

## CELL PROLIFERATION AND VOLUME-WEIGHTED MEAN NUCLEAR VOLUME IN HIGH-GRADE PIN AND ADENOCARCINOMA, COMPARED WITH NORMAL PROSTATE

FERNANDO TEBA<sup>1</sup>, ROCÍO MARTÍN<sup>2</sup>, VICENTE GÓMEZ<sup>3</sup>, LUIS M HERRANZ<sup>1</sup> AND LUIS SANTAMARÍA<sup>3</sup>

<sup>1</sup>Service of Urology, Hospital de la Princesa, Madrid, Spain; <sup>2</sup>Service of Pathology, Hospital N. Sra. de Sonsoles, Ávila, Spain; <sup>3</sup>Departament of Anatomy, Histology and Neuroscience, UAM, Madrid, Spain  
e-mail: luis.santamaria@uam.es  
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### ABSTRACT

High-grade prostate intraepithelial neoplasia (PIN) is considered a precursor of prostate adenocarcinoma. The aim of this study was to quantitate the differences between basal and luminal cells of PIN in relation to mean nuclear volume ( $v_{\text{v}}\text{nuc}$ ), and proliferating cell nuclear antigen labeling index ( $\text{LI}_{\text{PCNA}}$ ), and to compare these estimates with those obtained in normal prostate and carcinoma. The epithelium of both normal and PIN specimens was segmented in basal and luminal compartments, and the  $v_{\text{v}}\text{nuc}$  and  $\text{LI}_{\text{PCNA}}$  measured in both strata.  $v_{\text{v}}\text{nuc}$  was significantly lower in normal epithelium than in both PIN and carcinoma. The  $v_{\text{v}}\text{nuc}$  of basal layer of PIN was significantly higher than in luminal stratum. The luminal  $v_{\text{v}}\text{nuc}$  was similar in both PIN and adenocarcinoma. The  $\text{LI}_{\text{PCNA}}$  was greater in PIN and adenocarcinoma than in normal glands. The  $\text{LI}_{\text{PCNA}}$  of basal cells from PIN was similar to that observed in the basal stratum from normal prostate, whereas the luminal proliferation from PIN was similar to that observed in adenocarcinoma. The similarities in nuclear size between PIN and carcinoma are according to the premalignant character of PIN. The increase of basal  $v_{\text{v}}\text{nuc}$  in PIN indicates that the changes heralding the progression from PIN to carcinoma are produced in this layer, whereas the nuclear features of the luminal layer are the same to those of the carcinoma. These remarks make sense in reference to the progression of malignant changes from PIN basal layer to PIN luminal layer and from this to carcinoma.

Keywords: adenocarcinoma, cell proliferation, nuclear size, PIN, prostate, stereology

### INTRODUCTION

PIN is defined as an intraluminal proliferation of the secretory cells of the prostate duct-acinar system that displays a spectrum of dysplastic cytologic features ranging from minimal atypia to those that are indistinguishable from carcinoma cells. This intra-glandular proliferation is consistently surrounded by a basal cell layer that is often attenuated and disrupted, especially in high-grade PIN.

The relationship between PIN and carcinoma of the peripheral prostate zone in men has been well documented (Haggman *et al.*, 1997). A number of studies concluded that high-grade PIN represents a premalignant lesion that could evolve into invasive adenocarcinoma (Haggman *et al.*, 1997). Molecular and immunohistochemical markers have been found to be useful in characterizing the progression of human PIN. These markers include the increase of anti-apoptotic proteins (bcl-2, bcl-X, and bax) (Bostwick,

1996; Krajewska *et al.*, 1996), changes in several genes that encode the expression of cell proliferation regulators (*e.g.*, p53, bcl-2, p16, p22, and c-erbB2) (Bostwick, 1996; Myers and Grizzle, 1996), the increase in cell proliferation, and in genetic instability (Montironi *et al.*, 1994; Takahashi *et al.*, 1994). Nevertheless, few studies have been directed to evaluate the nuclear size and its variability in the preneoplastic epithelium of PIN, although some authors have suggested that the cytologic atypia and nuclear pleomorphism were more remarkable in the basal layer in comparison with the apical or columnar stratum of PIN lesions (Epstein, 1994).

The average volume-weighted mean nuclear volume ( $v_{\text{v}}\text{nuc}$ ) was performed using the point sampled intercept method (Howard *et al.*, 2005), that estimates from two-dimensional images, the volume of three-dimensional structures, giving greater chance of volume estimation to particles of greater size. This is of interest, since it is possible that the greater

particles have more information about changes that are taking place. Therefore, when studying cell samples containing a significant amount of great nuclei we could have a greater sensitivity in the detection of relatively small and precocious tumors.

Increased cell proliferation, evidenced by markers such as PCNA, Ki-67 or MIB-1, has been related to tumors poorly differentiated from different organs and to a worse prognosis (Bulten *et al.*, 1996; Martín *et al.*, 1997). The increase of the cell proliferation from benign prostate tissue to PIN, and to adenocarcinoma has been verified (Tamboli *et al.*, 1996; Tsuji *et al.*, 1999).

The aim of this study was to quantitate the relationship between the stereological estimation of mean nuclear volume weighted by volume ( $v_v$ nuc) and the cell proliferation measured by immunohistochemical detection of PCNA, in basal and luminal (columnar) cells of PIN, in comparison to normal prostate epithelium, and prostate carcinoma.

## MATERIAL AND METHODS

### SPECIMENS AND TISSUE PREPARATION

Ten lesions classified as high grade PIN, and 10 samples of prostate adenocarcinoma (PCA) were selected from 15 prostate biopsies and from 5 surgical pieces (radical prostatectomy), obtained from 16 patients studied for diagnosis of prostate cancer in the Hospital de la Princesa, Madrid, Spain. The age of the patients ranged from 60 to 86 years (mean: 68). Ten specimens of normal prostate obtained from autopsies of men 20 to 41 years old (mean: 33) without prostatic or endocrine disease were used as controls (CTR). The tissues were immediately fixed after surgery in 10% paraformaldehyde for 24 hrs. Afterwards, the samples were paraffin embedded, and serially sectioned at 5  $\mu$ m. Five sections per specimen were stained with haematoxylin-eosin.

### IMMUNOHISTOCHEMISTRY

Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% in phosphate-buffered saline (PBS) pH 7.4, to block endogenous peroxidase. A Mouse monoclonal antibody to PCNA was used (Biomedica, Foster City, CA, USA) diluted at 1:400 in PBS pH 7.4 containing 1% bovine serum albumin (BSA) plus 0.1% sodium azide. The incubation with primary antisera was overnight at 4 °C. The second antibody employed was a biotin-

caproyl-anti-mouse immunoglobulin (Biomedica, Foster City, CA, USA). The second antibody was diluted at 1/400 in PBS containing 1% BSA without sodium azide, and incubated for 30 min at room temperature. Thereafter, sections were incubated with a streptavidin-biotin-peroxidase complex (Biomedica). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3',4,4'-Tetraminobiphenyl, Sigma, St Louis, USA) in 200 ml of PBS, plus 40  $\mu$ L hydrogen peroxide.

After immunoreaction, sections were counterstained with Harris haematoxylin. All slides were dehydrated in ethanol, and mounted in a synthetic resin, Depex (Serva, Heidelberg, Germany). The specificity of the immunohistochemical procedures was checked by incubation of some sections not sampled for measurements with nonimmune serum instead of the primary antibody.

### QUANTIFICATION OF CELL PROLIFERATION

Five sections per specimen were systematically randomly sampled (Gundersen *et al.*, 1988) over the total sections obtained in each specimen. The percentage of PCNA-immunostained nuclei (PCNA labeling index,  $LI_{PCNA}$ ) (Martín *et al.* 2001), were calculated in each selected section for CTR, PIN, and PCA specimens, using the formula: number of labeled nuclei  $\times$  100/total number (labeled + unlabeled) of nuclei. For evaluation of  $LI_{PCNA}$ , the following cell compartments were considered:

In all the groups (CTR, PIN, PCA):  $LI_{PCNA} (TT) =$  Number of total (basal + columnar) labeled cells in relation to the number of total (basal + columnar) labeled and unlabeled cells.

For CTR and PIN:  $LI_{PCNA} (BT) =$  Number of labeled basal cells in relation to the total (unlabeled + labeled) columnar and basal cells.  $LI_{PCNA} (CT) =$  Number of labeled columnar cells in relation to the total (unlabeled + labeled) columnar and basal cells.

Measurements were carried out using an Olympus microscope equipped with a  $\times 100$  oil immersion lens (numerical aperture of 1.4) at a final magnification of  $\times 1200$ , and using the stereologic software CAST-GRID (Interactivision, Silkeborg, Denmark). This program allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. An average of 100 fields per section were scanned, and a total of 500 epithelial nuclei were evaluated per section in each group (CTR, PIN, PCA), using an unbiased frame (disector) superimposed on the fields selected (Howard *et al.*, 2005). PCNA immunostained nuclei were considered positive regardless of staining intensity.

## QUANTIFICATION OF VOLUME-WEIGHTED MEAN NUCLEAR VOLUME

The stereologic evaluation of the  $v_{\text{Vnuc}}$  was carried out on three systematically randomly sampled haematoxylin-eosin stained sections per specimen, using the stereologic software CAST-GRID. In the present study, an average of 100 nuclei were point sampled per case, because the number of nuclei to be estimated per specimen in order to obtain reliable results is considered within the range of 70-100 (Sørensen, 1991).

The sampling protocol for the normal prostate (CTR) and PIN was designed in order to estimate separately the  $v_{\text{Vnuc}}$  of the basal and columnar layers. The epithelial lining of both normal and PIN glands was segmented in two strata: a basal compartment 7  $\mu\text{m}$  wide from basal membrane, and a columnar (luminal) layer from the limit of the basal compartment to glandular lumen. In both strata  $v_{\text{Vnuc}}$  was independently estimated. All the measurements were carried out using an Olympus microscope equipped with a  $\times 100$  oil immersion lens (numerical aperture of 1.4) at a final magnification of  $\times 1200$ . The program used to evaluate the  $v_{\text{Vnuc}}$  enables the generation of random test-lines directions that were superimposed onto the microscope images. The nuclear intercepts can be measured along these test-lines. The length of nuclear intercepts ( $l_0$ ) was processed to obtain  $\pi \cdot l_0^3 / 3$ , an unbiased estimate of  $v_{\text{Vnuc}}$  independent of nuclear shape, which, because of point sampling, emphasizes larger nuclei rather than smaller ones. In addition, estimates of  $v_{\text{Vnuc}}$  combine information about the three-dimensional nuclear size with knowledge of variability of nuclear size (Gundersen *et al.*, 1988). The measurements obtained in PCA and PIN cases were compared with those obtained in CTR group. In CTR and PIN epithelium, the mean nuclear size measured in the basal layer was also compared with the measurements performed in the columnar stratum.

## STATISTICAL ANALYSIS

Means  $\pm$  SD were obtained for all the parameters evaluated in each group (CTR, PIN, PCA). The differences for  $v_{\text{Vnuc}}$  and  $\text{LI}_{\text{PCNA}}$  among the groups studied were evaluated by ANOVA, and the comparison between the means of the estimates was performed using the Newmann Keuls test ( $p < 0.05$ ).

The contribution of each of the three sampling levels (*i.e.*, nuclear intercepts and their measurements, fields of vision, and individual cases) to the total observed variance associated with the  $v_{\text{Vnuc}}$  estimates was investigated by nested analysis of variance. The relative contribution to overall variance is estimated by regarding the observed variance at each level of sampling (measurements, fields, and cases) as the sum of the true variance at that level plus the variance of the mean ( $\text{SEM}^2$ ), at the lower level of sampling. Thus, large variances at the lower levels are diminished in their contribution to totally observed variance by the number of observations at that particular level (Artacho *et al.*, 1995).

## RESULTS

### QUALITATIVE RESULTS

The prostatic acini affected by PIN showed a remarkable enlargement of the epithelial layer with pseudostratification and crowding of the nuclei in comparison with normal acini from controls. The nuclei from PIN lesions were larger in size than those observed in normal epithelium or in carcinoma, showing cytological atypia and frequent nucleoli. The nuclear size in PIN lesions decreases towards the glandular lumen (Fig. 1a). No mitotic figures were visualized in all the groups studied.

In PCNA immunostained samples, a small number of labeled nuclei were observed in the CTR prostate acini, most of them being located in the basal layer (Fig. 1b). An important amount of labeled nuclei was observed in the epithelium of glands with PIN, and these were predominantly located in the luminal compartment (Fig. 1c). The PCA cases show also a remarkable PCNA immunoreactivity, (Fig. 1d).

### QUANTIFICATION OF CELL PROLIFERATION INDEX

The  $\text{LI}_{\text{PCNA TT}}$  was significantly higher in both PIN and PCA groups than in CTR group, not significant differences were observed between PIN and PCA cases (Fig. 2a). The  $\text{LI}_{\text{PCNA B/T}}$  show a significant increase in comparison with  $\text{LI}_{\text{PCNA C/T}}$  in the PIN group, whereas no significant differences were observed for these estimates in the CTR group (Fig. 2b).

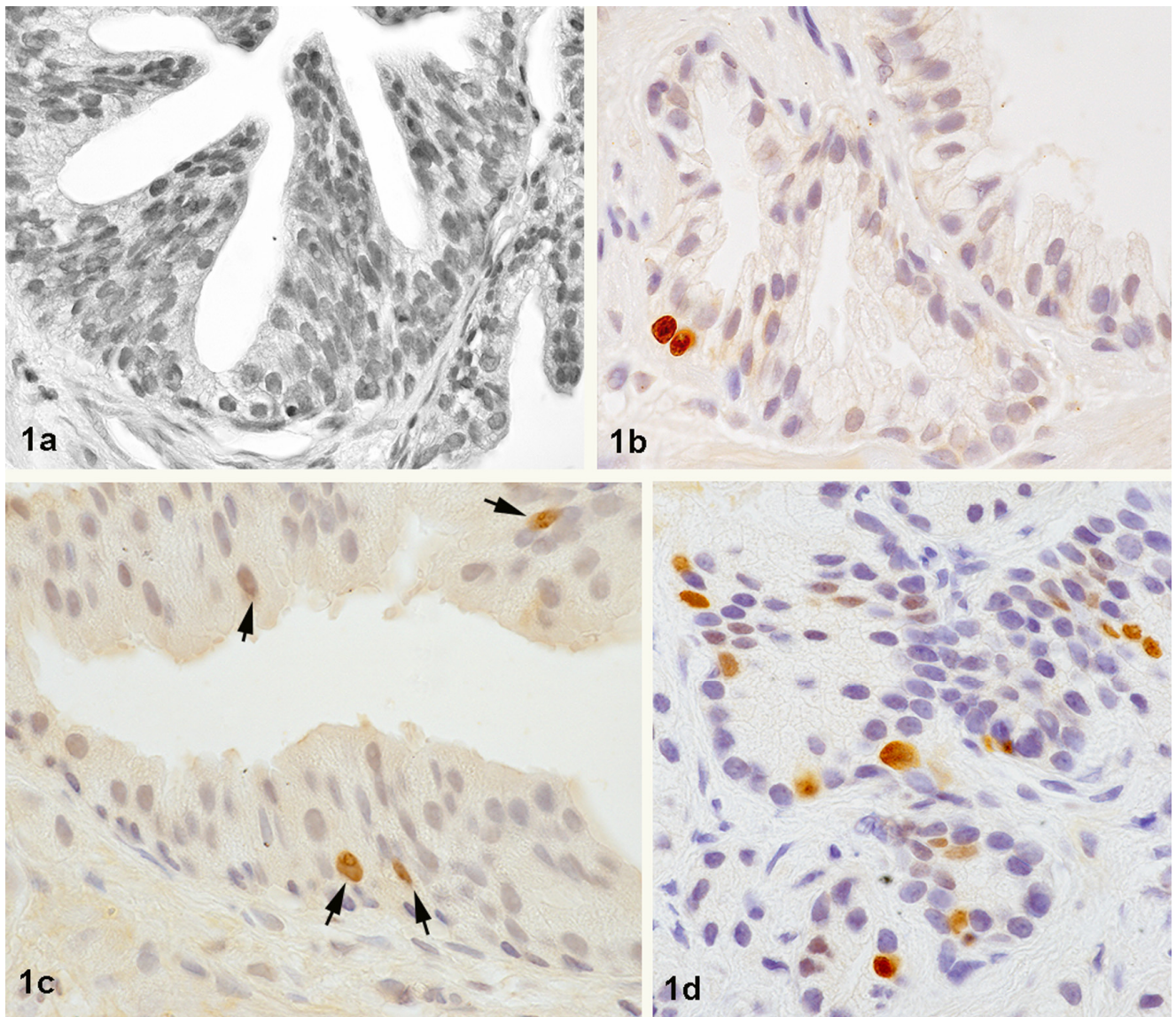


Fig. 1. a) Prostate intraepithelial neoplasia. Increase of cell stratification with crowded nuclei large in size, and with presence of prominent nucleoli. A discontinuous layer of basal cells is observed. Haematoxylin-eosin.  $\times 400$ ; b) Immunostaining to PCNA. CTR case. Two immunoreactive nuclei are placed in basal epithelial layer.  $\times 400$ ; c) Immunostaining to PCNA. PIN case. An increased number of immunoreactive nuclei is observed predominantly located in columnar layer (arrowheads).  $\times 400$ ; d) Immunostaining to PCNA. Prostate adenocarcinoma. A remarkable amount of immunoreactive nuclei with heterogeneous distribution is observed.  $\times 400$ .

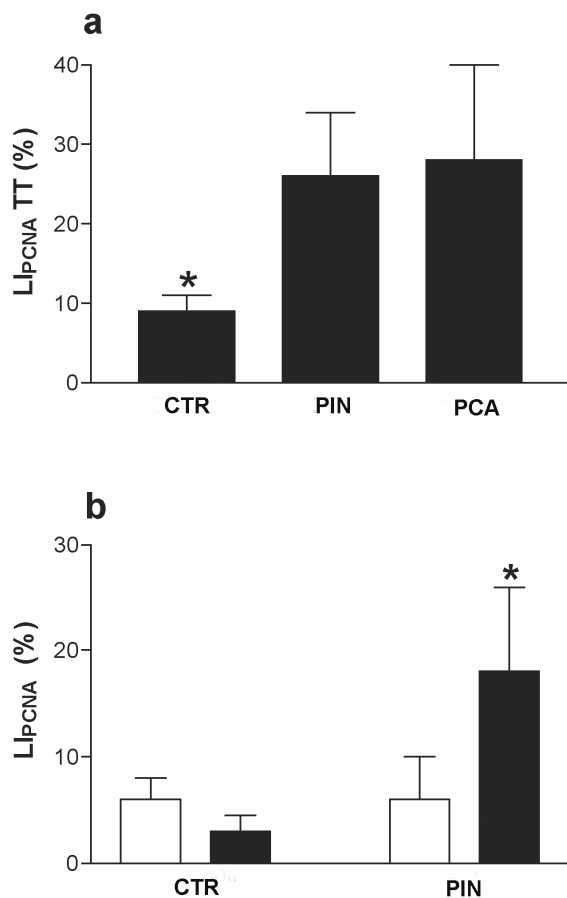


Fig. 2. Bar graphs are representing: a) Cell proliferation index respect to the total epithelial cells in in control group (CTR), PIN and adenocarcinoma (PCA). The asterisk onto the bar of CTR group indicates significant differences ( $p < 0.05$ ) with PIN and PCA; b) Cell proliferation index in CTR and PIN, once segmented glandular epithelium in basal layer (empty bar) and columnar layer (solid bar). The bar marked with an asterisk indicates significant differences ( $p < 0.05$ ) with the other bars within each diagram.

#### QUANTIFICATION OF $v_{\text{VNUC}}$

The  $v_{\text{VNUC}}$  of nuclei from basal compartment of PIN was significantly higher than  $v_{\text{VNUC}}$  of all other groups including PCA. On the other hand, the  $v_{\text{VNUC}}$  of PCA was significantly higher than in the CTR, and without significant differences when comparing with the columnar compartment of PIN. Moreover, both estimates from PIN columnar compartment and PCA were significantly higher than those observed in CTR basal and columnar layers. There was no significant differences between  $v_{\text{VNUC}}$  of CTR basal and CTR columnar compartments. In comparison with CTR, a greater dispersion around the mean was observed in both PIN and PCA measurements (Fig. 3).

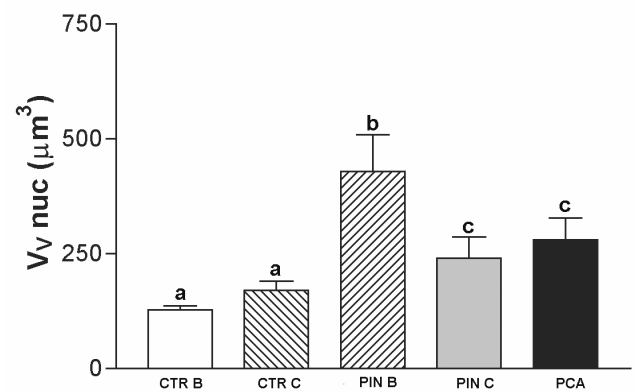


Fig. 3. Bar graphs are representing  $v_{\text{VNUC}}$ , for basal stratum of controls (CTR B), columnar stratum of controls (CTR C), basal stratum of PIN (PIN B), columnar stratum of PIN (PIN C), and carcinoma (PCA). The different letters on the error bars indicate significant differences ( $p < 0.05$ ).

The greatest contribution (expressed as percentage) to the totally observed variance for  $v_{\text{VNUC}}$  estimates in the columnar stratum of both control (53%) and PIN (64%) samples, basal compartment of PIN (71%), and carcinoma (77%), was provided by the highest level of sampling, *i.e.*, by the biological variation among the cases. This interindividual variability was higher in PIN (basal compartment) and carcinoma than in the other groups.

#### DISCUSSION

In the present study, it was observed that the cell proliferation index ( $\text{LI}_{\text{PCNA}}$ ) is greater in PIN and adenocarcinoma than in normal prostate tissue. Although proliferation was higher in adenocarcinoma, differences with PIN were not statistically significant. However, significant differences between cell proliferation in PIN and PCA have been shown by other authors (Tamboli *et al.* 1996; Santamaría *et al.* 2005). This disparity might be attributed to different proliferating markers employed in the studies cited (Ki-67 instead of PCNA). It has been observed that there were no significant differences in  $\text{LI}_{\text{PCNA}}$  from the basal layer between CTR and PIN specimens. However, the proliferation index, increased in the luminal layer of PIN, being the  $\text{LI}_{\text{PCNA}}$  in this location without significant differences when compared to that observed in adenocarcinoma. These data support, in PIN lesions, a possible path of progression relating to malignancy from basal to columnar cells and from these to carcinoma. It is interesting to note, that in spite of the findings of other authors (Tamboli *et al.*,

1996; Santamaría *et al.*, 2005), no significant differences in cell proliferation were detected between PIN and PCA. This fact might indicate that the invasive ability of PCA is not exclusively related to cell proliferation. The increase of  $v_{\text{v}nuc}$  in a number of neoplasias is related to several factors: the increase of histological grade (Fujikawa *et al.*, 1995), a poor prognostic (Martín *et al.*, 1997; Martín *et al.*, 1999), or the poor response to therapy (Fukuzawa *et al.*, 1995; Matsui, 2005).

In the present study, a significant increase of  $v_{\text{v}nuc}$  was detected in PIN when compared to normal prostatic epithelium. This fact could support the presumptive pre-malignant character of this lesion. Similar data have been reported by other authors, who found a progressive increase of nuclear volume from normal prostate acini to low grade PIN, high grade PIN, and carcinoma (López-Beltrán *et al.*, 2000).

It is interesting to note that the basal layer of PIN showed the highest values for both  $v_{\text{v}nuc}$  and inter-individual variability. This might indicate that basal cells of PIN correspond to cells in a stage of transient proliferation (with features of pluripotential cells), when these cells reach the luminal compartment their nuclear size decreases while maintaining their potential for proliferation (Tsuji *et al.*, 1999) and malignancy. In fact these basal cells are similar to those observed in invasive carcinoma. Thus, in contrast to those described in other neoplasias (Sørensen, 1989; Ladekarl *et al.*, 1995), the invasive features in prostate cancer are not immediately related to the increase of nuclear volume.

## CONCLUSIONS

The similarities in nuclear size and cell proliferation between PIN and carcinoma are according to the premalignant character commonly attributed to PIN. The increase of basal  $v_{\text{v}nuc}$  in PIN might indicate that the changes heralding the progression from PIN to carcinoma are produced in the basal layer, whereas the nuclear features of the luminal layer are the same as those of carcinoma.

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