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Nucleolar organiser regions in hyperplastic and neoplastic prostatic tissue

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Summary. Silver-stained nucleolar organiser regions (AgNORs) were studied in 10 hyperplastic, 5 intra-epithelial neoplastic and 30 malignant prostatic lesions. Total AgNOR counts and types were compared with histological features. The total AgNOR count per nucleus was significantly higher ($P<0.05$) in prostatic intra-epithelial neoplasia (PIN) and adenocarcinoma compared with prostatic hyperplasia. In addition, satellite AgNORs predominated in hyperplasia, while medium-sized and large nucleoli with granular AgNORs were only observed in PIN and adenocarcinoma. The results indicate that, despite statistically significant differences, AgNOR counts are of no use for diagnosis of any single case in the groups studied, because of considerable overlap. AgNOR typing, however, may contribute to the differential diagnosis between benign and malignant lesions. We propose a new AgNOR typing system.

Key words: Prostate tumours – Hyperplasia – Adenocarcinoma – Nucleolar organiser regions – DNA

Introduction

Silver-stained nucleolar organiser regions (AgNORs) contain loops of DNA with RNA genes, which may relate to such variables as cell proliferation rate, ploidy, transcriptional activity and to tumour malignancy potential quantitatively and qualitatively (Underwood and Giri 1988).

AgNORs are demonstrated by a quick one-step silver nitrate method for formalin-fixed tissue. Studies on different tissues, including skin (Howat et al. 1989), lymphoid (Crocker and Nar 1988), breast (Smith and Crocker 1988), gynaecological (Ramsden and Murray 1989), bladder (Ooms and Veldhuizen 1989) and gastrointestinal (Griffiths et al. 1989), have given conflicting results regarding the diagnostic and prognostic value of AgNORs in tumour pathology.

The grading of prostatic intra-epithelial neoplasia (PIN), as defined by McNeal and Bostwick (1986) and the WHO grading system for prostate adenocarcinomas (Mostofi et al. 1980), takes nuclear features into account. Consequently, we studied the presence of AgNORs in 45 hyperplastic and neoplastic prostate tissue samples from transurethrally resected prostate (TUR-P) lesions.

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Materials and methods

TUR-P specimens (45) were extracted from the files of our institute and examined. The PIN lesions were classified according to McNeal and Bostwick (1986) (Fig. 1). Adenocarcinomas were graded according to the WHO system, in which anaplasia refers to nuclear anaplasia (Mostofi et al. 1980). The cases were grouped as follows: hyperplasia ($n=10$), PIN I ($n=3$), PIN II ($n=1$), PIN III ($n=1$), well-differentiated carcinomas (slight anaplasia, $n=4$; moderate anaplasia, $n=6$), moderately differentiated carcinomas (slight anaplasia, $n=3$; moderate anaplasia, $n=7$) and poorly differentiated carcinomas (slight anaplasia, $n=2$; moderate anaplasia, $n=5$; marked anaplasia, $n=3$).

The NOR staining method described by Smith and Crocker (1988) was used: sections were cut at 3 μm from routinely processed paraffin blocks. These were dewaxed in xylene (5 min) and hydrated through ethanols to distilled, de-ionized water. The AgNOR staining solution was prepared by dissolving gelatin in 1 g/dl aqueous formic acid at a concentration of 2 g/dl. This solution was mixed, 1:2 volumes, with 50 g/dl aqueous silver nitrate solution, to give the final working solution. This was poured over the tissues and left for 30 min, at room temperature in the dark. The silver colloid was then washed off with distilled, de-ionized water and the sections were dehydrated through graded ethanols to xylene and mounted in synthetic medium, DPX. No counterstaining was applied.

In all cases, 100 nuclei per case were counted, using an oil-immersion lens at a magnification of ×1000. In each case 10 neighbouring cells in 10 randomly selected fields were examined, starting in the field-centre. AgNOR counting and typing was done after sharp focusing on the nuclear membrane and fine granular nuclear matrix in each nucleus. Only individual AgNORs with sharp non-blurred contours in this plane of focus were selected for description (Fig. 2). For AgNOR counting, recommendations set forth by

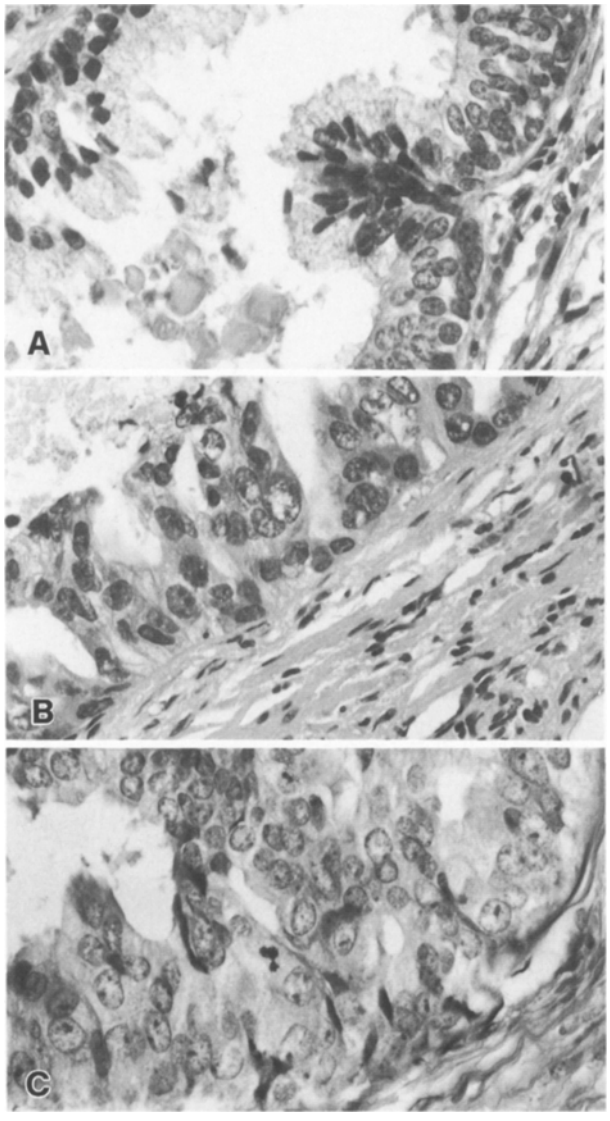

Fig. 1. A Prostatic intra-epithelial neoplasia (PIN) I with intraductal cell crowding, increase in nuclear size, anisokaryosis and normal chromatin. **B** PIN II with the features of PIN I and in addition, hyperchromatism and occasional small nucleoli. **C** PIN III with the features of PIN II and, in addition, numerous prominent nucleoli. There is incomplete intra-luminal bridging. H&E, ×400

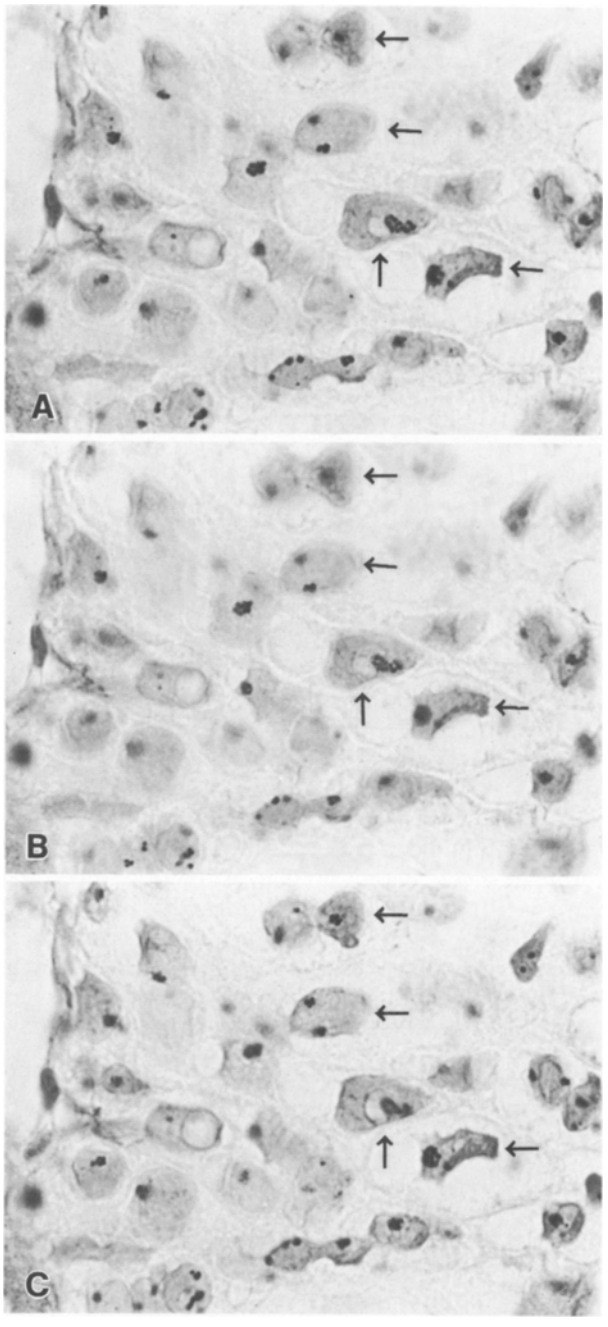

Fig. 2 A–C. Silver-stained nucleolar organiser regions (AgNORs) in PIN III, demonstrating correct focus plane. Note that further movements on the focusing micrometer result in simultaneous nuclear blurring and distortion of AgNORs. **A** Correct focus on vertically marked nucleus, but incorrect blurred focus on horizontally marked nuclei. **B** Blurring of all nuclei. **C** Correct focus on horizontally marked nuclei, but incorrect focus on vertically marked nucleus. Original magnification, ×1000

Crocker et al. (1989) were followed: for AgNOR enumeration, each AgNOR was counted as a unit when seen seperately within nucleoli, together with the smaller AgNORs seen outside the nucleolus. For AgNOR typing we used our own typing system according to Fig. 3: types A_{1-3} representing AgNOR satellites, types B_{1-3} representing fine granular AgNORs in nucleoli and types C_{1-3} representing large granular or semi-solid AgNORs in nucleoli (Fig. 3). Median values were calculated for each group. Statistical analysis was preformed using the Mann-Whitney U test. *P*-values of less than 0.05 were considered statistically significant.

Results

AgNORs were seen in all sections and the percentage of nuclei without AgNORs ranged from 0 to 5%, with no significant differences amoung the five groups. Figure 4 illustrates the AgNOR numbers per nucleus and the overlap of the values, mainly between hyperplasia and PIN. Lowest counts were obtained in the hyperplasia group (median, 1.98) and highest counts in the PIN (median, 4.26) and adenocarcinoma groups as follows: well-differentiated median 4.14, moderately differentiated median 4.24 and poorly differentiated median 5.04.

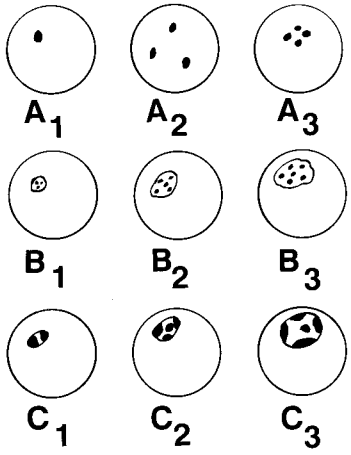

Fig. 3. AgNOR typing: **A** satellite AgNORs: single (A_1), scattered (A_2), grouped (A_3); **B** fine granular AgNORs in nucleoli: small (B_1), medium (B_2), large (B_3); **C** coarse to semi-solid AgNORs in nucleoli: small (C_1), medium (C_2), large (C_3)

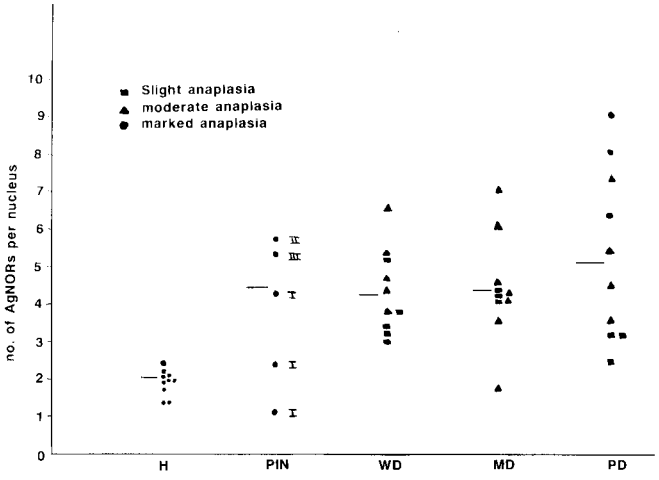

Fig. 4. Scattergram showing distribution of total AgNORs per nucleus (*bars* = median values). PIN and carcinomas generally have higher counts than hyperplasia, but there is considerable overlap. *H*, Hyperplasia; *PIN*, prostatic intra-epithelial neoplasia; *WD, MD, PD*, well-, moderately and poorly differentiated adenocarcinoma. *Roman numerals* indicate PIN grades. ■ Slight anaplasia, ▲ moderate anaplasia, ● marked anaplasia

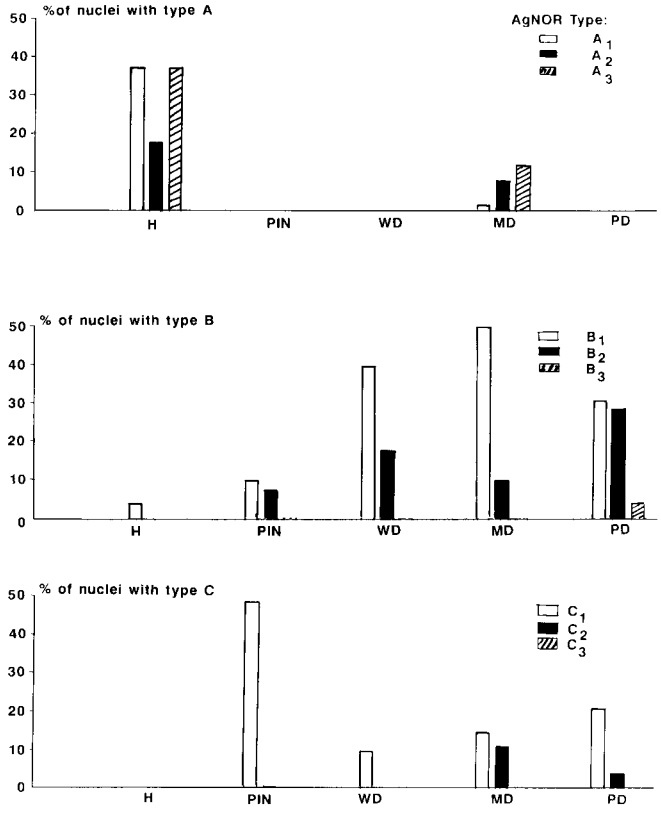

Fig. 5. Bar histogram showing relative distribution of AgNOR types (median values). Predominant types in hyperplasia were A_{1-3}, while types B_{2-3} and C_{1-2} were only observed in PIN and adenocarcinomas. Abbreviations as in Fig. 4

There were statistically significant differences ($P<0.05$) between the hyperplasia and the PIN groups as well as between the hyperplasia and the adenocarcinoma groups. Other comparisons were non-significant.

Figure 5 shows the correlation between the AgNOR types and histological diagnosis. Types A_{1-3} predominated in hyperplasia, while types B_{2-3} and C_{1-2} were only observed in PIN and carcinoma groups (Fig. 6). Type C_3 was seen in sporadic carcinoma nuclei.

Discussion

The grading systems for both PIN, as defined by McNeal and Bostwick (1986), and prostate adenocarcinomas (Mostofi et al. 1980; Murphy and Whitmore 1979) includes nuclear characteristics such as nuclear size, chromatin structure and nucleolar morphology. Earlier reports suggest that numerous or large nucleoli are characteristic features of PIN when compared with normal or hyperplastic prostatic tissue (McNeal and Bostwick 1986; Bostwick and Brawer 1987) and that nucleolar number and size increases with increasing degrees of malignancy (Helpap 1988).

Polyacrylamide gel electrophoresis, blotting- and RNA hybridisation studies (Buys and Osinga 1984) have shown that AgNORs may correspond to silver stained RNA polymerase I, C23 protein and B23 protein in NORs. The total number of AgNORs has been shown to correlate with other methods of analysis of cellular proliferative activity and ploidy such as Ki67 immunostaining (Raymond and Leong 1989) and DNA flow cytometry (Giri et al. 1988) in breast carcinomas.

In the present study we applied the one-step silver nitrate method at 20° C and with a 30 min reaction time for staining as described by Smith and Crocker (1988). We were thus able to resolve and count separate AgNORs within nucleoli, while longer reaction times resulted in blurring and lumping of the AgNOR dots. A prolonged staining time may explain why Howat et al. (1989) and Griffiths et al. (1989), using 40 min and 60 min, obtained a 12% mean inter- and intra-observer variation with this counting procedure. We found our

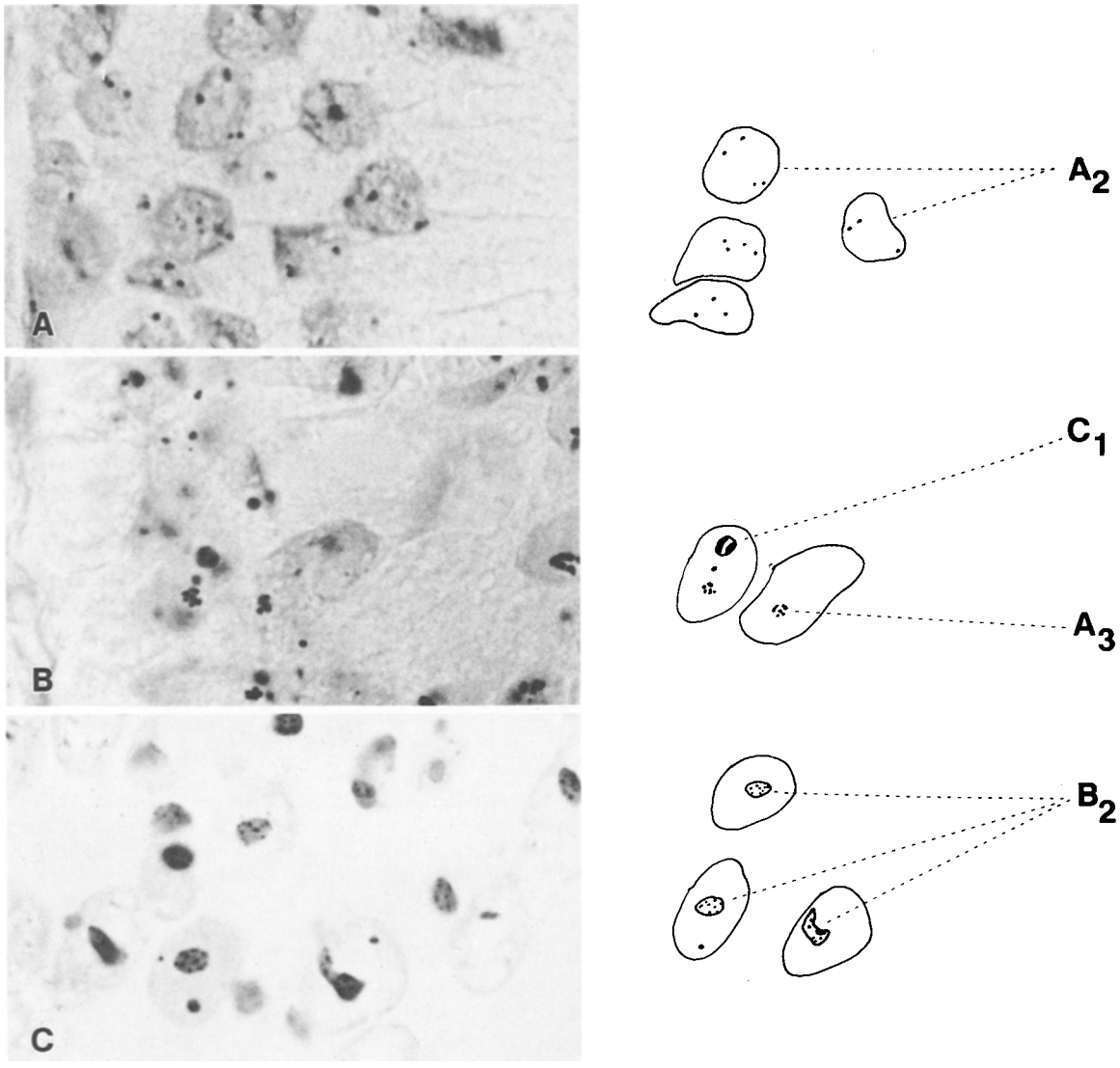

Fig. 6. **A** Prostatic hyperplasia, showing type A_2. **B** PIN II, showing types A_3 and C_1. **C** prostatic adenocarcinoma, poorly differentiated with moderate nuclear anaplasia, showing type B_2. Original magnification, $\times 1000$

own mean inter- and intra-observer variation of 6% acceptable. As mentioned earlier, AgNOR counting and typing was preformed after sharp focusing on the nuclear membrane and matrix, including only AgNORs with sharp contours in this plane of focus. This procedure avoided further movements of the focusing micrometer and hence variation of AgNOR morphology. Further focusing was practically always associated with nuclear blurring, before or simultaneously with change in AgNOR morphology (Fig. 2). Our typing system was devised to include two important features of NORs, namely size as pointed out by Ooms and Veldhuizen (1989) and degree of nucleolar disaggregation as stressed in previous reports (Croker and Nar 1987; Underwood and Giri 1988).

Because nucleolar characteristics are included in the grading of PIN and prostatic carcinoma, we hoped that the AgNOR technique would be helpful in the evaluation of these lesions. In our hands AgNOR counts are of no use for diagnosis of any single case in the groups studied, because in most instances a particular AgNOR count could place the TUR-P specimen in a number of different groups. AgNOR typing, however, differentiated between benign and malignant lesions and between benign and PIN lesions. Thus, types B_{2-3} and C_{1-2} were only observed in PIN and carcinomas, while types A_{1-3} were seen in both benign and malignant tissue. These findings were in accord with those of Fellowfield and Cook (1989) and Sinn et al. (1989).

It is concluded that qualitative typing of AgNORs may contribute to the differential diagnosis between benign and malignant prostatic lesions. Further studies are needed to determine the influence of preparation artefacts, observer variation and prognostic significance.

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