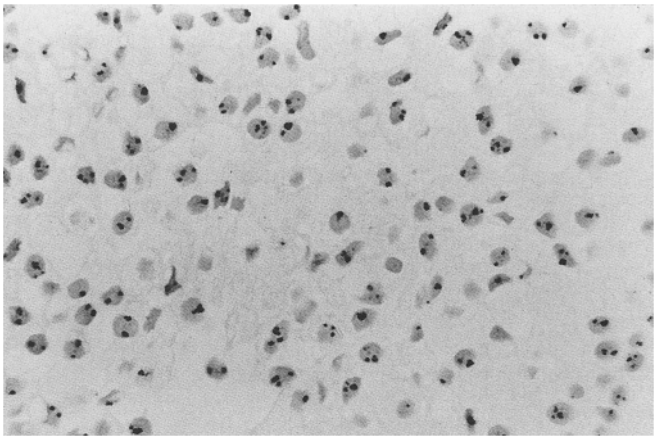

Fig. 5. Low number of argyrophilic nucleolar organizer regions (AgNORs; *black dots*) in grade IIa papillary urothelial carcinoma. Silver staining ×625

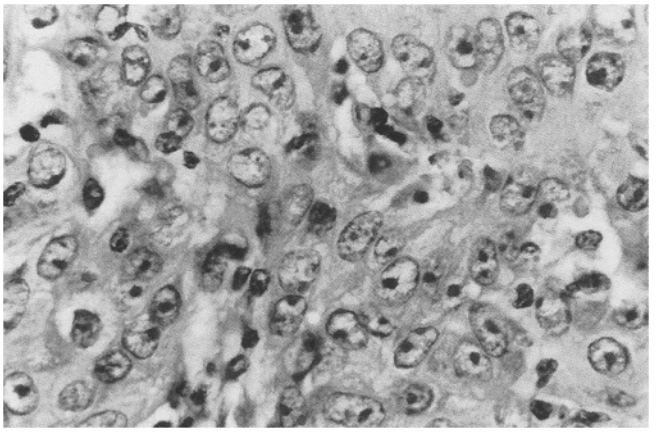

Fig. 4. Urothelial carcinoma (grade IIb) with multiple nucleoli in eccentric position within the nuclei. Haematoxylin and eosin ×625

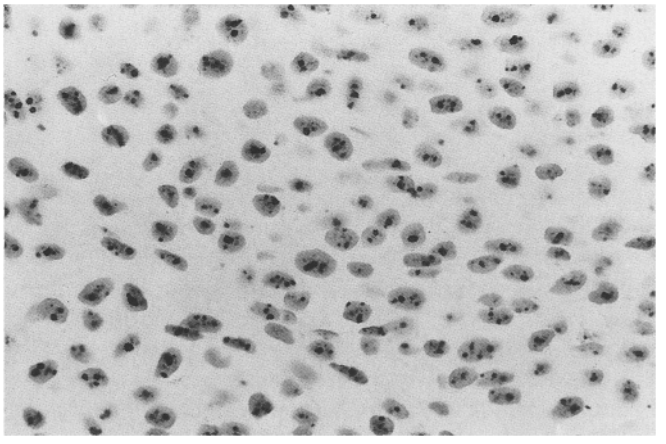

Fig. 6. Increased number of AgNORs in grade IIb papillary urothelial carcinoma. Silver staining ×625

The AgNOR analysis of the grade II carcinomas was performed within the two separated groups subdivided according to the nucleolar findings. The AgNOR value of the carcinomas characterised by low nucleolar frequency had an average of 6.8±2.3. The 17 cases with high nucleolar frequency had an average value of 10.9±3.2. This difference was highly significant ($P<0.001$). Therefore we subdivided the grade II carcinomas in two subgroups GIIa and GIIb (Figs. 5, 6, 7). Furthermore, when the tumour groups grade I and GIIa and GIIb as well as grade III were pooled respectively, statistics showed a highly significant difference between these groups ($P<0.001$).

Discussion

In this study we demonstrated significant differences in the nucleolar and AgNOR status between carcinomas of the urinary bladder with low or high grade malignancy (grade I or grade III). Urothelial carcinomas of low malignancy had only few nuclei with one single nucleolus

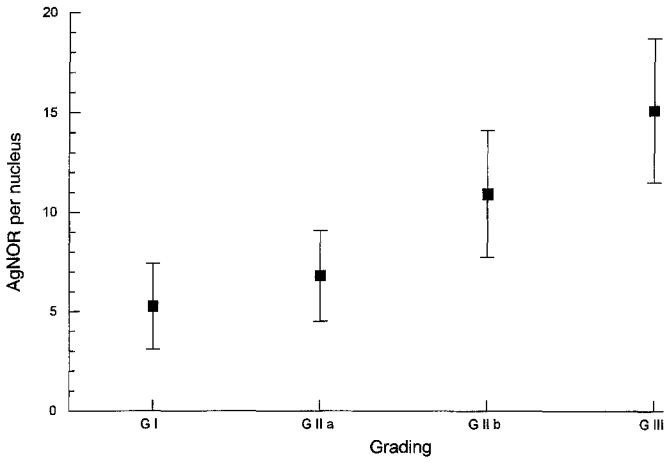

Fig. 7. Increasing AgNOR values of urothelial carcinomas with increasing grade of malignancy

in central position and were characterized by a small number of large AgNORs, while highly malignant tumours had a high frequency of nuclei with more than one prominent nucleolus in mainly eccentric position and a large number of small AgNORs per nucleus.

These results confirm the recently reported data of Pich et al. [21] and correspond also to results of morphometric studies. In these studies significant differences were found between normal urothelium, D2 and D3, and grade I, grade II and grade III carcinomas regarding to the percentage of nuclei with nucleoli and the number of AgNORs [17, 18, 23, 24]. Other studies showed considerable overlap between grades I, II and III transitional cell carcinomas without stromal invasion (pTa), regardless of recurrence rate [6], and also an overlap between dysplastic and neoplastic tissue [4]. In our study there was no overlap, but we found a heterogeneous AgNOR and nucleolar pattern within the group of carcinomas of grade II/pT1. In these tumours two subgroups could be separated. One group showed values similar to those grade I carcinomas, while the other was comparable to grade III carcinomas. The differences between the two groups examining either AgNORs or nucleoli were highly significant. These data are in accordance with the findings from cellular kinetic studies using autoradiographic analyses in combination with the immunohistochemical staining pattern of tissue polypeptide antigen and carcinoembryonic antigen [10, 12].

Furthermore, we support previous the findings of Skopelitou et al. [25] and Pich et al. [21]. These authors have studied cellular proliferation in bladder carcinomas emplyoing AgNOR analysis, proliferating cell nuclear antigen and Ki67 immunohistochemistry, nuclear morphometry, and DNA-flow-cytometry. While many overlaps of values were seen between grade I and grade II tumours, none were found between grade III and grade I/grade II tumours. This absence of overlap particularly with regard to AgNOR analysis, supports the suggestion that bladder carcinomas should be classified in two categories of malignancy only [21]. The difference of subgroups of grade II transitional cell carcinoma found using the AgNOR method has not been reported before.

A subdivision of grade II carcinomas into two groups is also supported by DNA cytometric analysis, where some grade II carcinomas were found to have values comparable with grade I carcinomas while others were similar to grade III carcinomas. Furthermore, 5-year survival rates of 92%–95% were found in diploid transitional cell carcinomas as opposed to 61.4%–62.5% in aneuploid carcinomas [1, 2, 26]. Numerous DNA analyses showed that grade I transitional cell carcinomas are predominantly diploid and grade III carcinomas are almost exclusively aneuploid. Approximately 30%–40% of grade II carcinomas are diploid and 60%–70% are aneuploid or polyploid [3, 16]. The heterogeneous nature of the grade II group was also demonstrated with respect to nuclear size and DNA pattern [7, 24]. A study performed by Kirkhus et al. [14] subclassified grade II carcinomas into two subgroups: one subgroup shows nuclear diploidy and a favourable prognosis while the other is characterized by aneuploidy and an unfavourable prognosis.

In conclusion, our results and those of Kirkhus et al. [14] and Pich et al. [21] suggest that patients with grade II transitional cell carcinomas should be divided into two groups (IIa and IIb) according to their different prognosis. Patients with transitional cell carcinomas grades I and IIa seem to belong to a low risk group requiring cautious surgical treatment, whereas patients with grade IIb and grade III urothelial carcinomas belong to a high risk group and should be treated more aggressively.

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