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Aneuploidy and Nuclear Features of Prostatic Intraepithelial Neoplasia (PIN)

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Abstract Quantitative analyses (QAs) of prostatic intraepithelial neoplasia (PIN) helped to objectively define some traditional features of the lesion because, first, changes in value are represented by numbers and not by subjective evaluation of morphologic clues. QAs have also helped to identify subtle abnormalities. For example, the degree of nucleolar margination is a new diagnostic feature which can be easily evaluated as the proportion of nucleoli touching the nuclear membrane. Thirdly, QAs have provided useful insights into understanding some morphologic changes. PIN, in fact, appears to be characterized by complex changes which involve the secretory cells as well as the basal layer and which affect the surrounding stroma. In the epithelial lining, two types of simultaneous changes take place, the first in the nucleus (expression of abnormal proliferative and/or renewal activity) and the second in the cytoplasm (expression of the disorder in cell differentiation), pointing out that only PIN samples of high grade can be considered as having acquired the characteristics of a neoplastic lesion. © 1992 Wiley-Liss, Inc.

Key Words: DNA analysis, morphometry, prostatic intraepithelial neoplasia (PIN)

The diagram of human prostate carcinogenesis by Bostwick and Brawer [1] surely represents one of the most accurate morphologic illustrations in recent pathology. This drawing was based on the strict criteria proposed by McNeal and Bostwick [2], useful for the histologic recognition and grading of prostatic intraepithelial neoplasia (PIN, intraductal or ductacinar dysplasia, atypical primary hyperplasia and atypical intraepithelial lesions are synonymous). Recently, Murphy [3] affirmed that "the lesions themselves have been defined sufficiently in light microscopic terms to be recognized readily by practicing pathologists without sophisticated instrumentation." This statement might appear to discourage the application of quantitative analyses (QAs) in the evaluation of PIN. However, QAs have helped to define qualitative impressions in quantitative terms, to detect subtle abnormalities, and to produce data amenable to statistical analysis [4].

USEFULNESS OF QAs

McNeal and Bostwick [2] observed that PIN is basically characterized by proliferation and anaplasia of the secretory cells of ducts and acini. The changes are based on the subjective evaluation of cytological, architectural and associated features. The literature includes some papers in which these groups of features were quantitatively evaluated, generally in histologic sections. The tissue architecture was considered fundamental in identifying objects to be measured.

QAs of Cytology-Related Features

Cytological features, such as nuclear enlargement and size variability, nucleolar frequency and prominence, and chromatin pattern, are all considered important for PIN diagnosis and grading [1,2,5–7]. The results of a quantitative study from our group [8] showed that the mean nuclear area of the lumenal cells increased from benign prostatic hyperplasia (BPH, 31.85 sq μm) through low-grade PIN (PIN1, 38.28 sq μm) and high-grade PIN (PIN2, 43.18 sq μm) up to invasive adenocarcinoma (AC, 50.18 sq μm), while the nuclei became slightly less round (in our research a two-grade system for PIN has always been adopted instead of the

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usual three grades). The "enlargement" of nuclei was associated with a decrease in the percentage of nuclei in the 2c (diploid) region from BPH to AC and an increase in the percentage of nuclei in other regions, mainly between 2c and 4c and in the tetraploid region, from BPH to AC; the ACs again showed values similar to those of the PIN2s. When the samples were classified as DNA diploid (with a major peak in the 2c range), DNA triploid (with a major peak in the region between 2c and 4c) or DNA aneuploid (with nuclei scattered over different ploidy regions), all of the BPH samples were diploid. Of the PIN1 samples, 80% were diploid, whereas 20% showed two major components, the first at 2c and the second in the 2c–4c (triploid) range. Of the PIN2 samples, 60% showed their major component in the triploid region; 40% were aneuploid. Of the AC samples, 20% showed a diploid peak; 55% had their major component in the triploid region and 25% were aneuploid. The AC samples classified as diploid showed a lower percentage of nuclei in the 2c range and a higher proportion of nuclei in the 2c–4c and 4c ranges than the BPH samples. Our results were recently confirmed by Patein *et al.* [9] in a study performed on cytospin slides. In particular, good correspondence existed between the proportion of nuclei in the DNA ranges greater than 2c evaluated in our study and Patein's proliferation index, indicating that the DNA proliferative activity increases with the severity of PIN.

Nuclear size variability was checked by calculating the standard deviation (SD) of the nuclear area in each case [8]. Progressively higher values for SD indicated non-heterogeneity in nuclear size between various disease stages. SD values were lowest for BPH (4.91 μm), intermediate in the PIN grades (PIN1, 6.08 μm; PIN2, 7.30 μm) and highest in AC (9.33 μm). A similar trend has been observed for the nuclear DNA content SDs; these changes can explain the nuclear area variability, at least in part. In fact, it can be assumed that variability is due either to a different percentage of nuclei in S-phase and G2-phase or to the presence of more than one population. We observed the latter in two PIN1 cases where the DNA histogram showed two distinct populations. The variability trend observed in our study is at odds with that reported when evaluating the diagnostic features subjectively. In fact, the size variability in the high-grade PIN was described as "some," *i.e.*, lower than in the low-grade, where it was reported as "marked" [5].

In addition to the DNA content QAs, Patein *et al.* [9] simultaneously performed evaluations of chromatin texture. They found that the variance in optical density values (a feature which evaluates the chromatin heterogeneity) decreased dramatically from BPH to low-grade PIN and then increased slightly in higher grade PIN and in AC. They considered that the values increase when chromatin displays various degrees of condensation, and decrease when chromatin clumps together into large regions or, conversely, when chromatin decondensation renders the nuclear texture very thin. They concluded that chromatin clumping was responsible for the decrease in chromatin heterogeneity. At the same time, by investigating the local mean of the co-occurrence matrix (a feature which measures the overall chromatin condensation level), they observed that there was a simultaneous increase in chromatin condensation. These results are partly in keeping with the grading criteria for PIN, in which chromatin of H&E-stained nuclei is "increased" and "more densely stained" in grade 2, and "markedly increased" in grade 3, although it is reported to be "normal" in grade 1 [5]. However, a slightly different description of the chromatin changes has been given by others: in grade 2 dysplasia, "increased density of chromatin staining was seen ... Chromatin was finely granular, with a variable proportion of cells showing chromatin condensation beneath the nuclear membrane"; in severe atypical hyperplasia, "nuclear chromatin was increased and appeared to be finely punctuated or coarse. Condensation of chromatin matter at the nuclear membrane could be clearly seen. Many nuclei seemed vacuolated" [2,6]. We have not yet studied the chromatin texture quantitatively. However, in agreement with some reports in the literature, the chromatin texture might follow a change similar to that of nucleoli: accumulation of irregularly shaped clumps of chromatin adjacent to the nuclear envelope (peripheral or marginal chromatin), while the chromatin in the remainder of the nucleus (probably corresponding to the chromatin centres) appears finely punctuated. The nucleolus-associated chromatin is less

visible, thus giving the nucleus a clear or vacuolated appearance.

The terms originally proposed for the subjective evaluation of nucleoli are as follows: infrequent; occasionally large and prominent; frequently large; or similar to invasive AC [1,2, 5–7]. Size-, frequency- and location-related nucleolar features were evaluated in H&E-stained histologic sections [10]. It has been observed that, going from BPH through PIN1 and PIN2 up to invasive AC, the values of the size-related features are progressively greater; the most evident change was expressed by the nucleolar hypertrophy index (BPH, 0.19; PIN1, 0.23; PIN2, 2.66; and AC, 3.09). The frequency-related features were characterized by a decrease in the proportion of mononucleolated nuclei and an increase in nuclei with two or more nucleoli (BPH, 3.99%; PIN1, 13.69%; PIN2, 15.06%; and AC, 16.38%). When considering the location-related features, the degree of shift towards the periphery of the nucleus increased, as shown by the percentage of nucleoli in the peripheral position and by the nucleolar eccentricity index, *i.e.*, a feature related to the degree of margination (BPH, 2.95; PIN1, 3.82; PIN2, 3.89; and AC, 4.13). Therefore, this study confirms (and expresses in quantitative terms) the concept that nucleoli are an important hallmark for the diagnosis and grading of PIN.

Garnett and Oyasu [11] graded nucleolar prominence in terms of its frequency and size. Although these authors were strict in defining nucleolar prominence (nucleoli greater than 1 μm in diameter), their evaluation of frequency (rare, occasional and common) and size (small, medium and large) was subjective. In the recent study by Kelemen *et al.* [12], nucleoli were investigated in benign and malignant prostate lesions together with PIN cases. It was found that the fraction of nuclei with prominent nucleoli (largest nucleolar diameter of 3 μm or greater) was the best histologic discriminator.

Helpap [13] investigated nucleolar location semiquantitatively and subjectively and found that, with increasing degree of malignancy, the nucleolus shifted to the periphery of the nucleus. Our observations [10] appeared to agree with Wachtler *et al.* [14] who postulated that nucleolus organizer regions change their position in order to find special sites where attachments to the nuclear membrane were possible and the development of one or a few large nucleoli more effective. QAs of silver-stained nucleolar organizer regions (AgNORs) have shown that the AgNOR count and AgNOR area increase from BPH through PIN to AC [15,16]. Interestingly, Deschenes and Weidner [16], who investigated the nucleolar size together with the AgNOR counts, observed that nucleolar size increased with increasing AgNOR counts, both becoming higher as diagnosis worsened. This has been our experience, too. In fact, prior to a previous study, we had found that nucleolar feature changes were related to changes in AgNORs.

QAs of proliferating cell nuclear antigen (PCNA) offer the unique possibility of investigating proliferative activity (1.60% of nuclei are PCNA-immunostained in BPH; 7.57% in PIN1; 11.42% in PIN2; and 18.60% in AC) together with its location. In BPH, scattered PCNA-immunostained nuclei are observed in the basal cell layer. In the PIN lesions, PCNA-positive cells are seen throughout the entire thickness of the epithelium, indicating that, when cells migrate towards the lumen, they retain their proliferative activity (unpublished data).

QAs of Architecture-Related Features

Architectural features, such as epithelial stratification, crowding and spacing, are all considered important for PIN diagnosis and grading [1,2,5–7]. As for stratification [7], the mean stratification index (*i.e.*, the ratio of the distance from the basement membrane to the basal margin of the nuclei of the secretory cells divided by the distance from the basement membrane to the lumenal cell border) had PIN values which were greater than in BPH, while in AC they were smaller than in BPH. In PIN1 and PIN2, the values were similar (BPH, 0.23; PIN1, 0.34; PIN2, 0.34; and AC, 0.16). The nuclear crowding index (expressed by the nuclear counts located on a constant length of the basal cell border) first increased while moving from BPH to PIN1, but subsequently decreased slightly in PIN2. In AC it returned to the BPH level (BPH, 1.06; PIN1, 2.68; PIN2, 2.47; and AC, 1.05). Spacing is linked to the cellular surface. Levels of mean cellular area (defined as the epithelial area divided by total number of

nuclei in the epithelial area) decreased from BPH to PIN. The cellular area was similar in the two PIN grades. The area in AC was intermediate between PIN and BPH, the mean category values being closer to the former (BPH, 151.04 sq μm; PIN1, 114.89 sq μm; PIN2, 111.38 sq μm; and AC, 126.94 sq μm). Therefore, the quantitative features accurately defined the degree of modification of the architectural features.

QAs of Associated Features

As for the associated features, the evaluation of intactness or disruption of the basal cell layer and basement membrane are included among the diagnostic features of PIN [1,2,5–7]. Considering the basal cell layer in particular, this was immunostained for the anti-keratin "basal cell-specific" antibody and subjectively quantitated by Bostwick and Brawer [1]. These authors showed that the frequency of disruption and the proportion of circumferential disruption of the basal cell layer increased with increasing grades of PIN. Our study of the basal cell layer has concentrated on the morphology of the nuclei adjacent to the basement membrane. In BPH, they are scattered and elongated. In PIN1, they become roundish and more frequent than in BPH. In PIN2, the nuclei in the basal position are mostly round, crowded and indistinguishable from the nuclei in the other layers. However, this observation is awaiting confirmation by means of QAs.

The stroma surrounding the ducts and acini with PIN has only been investigated to a small extent. In our experience, the stroma shows changes in lectin *Ulex europaeus* (UEA-1)-stained blood capillaries which parallel those present in the epithelial gland lining. When compared with those of BPH, capillaries become shorter and undulated (BPH, 17.40%; PIN1, 40.50%; PIN2, 42.00%; and AC, 56.60%), with open lumen (BPH, 54.40%; PIN1, 68.50%; PIN2, 69.70%; and AC, 81.60%) and visible endothelial nuclei (unpublished data).

QAs OF THE CONTINUUM OF CHANGES

PIN comprises a continuum of nuclear and cytoplasmic abnormalities in the cells lining preexisting ducts and acini [1,2,5–7]. In its most severe expression, dysplasia produces cells which individually are indistinguishable from those of invasive carcinoma [17]. Do QAs support the concept of the continuity of abnormalities? To verify this, linear discriminant analysis, a classification procedure used to assign objects to categories, was applied to the quantitative data. It is based on cases whose category membership is known and gives rise to a rule [canonical discriminant functions (CDFs)] which serves as the basis for classifying cases into one of the categories. One of the advantages of linear discriminant analysis is that only a small selection of features which best represent the lesions is included in the calculation of the CDF scores [4]. In our study [8] each PIN lesion is represented only by two CDF values or scores which summarize the information of the quantitative aspects. The plot of the two CDFs shows the separation achieved between the diagnostic categories and gives an idea of the spatial distribution of the samples. CDF No. 1 shows that BPH, PIN and invasive AC appear as contiguous categories, with some overlap, mainly between PIN and AC (Fig. 1; reproduced from Montironi *et al.* with permission from the Publisher) [8]. On the other hand, CDF No. 2 shows that, going from BPH through PIN to AC, the samples become more scattered. This could be viewed as an increasing variability among the samples in the higher categories. Moreover, the space occupied by the PIN samples in the scatterplot of Figure 1 can be subdivided into two parts (as indicated by the interrupted line), thus separating the PIN1 samples (closer to the BPH samples) from PIN2 (closer to AC samples). Therefore QAs, when associated with advanced statistics, clearly and graphically confirm the concept of the "continuum of abnormalities" [1,2,5–7].

QAs AND PIN GRADES

PIN forms a continuum of morphologic abnormalities. From the practical point of view, however, histologic grades are recognized. Originally three grades were adopted, although Helpap suggested four [1,2,5–7,18]. We have always preferred a two-grade system based on the results of cluster analysis. This multivariate method is adopted when the number of categories and category membership for all cases are

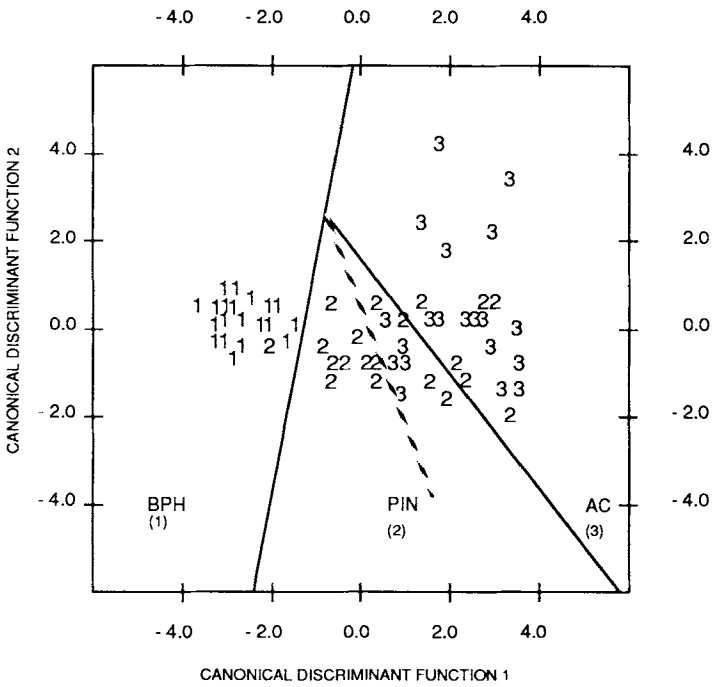

Fig. 1. Scatterplot of the spatial distribution of the cases of BPH, PIN and AC. The cases appear as continuous categories, with overlap mainly between PIN and AC. The two lines divide the scatterplot into three parts, corresponding to the three categories. The part corresponding to PIN can be subdivided into two parts (interrupted line), separating the PIN1 cases (close to the BPH cases) from PIN2 cases (close to the AC cases).

unknown, and may be employed to identify homogeneous categories on the basis of their "similarities." Hierarchical and non-hierarchical clustering methods gave similar results: PIN1 cases were basically clustered together with BPH cases, whereas PIN2 clustered with AC [4]. This corresponds to the morphologic observation that PIN1 is closer to BPH whereas PIN2 is closer to AC. Our results confirm the report by Drago *et al.* [19] in which the Bethesda Workshop Investigators agreed on a grading system that described low- and high-grade PIN by incorporating the previous grades "2" and "3" into the high grade. The two-grade system is probably preferable to three or four grades because the two PIN grades reflect the current understanding of premalignant or preinvasive malignant lesions in different organs where two successive phases can be observed. The first is characterized by euploid polyploidy, resulting from DNA duplication without nuclear division, or from an increased number of cycling cells. In the second, aneuploid elements appear, similar to those present in the invasive phase of the neoplasia [20].

ACHIEVEMENTS WITH QAs

First of all, the data reported in the literature show that QAs help to better define some traditional morphologic features. These are defined in an objective way because their changes are represented by numbers and not by the subjective evaluation of morphologic clues. Secondly, QAs help to identify subtle abnormalities, for example, in the nucleoli. The nucleolar size and frequency are objectively defined in numerical terms. Moreover, the degree of nucleolar margination is a new diagnostic feature, easily evaluated as the proportion of nucleoli touching the nuclear membrane. Even though not included in the original list of features identified by McNeal and Bostwick [2], marginated nucleoli have already been marked in the

original diagram by Bostwick and Brawer [1]. Thirdly, QAs provide useful insights into understanding some of the morphologic changes, such as nuclear enlargement. The changes in nuclear DNA content and in chromatin texture could contribute to the explanation of nuclear enlargement. This is in keeping with the concept that accelerated tumour growth is accompanied by an increase in the size and DNA content of the nuclei and by chromatin decondensation. In particular, "chromatin decondensation" should correspond to a change in the distribution and proportion of heterochromatin to euchromatin. The first is a condensed, presumably inactive form which is basophilic under the light microscope, and the latter is an active form, dispersed in the nuclear matrix [7].

Traditionally it is said that in BPH, ducts and acini are lined by a two-cell type epithelium, *i.e.*, the basal cell layer and the lumenal cell layer [1,2,5–7]. The basal cells have the capacity to divide and form daughter cells or to migrate towards the surface to form the differentiated lumenal cell layer. In PIN lesions this epithelial compartmentalisation is disrupted, starting from the basal layer. In fact, the proportion of cycling cells or cells able to divide has increased; cells with such a capacity are present in all layers. This increased "proliferation" [1] has been confirmed by the results of the nuclear DNA content, AgNOR and PCNA evaluations, and agrees with earlier studies by Helpap, who investigated atypical hyperplasia of the prostate using the thymidine labelling index [18]. QAs of PCNA offer the unique possibility of investigating proliferative activity and extension of the proliferative compartment.

In contrast, the differentiated compartment, which in BPH is represented by the secretory cells, is partly obscured, as indicated by the fact that the cells have a smaller cytoplasm with a defective presence of some prostatic markers and the expression of others not normally present. To the best of our knowledge, no QAs of cytoplasmic marker expression have been performed. However, in a semiquantitative study, differentiation markers such as prostate-specific antigen, prostatic acid phosphatase and Leu-7 (a differentiation antigen of human NK and K cells) have been found consistently reduced in dysplastic foci, the degree of reduction being proportional to the severity of PIN [21]. Both PIN and prostatic AC show UEA-1 binding, unlike normal secretory epithelium in BPH. Immunostaining for cytokeratins 14, 15, 16 and 19 has been reported to have a pattern of staining similar to UEA-1 [5].

PROSPECTS FOR QAs

Up to now, many morphologic and quantitative evaluations have been directed towards a better recognition of PIN and have pointed out that, similarly to the preneoplastic lesions of other body tracts, only PIN samples of high grade can be considered as having acquired the characteristics of a neoplastic lesion. As in the natural history of neoplastic lesions, one of the important questions is whether any particular intraepithelial lesion will become an invasive AC. QAs of nuclear DNA and of molecular biology may show that ploidy reduction and the expression of certain markers can all be important in the progression towards an invasive carcinoma. Therefore, in our opinion, the prostate too offers enough ground for future QAs aimed at recognising which PIN lesions progress to the invasive phase [20].

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