Urol Res (1995) 23: 27–32

ORIGINAL PAPER

H. Iwata · T. Otoshi · N. Takada · T. Murai
S. Tamano · T. Watanabe · Y. Katsura · S. Fukushima

Validation of silver-stained nucleolar organizer regions for evaluation of invasive character of urinary bladder carcinoma in rats and mice

Received: 11 April 1994 / Accepted: 3 November 1994

Abstract A series of 8 rat and 16 mouse invasive bladder carcinomas were investigated for the presence of silver-stained nucleolar organizer regions (AgNORs) to clarify whether this parameter is applicable to the estimation of their invasive character. With regard to number of AgNORs per cell, neither rat nor mouse carcinomas showed any difference between invasive and noninvasive sites within the same tumor. However, the frequency of cancer cells bearing bizarre dots, irregular in size and shape, was significantly higher at sites of actual invasion. Quantitative data generated using an image analyzer revealed significantly lower values for NOR roundness and significantly larger NOR size in invasive sites than in noninvasive sites in all groups. Double staining for the proliferation marker proliferating cell nuclear antigen (PCNA) and AgNORs was performed on eight rat carcinomas and a close correlation between the two was confirmed. Thus the number of AgNORs in PCNA-positive cells was significantly greater than in PCNA-negative cells. Furthermore, a particularly strong correlation was observed for incidences of PCNA-positive cells and bizarre dots ($P<0.01$). The quantitative data also demonstrated significant differences in size and shape of dots. It is concluded that AgNORs have diagnostic value for the invasive character of bladder carcinomas.

Key words Silver-stained nucleolar organizer regions Proliferating cell nuclear antigen · Experimental bladder carcinoma · Invasive character · Image analyzer

H. Iwata (✉) · T. Otoshi · N. Takada · T. Murai · S. Fukushima
First Department of Pathology, Osaka City University Medical School, 1-4-54 Asahi-machi, Abeno-ku, Osaka 545, Japan
Fax: (+81) (6)6463093

S. Tamano
First Department of Pathology, Nagoya City University Medical School, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan

T. Watanabe · Y. Katsura
Environmental Health Science Laboratory, Sumitomo Chemical Co. Ltd., 3-1-98, Kasugade-naka, Konohana-ku, Osaka 554, Japan

Nucleolar organizer regions (NORs) are loops of DNA located in the short arms of the acrocentric chromosomes (13–15, 21, 22) [1]. They bear ribosomal RNA (rRNA) genes which are transcribed by RNA polymerase I and are associated with various proteins such as B23 and C23 of unknown function which selectively bind silver nitrate [12]. The AgNOR technique visualizes argyrophilic non-histone proteins as black dots in the nucleus. It is considered that differences in number of these dots reflect the transcriptional activity of rRNA, in other words, levels of cellular and nuclear turnover. Indeed, the technique has been applied to various types of benign and malignant tumors, with an intimate association being indicated between AgNORs and histopathological findings. In the field of human urological malignancies, renal cell carcinomas, prostatic carcinomas and urinary bladder carcinomas have been found to show a correlation between AgNOR counts and grade of malignancy [9, 10, 15, 20].

The proliferating cell nuclear antigen (PCNA) was originally detected in systemic lupus erythematosus patients and subsequently proved to be an auxiliary protein of DNA polymerase delta which appears in late G1-phase and S-phase [2, 21]. Since Robbins first applied it to the immunohistochemistry of paraffin-embedded sections [22], it has been recognized that PCNA is a G1- and S-phase marker [13], which can be used to assess cell proliferation [17]. In the present study, we investigated AgNORs with reference to the invasive character of experimental urinary bladder carcinomas. Clinically, there are some bladder tumors that are difficult to judge for tumor stage and thus superficial or invasive type. The number, area and shape of AgNOR dots in invasive (over pT1) and noninvasive (Ta) regions of the same tumor were compared to assess their diagnostic potential. In addition, double staining of AgNORs and PCNA was undertaken to allow direct examination of the relationship between AgNOR counts, area or shape and PCNA expression as an index of cell proliferation.

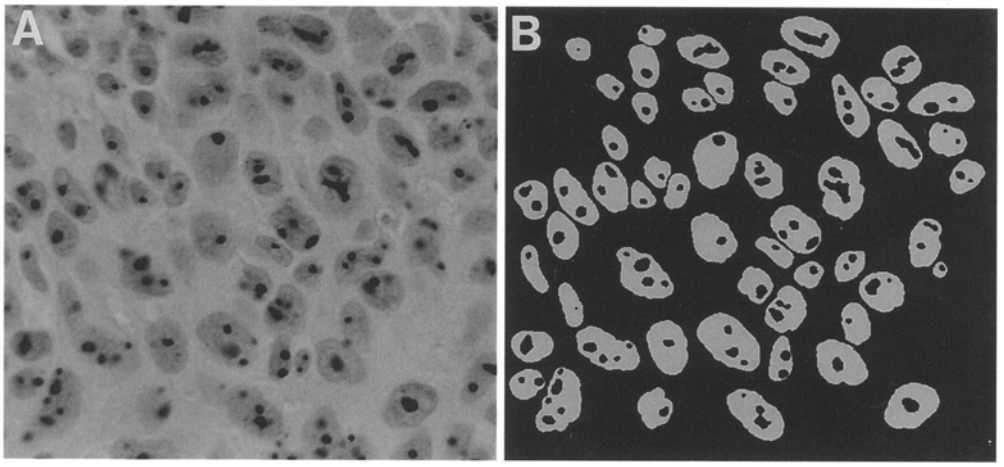


Fig. 1 Quantitative analysis was performed with an image analyzer (IPAP, Image processor for analytical pathology: Sumika Technos, Osaka, Japan) linked to a light microscope equipped with a ×40 objective lens. The microscopic images obtained (**A**) were scanned with a color television camera (DXC-930, Sony, Tokyo, Japan) as represented in **B**. The system software automatically calculated the area and roundness of AgNORs simultaneously

Materials and methods

Tumor materials

A total of 27 male F344 rats and male B6C3F1 mice (6 weeks old at the start of the study) were obtained from Charles River Japan, Atsugi, Japan. In order to induce carcinomas 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) for rats or 0.05% *N*-ethyl-*N*-(4-hydroxybutyl)nitrosamine (EHBN) for mice was given in the drinking water for a maximum of 32 weeks. Bladders were fixed in 10% neutral-buffered formalin and routinely processed for paraffin-embedding. Tumors obtained were eight rat papillary transitional cell carcinomas (TCCs), partially showing squamous cell differentiation, nine mouse TCCs and ten mouse squamous cell carcinomas (SCCs). All samples were diagnosed as histological grade 2 and all were of the invasive type, showing infiltration beyond the muscle into the serosa of the bladder wall.

AgNOR staining

Sections were cut at a thickness of 3 μm, dewaxed in xylene and hydrated through an ethanol series. Then AgNOR staining was performed following a modification of Chiu's method [4, 23]. The sections were treated with an acetic acid-ethanol (1:3) mixture for 5 min, rinsed with absolute ethanol, and transferred to 1% celloidin in equal parts of ethanol and ether for 1 min and placed in 70% ethanol. After this pretreatment, silver colloid solution was prepared by dissolving 2 g/dl gelatin in 1 g/dl aqueous formic acid and mixing this in a volume of 50% (w/w) aqueous silver nitrate solution. Specimens were incubated in silver colloid for 15 min at 45 °C and finally washed with deionized water and fixed with 5% sodium thiosulfate for 5 min.

Evaluation of AgNORs

For all specimens, the number of AgNOR dots/cell was counted for at least 200 tumor cells at two different sites in the same tumor. The region at which tumor cells infiltrated the muscle layer to the serosa was defined as an invasive site and the superficial region opposite to an invasive site was defined as a noninvasive site. These were selected under the light microscope at low magnification. The frequencies of cancer cells bearing at least one irregularly shaped bizarre dot were also examined in 500 cells at both sites. For quantitative analysis, the roundness and areas of AgNORs were measured with the aid of a color video image processor (IPAP, image processor for analytical pathology; Sumika Technos, Osaka, Japan) [29] in 100 cells each of invasive and noninvasive sites (Fig. 1). The value for roundness was calculated as $4\pi S/P^2$ (S, area of AgNORs; P, perimeter of AgNORs) and expressed per cell. The value of the AgNOR area was also expressed per cell.

Double staining for AgNORs and PCNA

First, PCNA staining of rat bladder carcinomas was performed. The primary monoclonal antibody (PC10, Dako Japan Co., Kyoto, Japan) was used at a dilution of 1:300 and the incubation time was 16 h. Strept ABcomplex/AP (Code No. K391, Dako) was selected to apply a new fuchsin substrate system, which stains the target antigen red. Next AgNOR staining was performed without celloidin pretreatment with an incubation period of 40 min at room temperature.

Evaluation of double staining

The number of AgNOR dots in both PCNA-positive and -negative cells in noninvasive sites was assessed in 500 cells for each sample. In addition, the frequency of bizarre dots in both populations was determined. Quantitative analysis was performed subsequently and the values for AgNOR roundness and area were calculated.

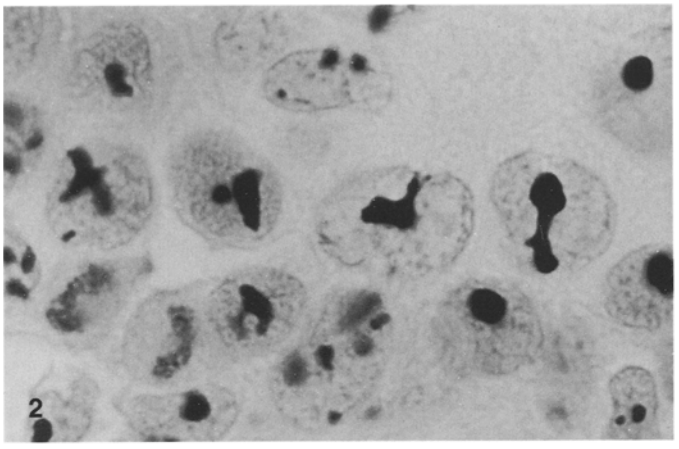
Statistical analysis

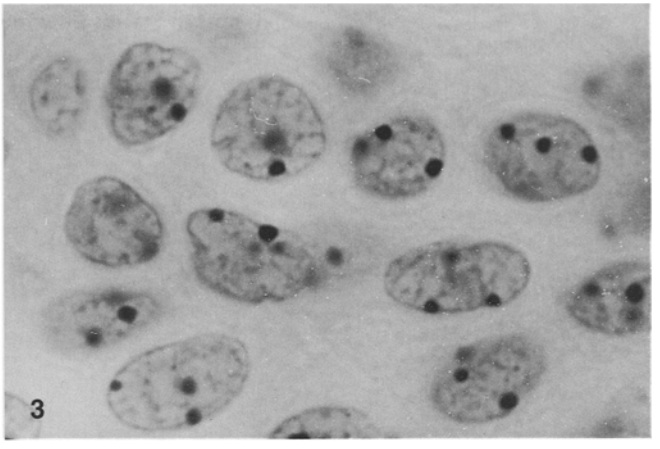
Statistical analysis was performed using the paired Student's t-test.

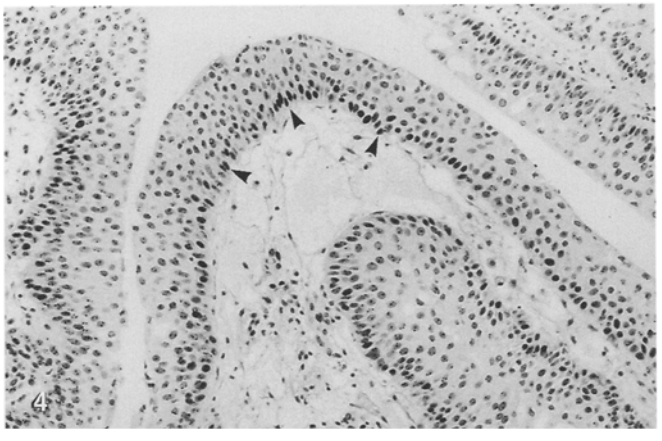
Results

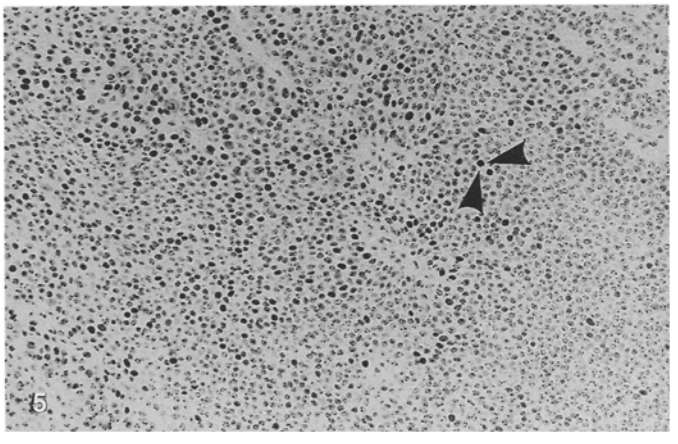
AgNOR dots could be observed clearly in the nucleus of all specimens. Data for the numbers of AgNOR dots/cell in rat and mouse invasive bladder carcinomas are summarized in Table 1. No statistically significant differences were observed in numbers of AgNORs between invasive and noninvasive sites of carcinomas in rats and mice.

Frequencies of cancer cells with bizarre dots in rat and mouse invasive carcinomas are shown in Table 2. AgNOR dots at invasive sites tended to be more elongated, elliptical or irregular in shape (Fig. 2). In contrast dots observed at noninvasive sites were mainly small and round in shape (Fig. 3). The frequencies of cancer cells with bizarre dots at invasive sites in both rat and mouse carcinomas were significantly higher than those at noninvasive sites ($P<0.01$).









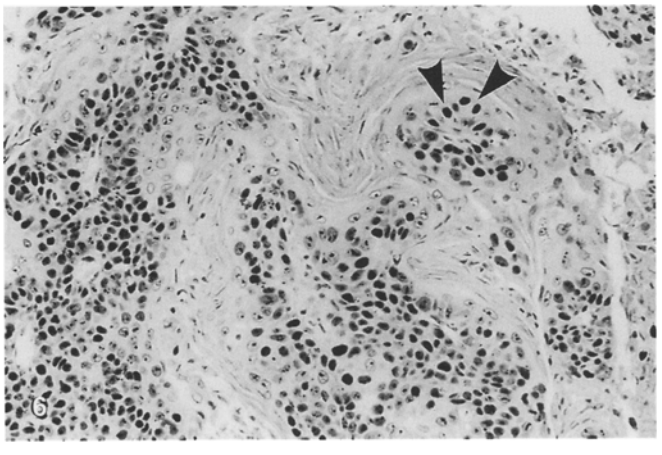


Fig. 2 AgNOR dots at an invasive site of a rat TCC are elongated, elliptical or irregular in shape, ×2000

Fig. 3 AgNOR dots at an noninvasive site of a rat TCC are small and round in shape, ×2000

Fig. 4 PCNA-positive cells are observed concentrated in the basal layer in a noninvasive site of a rat TCC, PCNA-positive cell, *arrowhead*, ×200

Fig. 5 PCNA-positive cells (*arrowhead*) are diffusely scattered in a noninvasive site of a rat TCC, ×200

Fig. 6 A large number of PCNA-positive cells indicated by *arrowheads* are present in an SCC region within a rat TCC, ×250

Table 1 Number of AgNOR dots/cell in invasive bladder carcinomas (values are mean ± SD)

	No. of cases	Invasive site	Noninvasive site
Rat TCC	8	1.93 ± 0.34	1.82 ± 0.22
Mouse TCC	9	1.92 ± 0.32	1.86 ± 0.33
Mouse SCC	10	1.90 ± 0.29	1.96 ± 0.32

Table 2 Percentage frequencies of cancer cells having bizarre dots in invasive bladder carcinomas (values are mean ± SD, * $P < 0.01$, invasive site vs. respective noninvasive site)

	No. of cases	Invasive site (%)	Noninvasive site (%)
Rat TCC	8	14.4 ± 12.7*	5.6 ± 5.8
Mouse TCC	9	13.8 ± 8.17*	7.6 ± 4.3
Mouse SCC	10	14.4 ± 7.4*	7.6 ± 4.7

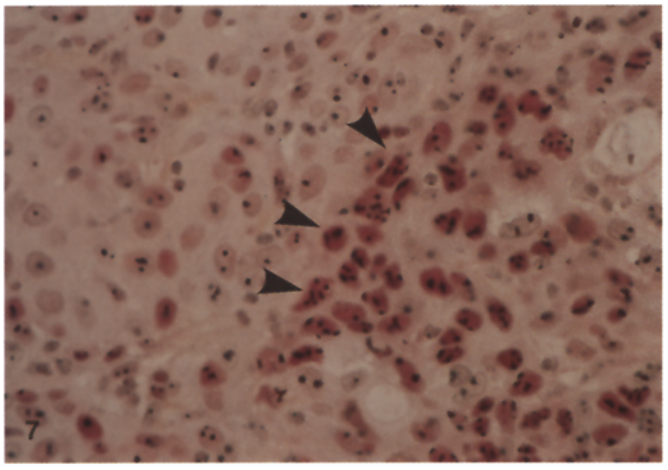


Fig. 7 In a rat TCC bizarre dots are mainly observed in PCNA-positive cells with red-stained nuclei, PCNA-positive cell, *arrowheads*, ×400

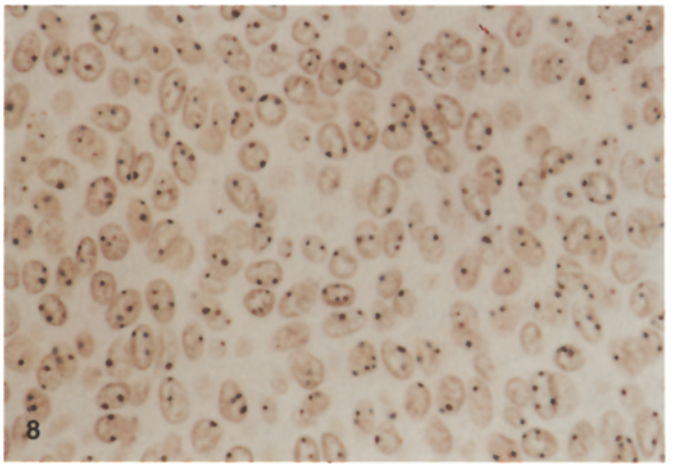


Fig. 8 In a rat TCC small and round dots are mainly seen in PCNA-negative cells. No PCNA-positive cell is present in this figure, ×400

Table 3 Number of AgNOR dots and percentage frequencies of PCNA-positive and -negative cancer cells having bizarre dots in noninvasive sites of invasive carcinomas in eight rats (values are mean ± SD, * $P<0.05$, PCNA-positive cells vs. -negative cells, ** $P<0.01$, PCNA-positive cells vs. -negative cells

	PCNA-positive cells	PCNA-negative cells
No. of dots	2.12± 0.34*	1.77±0.33
Frequency of cancer cells having bizarre dots (%)	29.0±11.7**	4.5 ±2.1

Table 4 Quantitative data for the roundness of AgNORs in invasive bladder carcinomas (values are mean±SD, * $P<0.01$, ** $P<0.005$, *** $P<0.0001$; invasive site vs. respective noninvasive site)

	No. of cases	Invasive site (%)	Noninvasive site (%)
Rat TCC	8	0.95±0.02*	0.98±0.01
Mouse TCC	9	0.96±0.02**	0.99±0.01
Mouse SCC	10	0.92±0.02***	0.98±0.01

Table 5 Quantitative data for the area of AgNORs/cell in invasive bladder carcinomas (values are mean±SD, * $P<0.05$, ** $P<0.0005$, *** $P<0.0001$; invasive site vs. respective noninvasive site)

	No. of cases	Invasive site (μm^2)	Noninvasive site (μm^2)
Rat TCC	8	5.18±1.75*	3.43±1.11
Mouse TCC	9	5.90±0.85**	2.99±0.72
Mouse SCC	10	8.86±1.31***	4.01±0.91

PCNA-positive cells showed different patterns in each lesion. In one case of rat TCC, PCNA-positive cells were concentrated in the basal layers of the tumors (Fig. 4), but in another area in same case they were diffusely scattered (Fig. 5). In another case, many more PCNA-positive cells were observed in a specific region, particularly in an SCC region within a TCC (Fig. 6) but, inversely, they were hardly observed in another area. Thus PCNA-positive cells demonstrated considerable heterogeneity within lesions in such cases.

Table 6 Quantitative data for the roundness of AgNORs in PCNA-positive and -negative cancer cells in noninvasive sites of eight rat invasive carcinomas (values are mean±SD, * $P<0.005$, PCNA-positive cells vs. PCNA-negative cells)

	PCNA-positive cells	PCNA-negative cells
Roundness of AgNORs	0.94±0.02*	0.99±0.00
AgNOR area (μm^2)	5.2±0.90*	1.69±0.25

Double staining of AgNORs and PCNA revealed a close relationship between these parameters. As shown in Table 3, both the number of AgNOR dots and frequencies of cancer cells with bizarre dots were significantly different between PCNA-positive and -negative cells. With regard to the number of dots, the value was clearly higher in PCNA-positive cells ($P<0.05$). Moreover, cancer cells with bizarre dots as shown in Fig. 7 were also mainly seen in PCNA-positive cells and the AgNOR areas appeared larger than in PCNA-negative cells. In the latter case the majority of dots were small and round in shape as shown in Fig. 8. The differences in frequency of cancer cells with bizarre dots between PCNA-positive and -negative cases was highly significant ($P<0.01$).

Quantitative data for the roundness and area of AgNORs measured by image analyzer supported the results obtained by light microscopic examination. The roundness of AgNORs in invasive sites demonstrated significantly lower values than noninvasive sites in all groups ($P<0.01-0.0001$, Table 4), and the areas of AgNORs in invasive sites were about twice as large as in noninvasive sites (Table 5). Similar relationships were evident for the roundness and areas of AgNORs between PCNA-positive and -negative cells (Table 6). In particularly the area

of AgNORs in PCNA-positive cells was significantly larger (more than 3 times) than in PCNA-negative cells. The data thus provided a definite indication that many more bizarre and large dots were in invasive sites and PCNA-positive cells.

Discussion

In this present study, comparison of the numbers, areas and shapes of AgNORs in invasive and noninvasive sites within the same tumors in a series of grade 2 rat TCCs and mouse TCCs and SCCs induced by BBN and EHBN revealed clear differences for bizarre forms and areas of AgNORs. Thus AgNOR shape and area rather than number reflected the invasive character of cancer cells. In addition, double staining with PCNA demonstrated a correlation for both number and particularly shape and area with this index of cell proliferation.

Since the silver-staining technique was first developed to demonstrate NORs as black dots in nuclei of paraffin-embedded tissues, the AgNOR approach has been applied to various benign and malignant tumors or normal tissues. Many pathologists have reported the numbers of AgNOR dots in high-grade malignant tumors to be much greater than in low-grade ones [5, 24]. Similarly, clear differences have been observed between malignant and benign tumors [7, 30]. Takeuchi et al. [27] reported experimental bladder tumors induced by BBN to show a good relationship between AgNOR count and malignancy grade. In addition, they described the sequence of changes from simple hyperplasia to carcinoma during carcinogenesis to be mirrored by increases in numbers of AgNOR dots. As for human urinary bladder TCCs, Lipponen et al. [2] indicated a prognostic value of AgNORs for superficial cancers. In our case we found the shape and area of AgNOR dots to be more important for evaluation of malignancy in urinary bladder carcinomas.

Crocker et al. [8] indicated an intimate association between the numbers of AgNOR dots and the percentage of cells in the S-phase of the cell cycle. Giri et al. [11] also observed a relationship to DNA ploidy using DNA flow cytometric analysis. AgNOR counts thus reflect cell proliferation as has also been confirmed in connection with Ki-67 and 5-bromo-2′-deoxyuridine (BrdU) immunostaining [18, 28], both of which are considered to be markers of DNA synthesis. Staining of PCNA has become one of the most popular approaches to detection of cell proliferation, because it can be applied to paraffin-embedded sections without the necessity of administration of chemicals such as BrdU before death of the animal. It was previously shown that PCNA expression and grading of human TCC are related [14]. However, recent application of a sequential staining technique revealed no correlation between AgNOR scores and proliferating cell counts [25]. We were able to document evidence of a direct association using double staining of both AgNORs and PCNA in the present study. The number of AgNOR dots and frequency of bizarre dots were clearly higher in PCNA-positive cells ($P<0.05$, $P<0.01$). Furthermore, quantitative analysis revealed conclusive differences in roundness and area of AgNORs between PCNA-positive and -negative cells ($P<0.005$). It is considered that NOR-associated protein is profusely produced in late G1- and S-phase and is seen as large and bizarre dots. Thus, we conclude that AgNORs reflect cell proliferation as well as invasive character.

On the other hand, Ooms et al. [19] suggested that AgNORs were of limited value due to the scatter of NOR numbers, and this might be caused by fixation artifacts, which could generate small fragments. But speculation only on the number of AgNORs as in this report is not sufficient and might mislead us. Therefore, systematic assessment of AgNOR parameters including number, area and shape is clearly required. Indeed large round and rod-shaped dots were increased in line with a high grade in their report. Recently it has often been discussed that not only the number but also areas of the AgNORs can be an indicator of malignant character [6, 16], but so far shapes have not been well defined [3] because of difficulties in their measurement. In the present study quantitative morphologic analysis was performed using a new image analyzer, IPAP, which allowed a more systematic assessment of AgNORs.

With regard to number, Suresh et al. [26] described two different counting techniques for estimating NOR clusters as discrete single AgNOR dots or separate AgNORs. Thus it is possible that bizarre dots may be composed of multiples of round dots. In this study the former method was selected for counting AgNORs because these dots could not be distinguished separately even under higher magnification. A distinction could not be made between invasive and noninvasive regions on the basis either of cell morphological features on hematoxylin-eosin staining or number of AgNORs; it proved possible in the present study to separate them in terms of the shapes and areas of the latter. As we regard noninvasive sites as Ta papillary tumors, this may be applied for judgment of whether a noninvasive papillary TCC close to the submucosa will acquire invasive ability and infiltrate into the muscle layer, and if we perform transurethral resection of bladder tumor and subsequent AgNOR analysis we should be able to diagnose whether a tumor is of the superficial or invasive type by observation of the state of the AgNORs. The existence of bizarre or large dots in resected portions would suggest deeper tumor growth over this portion.

References

1. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1983) Molecular biology of the cell. Garland Publishing, New York, p 424
2. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature 326:515

3. Bufo P, Franssanito F, Maiorano E (1991) Gastric carcinoma: histopathology, immunocytochemistry and variations of nucleolar organizer regions (AgNORs). Boll Soc Ital Biol Sper 67:707
4. Chiu KY, Loke SL, Wong KK (1989) Improved silver technique for showing nucleolar organiser regions in paraffin wax sections. J Clin Pathol 42:992
5. Crocker J, Nar P (1987) Nucleolar organizer regions in lymphomas. J Pathol 151:111
6. Crocker J, Egan MJ (1988) Correlation between NOR size and numbers in non-Hodgkin's lymphomas. J Pathol 156:233
7. Crocker J, McGovern J (1988) Nucleolar organiser regions in normal, cirrhotic, and carcinomatous livers. J Clin Pathol 41:1044
8. Crocker J, Macartney JC, Smith PJ (1988) Correlation between DNA and flow cytometric and nucleolar organizer region data in non-Hodgkin's lymphomas. J Pathol 154:151
9. Delahunt B, Ribas JL, Nancey JN, Bethwaite PB (1991) Nucleolar organizer regions and prognosis in renal cell carcinoma. J Pathol 163:31
10. Deschenes J, Weidner N (1990) Nucleolar organizer regions (NOR) in hyperplastic and neoplastic prostate disease. Am J Surg Pathol 14:1148
11. Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JCE (1989) Silver-binding nucleolar organiser regions (AgNORs) in benign and malignant breast lesions: correlations with ploidy and growth phase by DNA flow cytometry. J Pathol 157:307
12. Hernandez-Verdun D (1983) The nucleolar organizer regions. Biol Cell 49:191
13. Landberg G, Roos G (1991) Antibodies to proliferating cell nuclear antigen as S-phase probes in flow cytometric cell cycle analysis. Cancer Res 51:4570
14. Lipponen PK, Eskelinen MJ (1992) Cell proliferation of transitional cell bladder tumors determined by PCNA/cyclin immunostaining and its prognostic value. Br J Cancer 66:171
15. Lipponen PK, Eskelinen MJ, Nordling S (1991) Nucleolar organiser regions (AgNORs) as predictors in transitional cell bladder cancer. Br J Cancer 64:1139
16. Martin-DeLeon PA, Muneses C, Picciano S, Mealey J, Dickerson R (1988) Differential silver staining in lymphocytes and lymphoblastoid cell cultures. Cytobios 55:113
17. Matsuno Y, Hirohashi S, Furuya S, Sakamoto M, Mukai K, Shimosato Y (1990) Heterogeneity of proliferating activity in nodule-in-nodule lesions of small hepatocellular carcinoma. Jap J Cancer Res 81:1137
18. Mourad WA, Connelly JH, Sembera DL, Atkinson EN, Bruner JM (1993) The correlation of two argyrophilic nucleolar organizer region counting methods with bromodeoxyuridine-labeling index: a study of metastatic tumors of the brain. Hum Pathol 24:206
19. Ooms ECM, Veldhuizen RW (1989) Argyrophilic proteins of the nucleolar organizer region in bladder-tumors. Virchows Arch [A] 414:365
20. Pich A, Valente G, Azzoni L, Stramignoni A, Margaria E, Tasso M (1991) Argyrophilic nucleolar organizer region counts and Ki-67 scores in human renal cell carcinoma. Path Res Pract 187:482
21. Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature 362:517
22. Robbins BA, Vega D de la, Ogata K, Tan EM, Nakamura RM (1987) Immunohistochemical detection of proliferating cell nuclear antigen in solid human malignancies. Arch Pathol Lab Med 111:841
23. Sato M, Chida T, Watanabe H, Noda Y, Ajioka Y, Katayama A, Abe M (1991) A modified argyrophilic nucleolar organiser region (AgNORs) staining method for nucleolar staining in formalin-fixed, paraffin-embedded materials. Gastroenterol Jap 6:391
24. Sivridis E, Sims B (1990) Nucleolar organiser regions: new prognostic variable in breast carcinoma. J Clin Pathol 43:390
25. Smith FG, Murray P, Crocker J (1993) Correlation between PCNA and AgNORs scores in non-Hodgkin's lymphomas using sequential staining technique. J Clin Pathol 46:28
26. Suresh UR, Chawner L, Buckley CH, Fox H (1990) Do AgNORs counts reflect cellular ploidy or cellular proliferation? a study of trophoblastic tissue. J Pathol 156:233
27. Takeuchi T, Tanaka T, Ohno T, Yamamoto N, Kobayashi S, Kuruyama M, Kawada Y, Mori H (1990) Nucleolar organizer regions in rat urinary bladder tumor induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Virchows Archiv [B] 58:383
28. Trere D, Farabegoli F, Cancellieri A, Ceccarelli C, Eusebi V, Derenzini M (1991) AgNOR area in interphase nuclei of human tumors correlations with the proliferative activity evaluated by bromodeoxyuridine labeling and Ki-67 immunostaining. J Pathol 165:53
29. Watanabe T, Katsura Y, Yoshitake A, Masataki H, Mori T (in press) IPAP: Image processor for analytical pathology. J Toxicol Pathol
30. Yang P, Huang GS, Zhu XS (1990) Role of nucleolar organiser regions in differentiating malignant from benign tumours of the colon. J Clin Pathol 43:235