AgNOR quantity in needle biopsy specimens of prostatic adenocarcinomas: correlation with proliferation state, Gleason score, clinical stage, and DNA content

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Abstract

***Aims*—To define the relation between the quantity of silver stained nucleolar organiser regions (AgNORs) and histological grade, clinical stage, DNA content, and MIB-1 immunostaining in needle biopsy specimens of prostatic adenocarcinomas.**

***Methods*—Histological grade was determined according to the Gleason system. AgNOR quantity, DNA content and MIB-1 immunostaining were evaluated by image cytometry on routine histological sections stained with silver, Feulgen reaction and MIB-1 antibody, respectively.**

***Results*—The mean AgNOR area increased with increasing Gleason score. A significant difference was found in the AgNOR values between low, intermediate and high grade tumours. Patients with clinically localised tumour (stages A and B) had lower AgNOR values than patients with advanced disease (stages C and D), but the difference in the mean AgNOR values between the two groups was not statistically significant. Non-diploid tumours had a significantly higher mean (SD) AgNOR area than diploid tumours (3.68 (1.04) μm^2 *v* 2.73 (0.60) μm^2, respectively), while no significant difference was observed in the mean AgNOR values between aneuploid and tetraploid tumours (3.68 (1.04) μm^2 *v* 3.70 (1.05) μm^2). When AgNOR and MIB-1-PI values were compared using linear regression analysis, a highly significant correlation was found.**

***Conclusions*—These data demonstrate that AgNOR quantity reflects the proliferative potential of prostatic adenocarcinomas, and is significantly related to histological grade and DNA content. The ease of application on routine sections, maintaining the morphological integrity of the tissue, the ability to evaluate selected histological areas of limited size and objective quantification by image cytometry make the AgNOR method particularly suitable for cell kinetic analysis in prostatic needle biopsy specimens.**

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Prostatic carcinoma has become the most common malignant tumour among men in the USA and the second leading cause of death from cancer.[1] The optimal treatment for patients with prostatic carcinoma depends on the capability to predict the natural course of the disease; while some tumours are indolent and require delayed or no treatment, other clinically localised cancers may have very aggressive behaviour, requiring immediate treatment. Continuous attempts are being made to develop objective markers to define the progression of the disease accurately. At present, histological grade, clinical stage and DNA content are used to predict biological aggressiveness and survival, and to define the appropriate treatment.[1]

The nucleolar organiser regions (NORs) are defined nucleolar components where ribosomal genes are complexed with a set of non-histone proteins characterised by a high affinity for silver (AgNOR proteins).[2] NORs can be selectively visualised by silver staining in routinely processed histological samples[3] and quantified by image analysis.[4 5] Extensive evidence shows that the quantity of AgNOR protein reflects the state of cell duplication and represents an independent marker with highly significant predictive value in numerous human tumours.[6]

In the present investigation we have analysed cell AgNOR quantity in 194 needle biopsy specimens from prostatic carcinomas. We have also correlated the AgNOR data with histological grade, clinical stage and DNA content. In order to demonstrate the ability of AgNOR quantitation to define cell proliferative activity, AgNOR scores have been compared with MIB-1 immunostaining.

Methods

Core needle biopsy specimens (n = 206) obtained from 156 patients with prostatic adenocarcinoma diagnosed between 1991 and 1993 at the Albert Einstein Medical Center were studied. The specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Three serial sections (4–5 μm) were cut from each paraffin wax block: the first was stained with haematoxylin and eosin for histological diagnosis and tumour grading, the second with Feulgen reagent for DNA quantitation and the last with silver to visualise the NORs. A fourth section was cut in 50 selected

Table 1 Allocation of Gleason score to the 206 needle core biopsy specimens

Gleason score	Number of specimens (%)
1	–
2	–
3	1 (0.49)
4	38 (18.44)
5	34 (16.50)
6	56 (27.28)
7	47 (22.71)
8	17 (8.26)
9	11 (5.35)
10	2 (0.97)

biopsy specimens with extensive tumour involvement for MIB-1 immunostaining.

The Gleason system was used to grade the tumours.[7] All biopsy specimens were scored independently by three pathologists to reduce subjectivity in the final grade (table 1).

Clinical information was available for 92 patients. Clinical stage was assessed according to the AUA (American Urologic Association) system.[8] Twelve (13%) tumours were stage A, 54 (58.74%) stage B, 13 (14.13%) stage C, and 13 (14.13%) stage D.

DNA STAINING AND QUANTIFICATION

For analysis of DNA content, sections were deparaffinised in xylene, then processed to ethanol and rehydrated. After hydrolysis in SN hydrochloric acid for 60 minutes, slides were stained using the CAS (Cell Analysis System; Elmhurst, Illinois, USA) Feulgen DNA kit. Each batch was prepared with a CAS calibration slide containing cultured rat hepatocytes. DNA content analysis was performed by using a specific program (QDA: Quantitative DNA Analysis) of the CAS 200 image analysis system. The instrument was calibrated for each staining run by using the CAS calibration slides provided with the kit. At least 20 rat hepatocytes were measured on each CAS calibration slide, accepting a maximum coefficient of variation of 5%. For each needle biopsy specimen, at least 30 control cells (lymphocytes, fibroblasts and epithelial cells from non-neoplastic glands) and 100 cancer cells were evaluated. Neoplastic and non-neoplastic areas were identified carefully in the haematoxylin and eosin sections and then localised in the corresponding Feulgen stained section prior to measurement. The integrated optical density (IOD) of individual nuclei was measured on a 620 nm wavelength and computed on 256 densitometric levels for each pixel. DNA histograms were generated by plotting the IOD versus number of nuclei. The DNA index (DI) was given by the ratio of the mean peak value of the tumour nuclei to the mean peak value of the control nuclei. The histograms were classified as diploid when a single peak was found in the G_1/G_0 (diploid) position, with a DI ranging from 0.8 to 1.2 (2N ± 20%),[9] and fewer than 20% of the cells were present in the G_2/M (tetraploid) position. A tumour was considered tetraploid when there was a peak in the diploid region and a second peak with more than 20% of cells in the tetraploid region with a DI ranging from 1.85 to 2.15 (4N ± 15%). A tumour was considered aneuploid when at least 20% of cells showed one or more distinct abnormal peaks outside the 2N or 4N compartments.

NOR SILVER STAINING AND AGNOR QUANTITY MEASUREMENT

AgNOR quantity was measured in 194 of the 206 needle biopsy specimens selected for the study. Five (0.24%) cases could not be evaluated because of tissue detachment during microwave oven pre-treatment and in seven (0.34%) of the silver stained sections it was not possible to identify the neoplastic area identified in the haematoxylin and eosin sections. In order to obtain better AgNOR visualisation, slides were pre-heated in sodium citrate buffer, as recently proposed by Ofner *et al.*[10] Sections were cleared in xylene and rehydrated through alcohol to distilled water. Slides were then removed from water into microwave-safe plastic slide holders and immersed fully in 10 mM sodium citrate buffer (pH 6.0). Slide holders were placed in the centre of a plastic pan containing about 500 ml water. The pan was always placed in the same position of the same microwave oven (Specemaker II). Slides were microwaved for 10 minutes at high setting (800 W) and cooled at room temperature for 20 minutes. NOR silver staining was then carried out for 20 minutes at room temperature in the dark using a solution of one volume 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate.[11]

The neoplastic area to be evaluated was identified in the haematoxylin and eosin section and located carefully in the silver stained section. For each case, the AgNOR area within 100 nuclei was measured using the Cell Measurement Program (CMP) of the CAS 200 image analysis system, and the mean value and the standard deviation (in μm^2) were obtained automatically. We limited our morphometric analysis to 100 nuclei per biopsy specimen as after 75–80 measurements both the mean value and the standard deviation were nearly constant, even in heterogeneous specimens.

MIB-1 IMMUNOSTAINING AND QUANTIFICATION

Sections for MIB-1 immunostaining were deparaffinised in two changes of xylene for 10 minutes each and hydrated in graded alcohols. Slides were then microwaved for five minutes, as described previously, on maximum power (800 W) and for a further eight minutes on 30% power. After cooling at room temperature for 20 minutes, slides were processed with the Ventana ES Immunostainer Automated System for MIB-1 immunostaining (clone MM1, Ventana Medical System Inc., Tucson, USA). The immunoreaction was developed with 3,3'diaminobenzidine (DAB) and counterstained with haematoxylin. Quantitative analysis of MIB-1 immunostaining was obtained by image analysis, using a specific program (QPI: Quantitative Proliferation Index) of the CAS 200 image analysis system. The QPI program uses two sensing channels. After the definition of the specific grey thresholds, the first channel (at 620 nm) was used for the identification of all the nuclei and the second one (at 500 nm)

Table 2 AgNOR area and DNA content according to Gleason score

Gleason score	AgNOR area (mean (SD) μm²)	DNA content (n (%))* Diploid	Aneuploid	Tetraploid
3 and 4	2.51 (0.54)	32 (82%)	2 (5.2%)	5 (12.8%)
5	2.80 (0.65)	21 (61.7%)	9 (26.5%)	4 (11.8%)
6	3.10 (0.67)	31 (55.3%)	16 (28.6%)	9 (16.1%)
7	3.47 (0.97)	15 (31.9%)	20 (28.5%)	12 (25.6%)
8	3.77 (1.01)	4 (23.5%)	9 (53%)	4 (23.5%)
9 and 10	4.59 (1.34)	2 (15.4%)	9 (69.2%)	2 (15.4%)
Total	3.19 (0.96)	105 (50.9%)	65 (31.6%)	6 (17.5%)

*Number of cases (per cent of total).

Table 3 AgNOR area and DNA content according to clinical stage

	Clinical stage A and B		C and D	
AgNOR area (mean (SD) μm²)	3.26 (0.96)		3.52 (0.86)	
DNA content				
Diploid	34	(51.6%)*	7	(26.9%)
Aneuploid	16	(24.2%)	10	(38.5%)
Tetraploid	16	(24.2%)	9	(34.6%)

*Number of cases (per cent of total).

for the identification of the DAB stained nuclei. Image analysis of MIB-1 immunostaining was performed at ×40 magnification in consecutive fields of the most "proliferatively active" tumour area until at least 50 000 μm² cancer nuclei were evaluated.[12] The PI (Proliferation Index—that is, the ratio between the area of positive nuclei to the area of the total nuclei × 100) was automatically obtained by the computer in each selected field after any non-tumour cells (stromal and inflammatory cells) were interactively excluded by the operator using a specific "draw function".

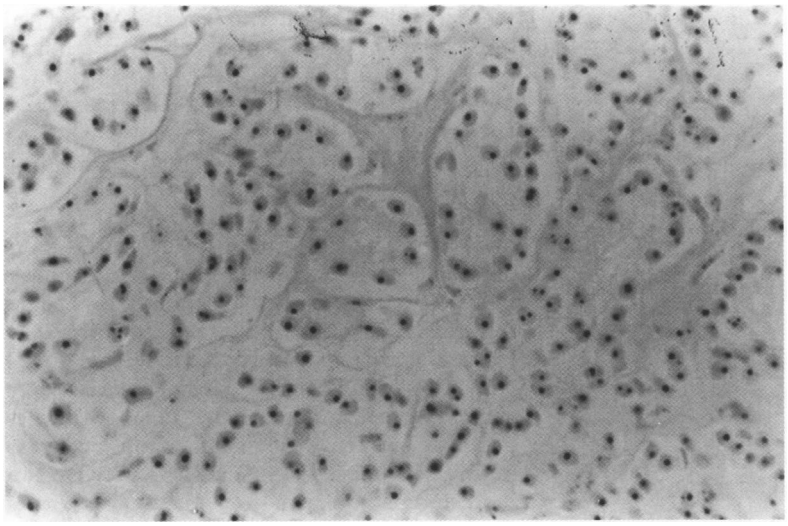

Figure 1 Histological section of a needle biopsy specimen of a low grade prostatic adenocarcinoma (Gleason score 4). Mean (SD) AgNOR area 2.38 (1.05) μm². Selective staining for AgNOR proteins. No counterstaining (×400).

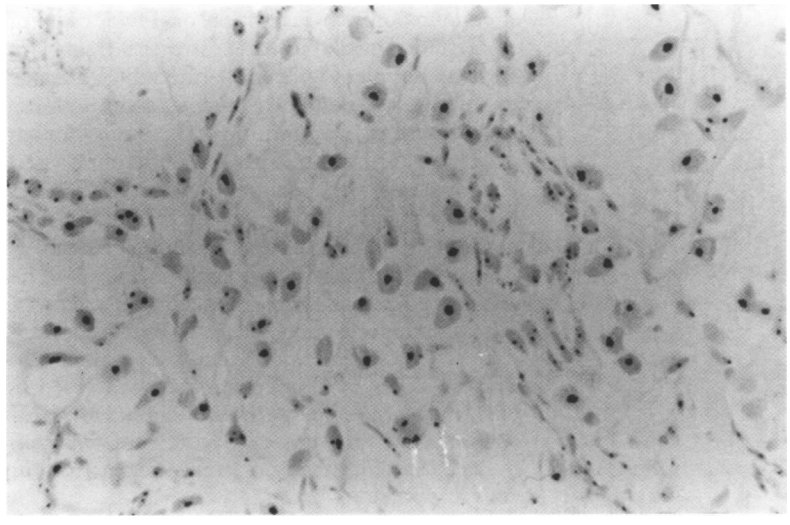

Figure 2 Histological section of a needle biopsy specimen of a high grade prostatic adenocarcinoma (Gleason score 9). Mean (SD) AgNOR area 4.12 (1.63) μm². Selective staining for AgNOR proteins. No counterstaining (×400).

STATISTICAL ANALYSIS

Differences between groups were tested by Mann-Whitney and χ^2 analysis. Correlations were evaluated by linear regression analysis.

Results

AGNOR QUANTITY

The mean AgNOR areas of the 194 evaluated needle biopsy specimens ranged from 1.60 to 7.56 μm², with median and mean (SD) values of 2.94 and 3.19 (0.96) μm², respectively. The mean AgNOR area values gradually increased with increasing Gleason score (table 2). For statistical analysis the 194 cases were compressed into three clinically relevant groups. The first group included 34 low grade tumours (Gleason scores of 3 and 4) with a mean (SD) AgNOR area of 2.51 (0.55) μm²; the second group included 131 intermediate grade tumours (Gleason scores ranging from 5 to 7) with a mean AgNOR area of 3.16 (0.83) μm²; and the third group included 29 high grade tumours (Gleason scores ranging from 8 to 10) with a mean AgNOR area of 4.11 (1.21) μm². A highly significant difference in the AgNOR values was found between both low versus intermediate and intermediate versus high grade tumours (Mann-Whitney test: $p < 0.001$).

In routine sections pre-heated in a microwave oven and stained with silver, the NORs appear as dark staining regions. Figures 1 and 2 show silver stained sections of two prostatic carcinomas; fig 1 shows a low grade carcinoma (Gleason score 4) with a very low AgNOR area per cell; fig shows a high grade tumour (Gleason score 9) with a greater number and more irregular distribution of AgNORs.

Patients with stage A and B tumours (n = 62) had lower AgNOR values than patients with stage C and D disease (n = 22), but the difference in the mean (SD) AgNOR values between the two groups was not statistically significant (3.26 (0.96) μm² *v* 3.52 (0.86) μm², respectively; Mann-Whitney test: p = 0.14) (table 3).

DNA CONTENT

The DIs of the 206 needle biopsy specimens evaluated in this study ranged from 0.98 to 2.41, with a median value of 1.27. One hundred and five (50.9%) cases were diploid, 65 (31.6%) were aneuploid and 36 (17.5%) were tetraploid. The distribution of DNA content according to histological grade is presented in table 2. Interestingly, the percentage of diploid tumours decreased progressively with increasing histological grade. For statistical analysis, the 206 cases were divided into three groups. The first group included 39 low grade tumours with Gleason scores of 3 and 4.

The second group included 137 intermediate grade tumours with Gleason scores ranging from 5 to 7 and the third group included 30 high grade tumours with Gleason scores ranging from 8 to 10. When the percentages of diploid, aneuploid and tetraploid tumours were compared among different groups, a highly significant difference was found between low and intermediate grade ($\chi^2 = 15.72$; $p = 0.0004$) and between intermediate and high grade tumours ($\chi^2 = 9.55$; $p = 0.0084$).

The percentage of diploid tumours decreased progressively with increasing clinical stage (66.7% in stage A, 48.1% in stage B, 38.6% in stage C, and 15.4% in stage D tumours) with a significant difference in the percentage of diploid and non-diploid tumours between stages A and B compared with stages C and D ($\chi^2 = 4.57$; $p = 0.032$; table 3).

Diploid tumours (n = 100) had lower AgNOR areas than non-diploid tumours (n = 94), with a significant difference in the mean AgNOR values between the two groups (2.73 (0.60) μm^2 *v* 3.68 (1.04) μm^2, respectively; Mann-Whitney test: $p < 0.0001$). AgNOR area values did not differ significantly between aneuploid and tetraploid tumours (3.68 (1.04) μm^2 *v* 3.70 (1.05) μm^2, respectively; Mann-Whitney test: $p = 0.83$).

MIB-1 IMMUNOSTAINING

MIB-1-PI of the 50 selected biopsy specimens ranged from 0.34 to 18.73, with a median and mean (SD) value of 3.24 and 4.67 (3.72), respectively. High grade tumours (Gleason scores ranging from 8 to 10) had greater MIB-1-PIs than low and intermediate grade tumours (Gleason scores ranging from 4 to 7) (7.97 (4.78) *v* 3.25 (1.91), respectively; Mann-Whitney test: $p = 0.0005$) and non-diploid tumours had greater MIB-1-PIs than diploid tumours (5.25 (3.97) *v* 2.47 (1.81), respectively; Mann-Whitney test: $p = 0.0009$).

Patients with stage A and B tumours had lower MIB-1-PIs than patients with stage C and D disease, but the difference between the two groups was not statistically significant (4.70 (3.03) *v* 5.79 (5.03), respectively; Mann-Whitney test: $p = 0.53$).

When MIB-1-PIs were compared with the mean AgNOR area values by linear regression analysis, a highly significant correlation was found ($r = 0.83$; $p < 0.001$; fig 3).

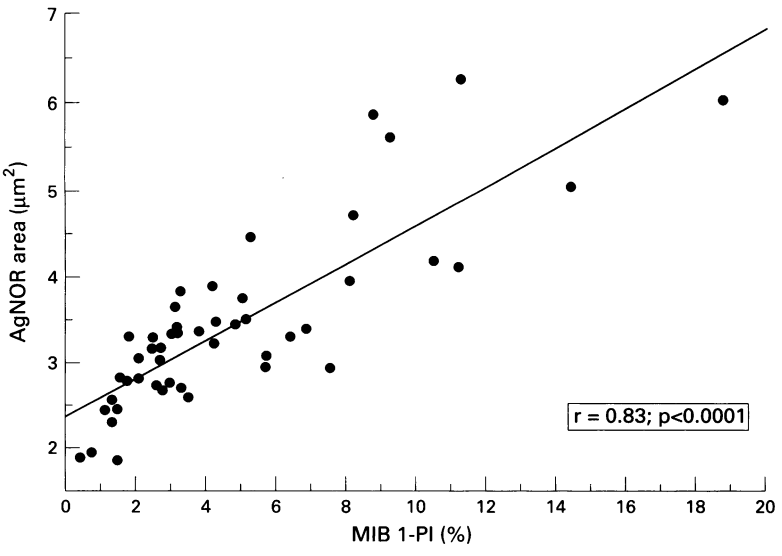

Figure 3 Regression analysis of MIB-1-PI and AgNOR area values.

Discussion

Histological grade is the most important independent parameter for predicting prognosis of patients with prostatic cancer.[1] Of the different methods proposed for grading prostatic tumours, the Gleason system is the most frequently used.[7] However, the criteria used for histological grading are subjective and several authors have reported significant intra- and inter-observer variability for the Gleason system.[13] In the present study we have demonstrated that AgNOR area increases progressively with increasing Gleason score, showing a highly significant difference in the mean AgNOR values between low and intermediate and between intermediate and high grade tumours. Our results, obtained by image cytometry, confirm previous small studies in which AgNOR evaluation was performed using the counting method, consisting of the enumeration of each silver stained dot per cell directly at the microscope at high magnification.[14 15] Compared with the counting method, morphometric analysis permits more objective and reproducible AgNOR quantification in histological sections, as shown in a recent study in which the two methods were compared in the same series of breast carcinomas.[4]

DNA content is also regarded as an important prognostic parameter in prostatic cancer. In a study performed on 494 radical prostatectomies, Lieber *et al*[16] have defined the prognostic significance of ploidy, showing that DNA content is significantly related to disease-free and overall survival, and gives a high and independent predictive value when entered into multivariate analysis together with the Gleason score and pathological stage. Flow cytometry is the standard method for measuring DNA content in prostatic cancers. However, as a large number of cells is required for analysis, it is generally performed on radical prostatectomy specimens only. Information on DNA content is therefore available only after the treatment strategy has already been planned. DNA image cytometry on Feulgen stained sections, however, requires a minimum of 100 cells for an interpretable histogram and can be performed on needle biopsy specimens. In a recent paper Pindur *et al*[17] compared the DNA data obtained by flow cytometry and image analysis using both imprints and tissue sections. The authors concluded that image analysis is more sensitive than flow cytometry in detecting non-diploid populations, and tissue sections are as reliable as imprint preparations. Image cytometry therefore is the method of choice for measuring DNA content in needle biopsy specimens, particularly when small foci of tumour cells are present.

In the present study we have demonstrated a significant association between DNA content and both histological grade and clinical stage.

Moreover, when DNA content was related to AgNOR quantity, non-diploid tumours had greater AgNOR scores than diploid tumours.

Clinical stage is another important prognostic variable in prostatic tumours. However, the natural history of patients with a given stage, particularly with clinically localised tumours, may vary considerably. In our series we have found a strong association between DNA content and clinical stage. The percentage of non-diploid cases was significantly lower in pathologically confined tumours (stages A and B) than in tumours extending to seminal vesicles (stage C) or those with metastases (stage D). Conversely, no significant correlation was demonstrated between clinical stage and both AgNOR and MIB-1-PI values. The independence of cell proliferation from the extent of the disease suggests that kinetic markers may predict prognosis of patients stratified according to clinical stage. For example, in breast tumours cell proliferation is independent of lymph node status,[11] but is significantly correlated with survival both in node negative and node positive patients.[18 19]

To demonstrate the actual use of the AgNOR parameter in assessing the proliferative activity of prostatic needle biopsy specimens, the AgNOR data were also correlated with MIB-1 immunostaining. MIB-1 is a recently developed monoclonal antibody which reacts with an epitope of Ki-67 antigen in formalin fixed, paraffin wax embedded tissues which have been pre-heated in a microwave oven.[20] The Ki-67 antigen is expressed selectively during G_1, S and G_2/M phases of the cell cycle, but not in G_0 cells. Its detection represents a useful method for assessing tumour growth state in cytological and histological samples. However, for a reliable determination of MIB-1-PI, a large number of tumour cells should be evaluated. This is not always possible in needle biopsy specimens, where sometimes only a few neoplastic foci are present. Therefore, in the present study the relation between AgNOR area and MIB-1 immunostaining was tested on 50 selected biopsy specimens extensively infiltrated by tumour. Regression analysis showed a significant correlation between the AgNOR area and MIB-1-PI values. Moreover, as demonstrated for AgNOR quantity, MIB-1 immunostaining was related to histological grade and DNA content, but was independent of clinical stage.

In conclusion, our results demonstrate that AgNOR quantity is related to the aggressiveness of prostatic carcinomas, and may provide pathologists with a reliable parameter for an objective assessment of the malignant potential of these cancers. The AgNOR method is particularly suitable for cell proliferation analysis in needle biopsy specimens.[21] Advantages of the AgNOR technique over the other proliferation markers include ease of staining procedure, objective quantification by image analysis, applicability on routine sections preserving of the architectural integrity of the tissue examined and, therefore, enabling the evaluation of selected histological areas of limited size.[22] Moreover, as AgNOR data can be acquired at diagnosis, they may have an impact on treatment.

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