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Immunohistochemical detection of cell-cycle associated markers on paraffin embedded and formalin fixed needle biopsies of prostate cancer: correlation of p120 protein expression with AgNOR, PCNA/Cyclin, KI-67/MIB1 proliferation-scores and Gleason gradings

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SUMMARY

Paraffin embedded and formalin fixed needle biopsies of prostate cancer (PC) were used to immunocytochemically detect the p120 nucleolar protein in relation to the Gleason histological gradings (GHG), the labelling indices of proliferating nuclear immunocytochemical markers (PCNA/Cyclin, Ki-67/MIB1) and the argyrophilic nucleolar region (AgNOR) rate. The twenty-six cases of PC (6 from large histological samples and 20 from needle biopsies) were equally distributed into low (≤ 6) or high (≥ 7) GHG groups.

The p120 nucleolar protein immunocytochemical reaction was randomly expressed in large histological sections but uniformly distributed without gaps in needle biopsy sections. Only on the latter were quantitative values of PCNA/Cyclin (23.2 in low and 45.3 in high GHG), Ki-67/MIB1 (13.8 in low and 43.3 in high GHG) and AgNOR (5.0 in low and 7.5 in high GHG) related to those of p120 nucleolar protein (0.8 in low and 3.8 in high GHG). The values of all these cell cycle markers increased from low to high GHG of PC, all four reaching high statistical significance between the two groups (ANOVA - two tailed $p < 0.0001$). The PCNA/Cyclin index showed a higher positivity than the Ki-67/MIB1 index in PC with low GHG but not in PC with high GHG.

In conclusion, paraffin embedded and formalin fixed PC needle biopsies exhibit a higher diagnostic PCNA/Cyclin than Ki-67/MIB1 index for cases presenting differentiated features, whereas p120 nucleolar protein detection seems to be a suitable marker of poorer outcome of PC.

INTRODUCTION

Rates of cell proliferation by changes in DNA *status,* investigated by flow cytometry analysis (Ring *et al.*, 1990; Losi *et al.*, 1991; Daskal and Trerè,

1996), H^3-thymidine autoradiography (Meyer *et al.*, 1982) and 5-bromodeoxyuridine (Nemoto *et al.*, 1990) immunohistochemistry, to date best predict the clinical outcome of prostate cancer (PC). Nevertheless, in histopathological diagnostic practice, Gleason (1992) histological gradings - **GHG**, immunocytological marker labelling indices of the proliferating cell nuclear antigen - **PCNA/Cyclin** (Harper *et al.*, 1992; Visokorpi, 1992; Botticelli *et al.*, 1993) - proliferation associated **Ki-67** antigen (Galle *et al.*, 1989; Nilsson *et al.*, 1991; Oomens *et al.*, 1991; Daskal and Trerè, 1996) and argyophilic nucleolar regions - **AgNOR** (Hansen and Ostergard, 1990; Botticelli *et al.*, 1991; Mamaeva *et al.*, 1991; Marandola *et al.*, 1992; Ferrari *et al.*, 1993; Botticelli *et al.*, 1995; Chiusa *et al.*, 1996; Daskal and Trerè, 1996) - are the most important histocytological factors predicting PC progression (Botticelli, 1994; Botticelli *et al.*, 1996).

The proliferating cell nuclear antigen (**PCNA**) is associated with DNA damage repair mechanisms (Shivji *et al.*, 1992). This antibody labels nuclei in G1 (Gap 1), S (DNA replication), G2 (Gap 2) and M (Mitosis) phases of the cell cycle and persists in the early non-proliferating stage (G0); but afterwards it becomes completely negative after the cell is in G0 for a long period of time (Bravo and Macdonald-Bravo, 1985). The **Ki-67/MIB1** nuclear antigen is associated with cell proliferation and is expressed as a fine granular component of the nucleus throughout the cell cycle (late G1, S, G2, M phases); but it is negative in resting (G0) cells (Gerdes *et* al., 1984). Both PCNA/Cyclin (Harper *et al.*, 1992; Visokorpi, 1992; Botticelli *et al.*, 1993; Botticelli, 1994; Botticelli *et al.*, 1996) and Ki-67/MIB1 (Galle *et al.*, 1989; Nilsson *et al.*, 1991; Oomens *et al.*, 1991; Botticelli, 1994) have been indicated as significant prognostic markers predicting malignant progression of PC.

AgNOR protein detection is independent of DNA content (Derenzini *et al.*, 1994; Trerè *et al.*, 1996), but is related to cellular proliferation (Crocker, 1990), particularly in malignant tumours (Derenzini *et al.*, 1990; Derenzini and Ploton, 1991; Derenzini and Trerè, 1991; Rüschoff, 1992; Derenzini *et al.*, 1994), where a greater number of positive AgNOR dots is observed than in normal or benign lesions, and is considered an independent prognostic factor of malignancy (Derenzini, 1996). A significant correlation between AgNOR protein value and PCNA/Cyclin immunostaining labelling index has been demonstrated in malignant tumours of different origin (Skopelitou *et al.*, 1992; Pich *et al.*, 1992; Risio and Rossini, 1993). AgNOR counts in PC correlate well with pTNM (pathologist Tumor Node Metastasis) and cytohistological grades (Botticelli *et al.*, 1991), serum prostate specific antigen (PSA) values (Marandola *et al.*, 1992; Botticelli *et al.*, 1995), clinical stages (Chiusa *et al.*, 1996) and DNA content (Daskal and Trerè, 1996).

Recently, the **p120 antigen** has been introduced as a cell cycle marker, being confined to nucleoli of proliferating cells. The considerable increase in p120 nucleolar protein expression at the G1 (Freeman *et al.*, 1988) to S phase and during re-entry of G0 cells into the cell cycle (Bolton *et al.*, 1994) suggests that the p120 nucleolar protein may play a role in the regulation of cell proliferation (Fonagy *et al.*, 1995). The higher labelling index of p120 nucleolar protein is described in rapidly dividing cells or in malignant tumour cells in close relation to activation of proliferative stages of the cell cycle (Rüschoff *et al.*, 1996). Since p120 nucleolar protein is found significantly higher in neoplastic cells than in normal cells, this antigen is considered a morphological expression of a poorer prognosis for progression of malignant tumours. It is to be noted that most of p120 nucleolar protein detections have been made on fresh (frozen) specimens because fixed specimens give very poor results (Rüschoff *et al.*, 1993). Nonetheless, the p120 nucleolar protein score on frozen prostate sections ranges from 12% in benign prostatic hyperplasia to 76% in PC and from 18.1% in WHO histological grade 1 to 82.2% in WHO histological grade 3 of PC (Rüschoff *et al.* 1993; Bocker *et al.* 1995).

The aim of this study was to evaluate the significance of p120 nucleolar protein immunohistochemical expression in comparison with AgNOR, PCNA/Cyclin and Ki-67/MIB1 proliferation labelling indices on routinely paraffin-embedded and formalin-fixed needle biopsies of PC with low and high GHG.

MATERIALS AND METHODS

Twenty-six cases of PC were selected; patients had an average age of 72 years (range 65-75 years). The specimens (6 large histological sam-

ples from radical prostatectomies and 20 by needle biopsies) were fixed in 10% neutral buffered formalin and processed for routine histological procedures up to paraffin embedding. Four to five μm thick histological sections were dewaxed by serial baths in xylol and ethanol, then rehydrated in running tap water and stained with Mayer's Haematoxylin-eosin, Ploton's AgNOR method (Ploton *et al.,* 1986) and immunohistochemical techniques for PCNA/Cyclin, Ki-67/ MIB1 (Botticelli *et al.,* 1996) and p120 nucleolar protein.

AgNOR staining was performed with a freshly prepared AgNOR solution obtained by mixing two parts of 50% $AgNO_3$ stock solution and one part of 2% gelatin in 1% formic acid (both solutions were maintained 1 hour in a thermostatic chamber at 37°C before mixing). The pre-heated histological sections (37°C) were put in warm AgNOR solution for 9 minutes in a dark thermostatic chamber at 37°C, washed twice in distilled water, toned in 0.1% gold chloride solution for 1 minute and then washed again in distilled water. The histological sections were fixed in 1% sodium sulphate for 5 minutes, washed in several changes of warm tap water, counterstained with nuclear fast red for 5 minutes, dehydrated in ethanol, clarified in xylol and mounted in synthetic mounting media.

Immunohistochemical stained sections were placed in Coplin glass jars filled with 0.01 M citrate buffer (pH 6.0). Slides for Ki-67/MIB1 were microwaved once and those for p120 nucleolar protein six times, for 5 minutes each at 750 W, adding buffer to keep the slides immersed. The sections were incubated with 5% normal horse serum in 0.1 M.phosphate-buffered saline at pH 7.4 (PBS) for 40 minutes. Primary antibodies (PCNA clone PC10, DAKO, Denmark; monoclonal Ki-67, clone MIB1, Immunotech Int, France; monoclonal p120 nucleolar protein, Neumarkers, Freemont, CA, USA), diluted in a 1:50 solution were incubated overnight at 4°C in a moist and darkened chamber. After washing with PBS for 15 minutes, all sections (for PCNA/Cyclin, Ki-67/MIB1 and p120 immunostainings) were incubated with 1:200 horse anti mouse biotinylated secondary antibody (DAKO, Denmark) for 30 minutes at room temperature. The sections were then washed and incubated with a 1:100 streptavidin biotinylated complex (DAKO, Denmark) for 60 minutes. After a final wash in PBS, the chromogen reaction was developed with diaminobenzidine to detect the dark brown stain of the nuclei. The sections were counterstained with Mayer's Haematoxylin-eosin for 2 min, dehydrated in ethanol, clarified in xylol and cover-slipped with synthetic mounting media.

Under the optical microscope (Axiophot Zeiss, Germany), AgNOR and p120 nucleolar protein scores per nucleus were obtained by counting the number of positive dots in 100 randomly selected nuclei on 20 fields at 1,000 x magnification, whereas the labelling indices of PCNA/Cyclin and Ki-67/MIB1 were computed as percentages of positive nuclei out of 1,000 cells on 20 fields at 400 x magnification.

Analysis of variance (ANOVA) was used to calculate the statistical data.

RESULTS

Sections of 26 cases of PC were graded histologically according to GHG and divided in two grou-

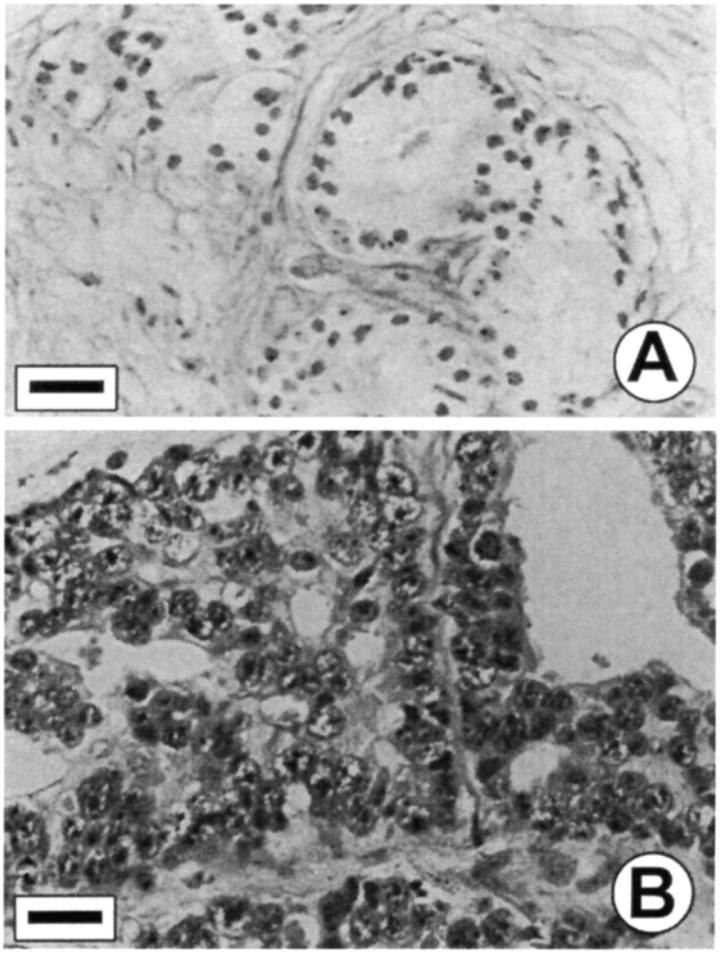

Fig. 1 – Prostate cancer. Large and small dots in the nuclei of prostate cancer with low (**A**) and high (**B**) Gleason histological grading as stained by the AgNORs method. Bar = 25 μm.

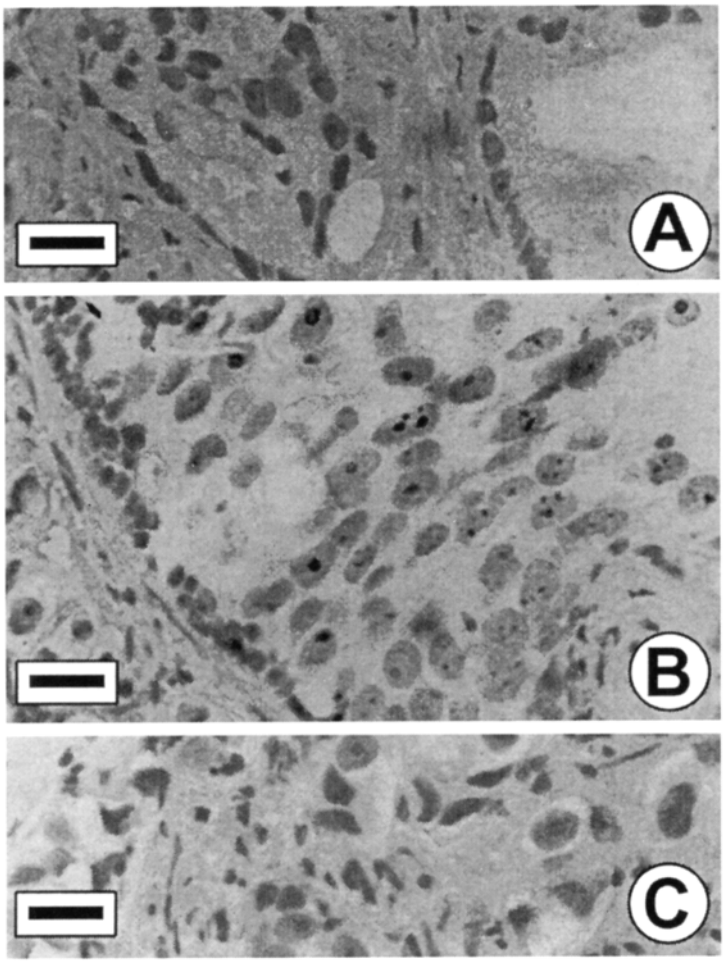

Fig. 2 – Several nucleoli of prostate cancer cells with low (**A**) and high (**B** and **C**) Gleason histological grading as immunostained by p 120 nucleolar protein. Bar = 25 μm.

ps, the first including cases with low (≤ 6) (13 cases) and the second with high (≥ 7) (13 cases) GHG.

The stains for AgNOR and immunoreactions for PCNA/Cyclin and Ki-67/MIB1 cell cycle markers were variously positive in both large and needle biopsy sections of PC. In large histological slides, the mean number of positive dots per nucleus for AgNOR increased from 6.4 in low GHG to 8.6 in high GHG, whereas the percentage of positive nuclei per 1,000 cells for Ki-67/MIB1 and PCNA/Cyclin rose from 12.6 and 23.2, respectively, in low GHG to 40.2 and 45.2, respectively, in high GHG. Histological sections from large samples of PC (6 cases) were randomly positive or totally negative after immunocytochemical reaction for p120 nucleolar protein, whereas those from needle biopsies of PC (20 cases) were uniformly positive.

Quantitative rates of p120 nucleolar protein were compared with the other three cell cycle markers only on PC needle biopsy sections. Nucleolar dots per nucleus positive to AgNOR (Fig. 1) and immunoreactive to p120 nucleolar protein (Fig. 2), and the number of nuclei immunoreacting PCNA/Cyclin (Fig. 3) and Ki 67/MIB1 (Fig. 4) stained out of 1,000 cells were greater in the group of PC with high GHG (Fig. 5). The percentage

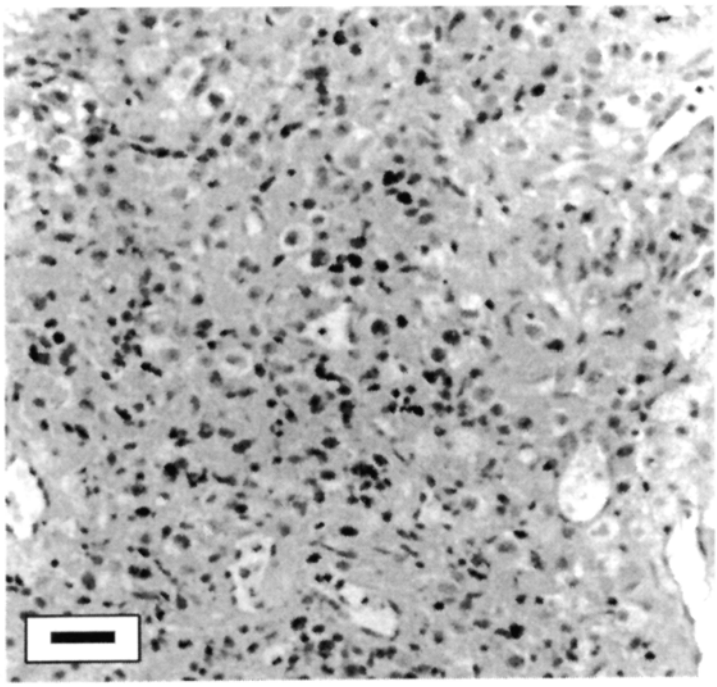

Fig. 3 – Proliferating cell nuclear antigen (PCNA/Cyclin) immunostaining of histological sections from needle biopsies of prostate cancer with high Gleason histological grading. Bar = 25 μm.

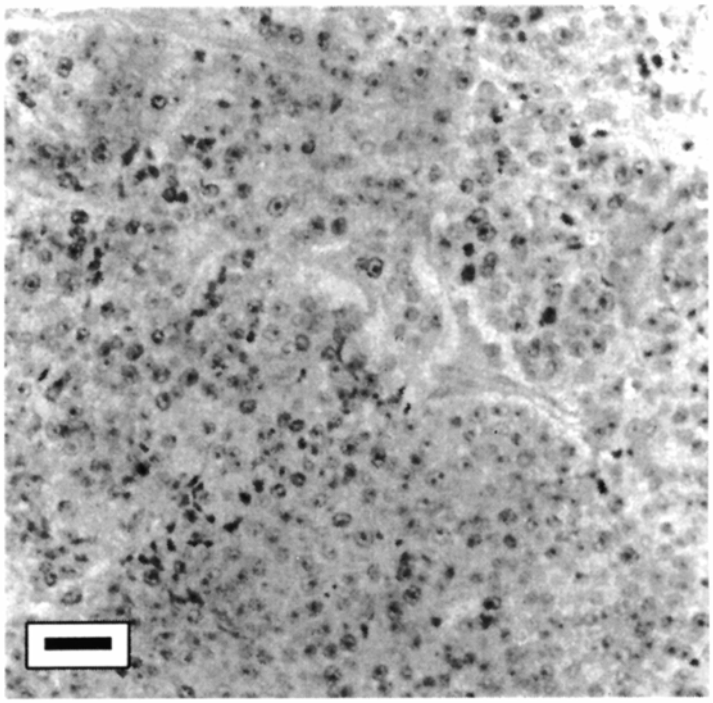

Fig. 4 – Ki-67/MIB1 immunostaining nuclei of prostate cancer with high Gleason histological grading. Bar = 25 μm.

rate out of 100 neoplastic prostatic cells for these 4 cell-proliferation activity markers increased from low to high GHG of PC (Fig. 6).

ANOVA showed extremely significant differences between the means of each of the four markers, with two-tailed p values <0.0001 when proliferation indexes of low GHG were compared with high GHG of PC needle biopsies.

DISCUSSION

The TNM staging system, GHG and serum PSA levels are the only prognostic factors of PC recommended by the Prostate Cancer Working Group of the College of American Pathologists, because there is not sufficient evidence from other markers for routine use (Grignon and Hammond, 1995). Nevertheless, in histopathological diagnostic practice, rates of cell proliferation by changes in DNA *status,* AgNOR, PCNA/Cyclin and Ki-67 indices, have to date yielded more definite information on the biological behaviour and clinical outcome of PC (Botticelli *et al.*, 1996).

Among the predictive indices of PC progression, the GHG is not advised for evaluating histological sections of needle biopsies because the very small amounts of prostatic tissue do not allows correct identification of many histopathological patterns. In contrast, our results on needle biopsies of PC suggest that GHG may be useful when only two GHG groups, ≤ 6 (low grade) and ≥ 7 (high grade), are considered. Moreover, due to its simple histological execution and cost effectiveness (Botticelli *et al.*, 1996), AgNOR staining offers practical advantages and reliability in routine histocytopathology of needle biopsies of PC (Botticelli *et al.*, 1995).

As previously reported (Bocker *et al.*, 1995), the expression of p120 nucleolar protein appears to correlate with hyperactivity of the nucleolus and malignant tumour activity. In frozen sections of

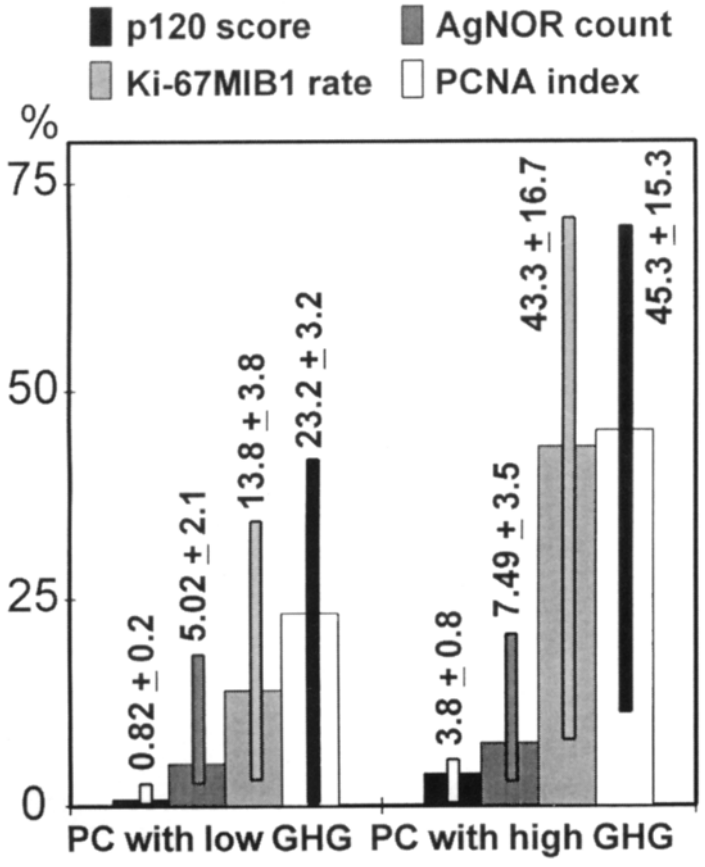

Fig. 5 – Labelling rates (p120 nucleolar protein, AgNOR, Ki-67/MIB1 and PCNA/Cyclin) on needle biopsies of prostate cancer of low and high Gleason histological grading. The bars represent the mean percentage of the labelling rate, and the error bars represent the range (min, max). The mean and standard deviation are reported above each bar.

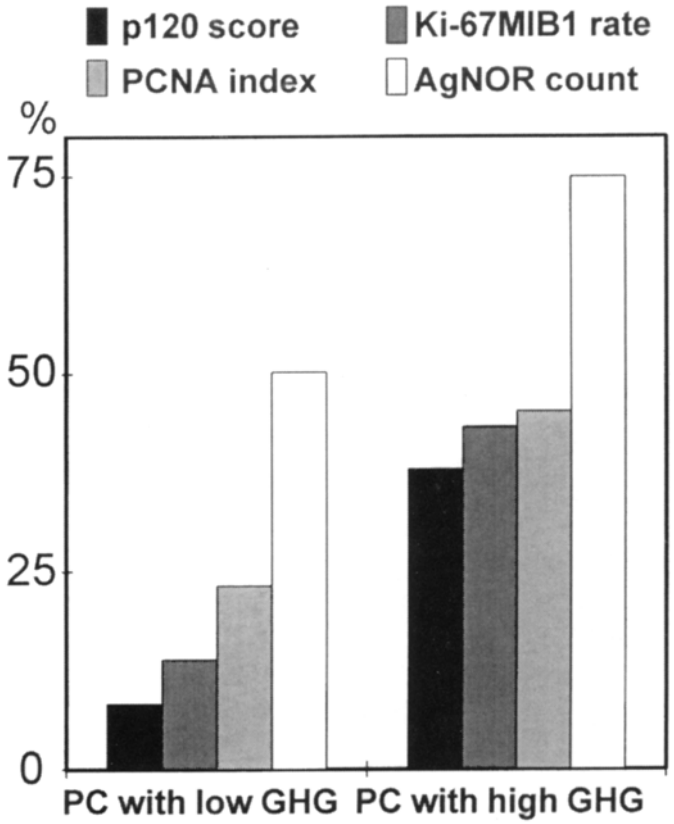

Fig. 6 – Pattern of mean number of nucleolar dots (AgNOR and p120 nucleolar protein) and positive nuclei (PCNA/Cyclin and Ki-67/MIB1) out of 100 prostatic neoplastic cells between prostate cancer with low and high Gleason histological grading.

PC (Rüschoff *et al.*, 1993; Bocker *et al.*, 1995), positive immunoreactivity of p120 nucleolar protein has been observed in 12% of benign prostatic hyperplasia and in 76% of PC specimens, and there is a highly significant rise in the labelling index ($p < 0.0001$) from 18.1% in WHO histological grade 1 to 82.2% in grade 3 PC. Conversely, in paraffin-embedded and formalin-fixed prostate tissue (Rüschoff *et al.*, 1993), immunohistochemical detection of p120 nucleolar protein seems less evident than in frozen sections, however the poor expression of p120 nucleolar protein rises from zero in WHO histological grade 1, to 35.5% in grade 2, and to 62.2% in grade 3 PC.

This behaviour might be due to two essential technical factors:

(a) the prostate gland from radical prostatectomy may normally be insufficiently fixed. The antigen-antibody reactions strictly depend on quality, quantity, time and type of fixative solutions and embedding media. Due to the presence of the capsule, large samples of neoplastic prostatic tissues need more accurate fixation, large amounts of fixative and adequate fixing times to test p120 nucleolar protein immunoreactivity. Cellular alterations may intervene before the action of the fixative, particularly in the inner part of the gland. To improve results, the entire prostate gland should be perfused or dissected into slices with prompt immersion in fixative solution. Indeed, delayed tumour fixation is associated with a decrease of proliferative events and cell cycle marker positive reactions (Laroye, 1996). Due to the small amount of tissue, needle biopsies allow a better and uniform penetration of fixative solutions throughout the whole sample, allowing a more regular immunocytological response.

(b) Immunocytochemical techniques for paraffin embedded and formalin fixed tissues, requiring the use of autoclaves, microwave ovens and proteolytic enzymes, produce histocytological denaturation of normally fixed tissues, and may produce perturbing effects on insufficiently fixed tissues.

The results of our study on needle biopsies of PC allow the following conclusions:

1 - The histo- and immunohisto-chemical detection of nucleolar (AgNOR and p120 nucleolar protein) and nuclear (PCNA/Cyclin, Ki-67/MIB1) proliferation associated antigens may be methods particularly well suited for cell kinetic analysis in prostate needle biopsies.

2 - The rates of all the investigated markers increase with decreasing PC differentiation and rising GHG of PC.

3 - Upon statistical analysis, all four markers were highly significant when low and high GHG of PC ($p < 0.0001$) were compared, thus proving to be very important in enacting and following the effectiveness of therapy in these patients.

4 - No differences were observed between PCNA/Cyclin and Ki-67/MIB1 immunoreactivity in the low and high GHG groups of PC, whereas the PCNA/Cyclin index showed a higher positive reaction than Ki-67/MIB1 index in low GHG, thus appearing to be more useful for distinguishing cases presenting differentiated features from those with malignant progression of PC (Botticelli *et al.*, 1993).

5 - The p120 nucleolar protein appears to be related to cell proliferative activity and, like PCNA/Cyclin, Ki-67/MIB1 antigens and AgNOR, is particularly expressed in high GHG of PC. Although the immunohistocytological technique for p120 nucleoloprotein on paraffin-embedded and formalin-fixed tissues might appear to be a complex technique, paraffin-embedded and formalin-fixed needle biopsies of PC seem be a suitable approach for the detection of this biological marker, indicative of tumour aggressiveness. The p120 nucleolar protein expression may play an important role in the identification of patients who would most benefit from adjuvant therapy against PC progression.

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