Original contribution

DNA and kinetic heterogeneity during the clonal evolution of adrenocortical proliferative lesions

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Received 1 March 2006; revised 21 April 2006; accepted 21 April 2006

Keywords:

Adrenocortical nodular hyperplasias;
Adrenocortical;
Neoplasm;
Cell heterogeneity;
Kinetics;
Clonality

Summary Monoclonal adrenocortical lesions show inverse correlation between proliferation and apoptosis, with proliferation being the single most important criterion of malignancy in adrenal lesions. No study yet has evaluated the variability of proliferation regarding the clonal pattern and diagnosis in adrenocortical nodular hyperplasias (ACNHs), adrenocortical adenomas (ACAs), and adrenocortical carcinomas (ACCs). We studied 69 ACNHs, 64 ACAs, and 23 ACCs (World Health Organization criteria) from 156 females. Clonality HUMARA test (from microdissected DNA samples), DNA content and proliferation analysis (slide and flow cytometry), and mitotic figure (MF) counting/50 high-power fields (HPFs) were performed in the same areas. Heterogeneity was assessed by 5cER (percentage of nonoctaploid cells with DNA content exceeding 5c) and standard deviation of MF/HPF. Statistics included analysis of variance/Student *t* tests regarding the clonal patterns and diagnosis. Polyclonal patterns were observed in 48 of 62 informative ACNHs and 7 of 56 informative ACAs, and monoclonal in 14 of 62 ACNHs, 49 of 56 ACAs, and 21 of 21 ACCs, with all hyperdiploid lesions (14 ACCs and 13 ACAs) being monoclonal. The standard deviation of MF/HPF progressively increased in ACNH-ACA-ACC (0.048 ± 0.076 , 0.110 ± 0.097 , 0.506 ± 0.291 , respectively; $P = .0023$), but did not differentiate ACNH/ACA. Only tetraploid percentage ($P = .0496$) and 5cER ($P = .0352$) distinguished polyclonal (3.64 ± 2.20 and 0.14 ± 0.15) from monoclonal (7.25 ± 7.52 and 1.00 ± 1.74) benign lesions. Malignancy significantly correlated with a low diploid percentage and high tetraploid percentage. Cell kinetic heterogeneity is the hallmark of adrenocortical neoplasms: tetraploid/hypertetraploid cell accumulation characterizes monoclonal lesions (suggesting nondisjunctional mitoses), whereas heterogeneously distributed mitotic figures and decreased diploid percentage define ACCs.

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This manuscript was presented in part at the Annual Meeting of the United States and Canadian Academy of Pathology (Washington DC, March 1996 and March 2003), and at the International Congress of the International Academy of Pathology, Amsterdam, September 2002.

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1. Introduction

Proliferation can assess endocrine neoplasm progression and is the single most important malignancy criterion in the adrenal cortex [1–3] and provides helpful prognostic information for grading adrenocortical carcinomas (ACCs) [4]. However, there is no consensus on an accurate and

reliable method for evaluating the mitotic rate [3,5,6], and no study yet has determined the significance of mitotic figure (MF) counting variability. Proliferation is also closely related with DNA content and has been studied in multiple conditions, both reactive and neoplastic.

Neoplasms result from the progressive and convergent selection of cell populations for which clonality is still the hallmark indicative of acquired somatic mutations [7-10]. However, the molecular events during neoplastic transformation are not completely understood and remain essentially unknown, which leaves X-chromosome inactivation assays as the best molecular option, because this test is not based on any tumor-related genetic alteration [11,12]. Clonality offers a better understanding of tumors if it is combined with proliferation and apoptosis [13-15]: polyclonal patterns predominate in adrenocortical nodular hyperplasias (ACNHs) and show increasing apoptosis as a counterpoise to rising proliferative rates, whereas monoclonal patterns predominate in adrenocortical adenomas (ACAs) and have progressively lower apoptotic rates as proliferation increases. That inverted proliferation/apoptosis relationship provides the best selective mechanism, resulting in dominant clone expansion and monoclonal patterns in ACAs, whereas direct correlation gives a less selective mechanism, allowing balanced expansion of clones and polyclonal patterns in ACNHs. Nothing yet has been done to this respect in ACCs.

The sequence hyperplasia⇒adenoma⇒carcinoma has been proposed as a model for neoplastic transformation in endocrine organs. However, the results reported for DNA ploidy and clonality patterns are heterogeneous and do not show a consistent relationship with the histologic diagnosis [16-19]. In addition, there is no information about the relative timing of proliferation, DNA content abnormalities, and clonality profile. The reason for this disagreement is partly related with tissue heterogeneity, which affects both kinetic and genetic features [15,20-22].

This study evaluates the kinetic features of ACNHs, ACAs, and ACCs, by clonal patterns (X-chromosome inactivation) in microdissected tissue samples. We also assess DNA and kinetic variability in these conditions by slide and flow cytometry of DNA ploidy.

2. Materials and methods

2.1. Case selection and sampling

Consecutive adrenocortical proliferative lesions in females (156) were selected and histologically evaluated, including 69 ACNHs, 64 ACAs, and 23 ACCs. All surgical specimens were routinely processed for histologic diagnosis and sampled with at least 1 block per centimeter of maximum lesion diameter. The cases were classified according to the World Health Organization and Armed Forces Institute of Pathology criteria [23,24], incorporating

the scoring diagnostic systems and follow-up data (mean, 159 months) [3,5,6], being lesions with metastases classified as malignant.

The most cellular areas from the biggest nodule in each case of ACNHs and from every ACA and ACC were screened and selected for further analysis. The same areas were used in each analysis; hematoxylin and eosin-stained sections taken before and after the specimen samples were used to check the cellular composition of each sample.

2.2. X-Chromosome inactivation assay

Two 20- μ m unstained paraffin sections were used for microdissection under microscopic control. Adrenocortical cells and controls (histologically normal adrenal cortex, adrenal medulla, and periadrenal soft tissue from the same slide) underwent DNA extraction. At least 2 separated areas of 0.25 mm², containing about 100 target cells each, were harvested from both peripheral and internal areas of the biggest nodule in ACNHs, ACAs, and ACCs.

DNA was extracted from the samples using a modified phenol-chloroform protocol, as described [25]. Half of each sample underwent *Hha*I (New England Biolabs, Beverly, Mass) digestion (0.8 U/ μ L), with the remaining half kept undigested and both processed identically. The samples were digested under appropriate buffer conditions (50 mmol/L potassium acetate, 20 mmol/L Tris acetate, 10 mmol/L magnesium acetate, 1 mmol/L dithiothreitol, pH 8.0, 100 μ g/mL bovine serum albumin, 100 μ g/mL mussel glycogen) at 37°C for 4 to 16 hours. A mimicker (0.3 μ g of double-stranded and *Xho*I-linearized ϕ X174-RII phage; Life Technologies, Gaithersburg, Md) was included in each reaction mixture to check digestion completion by gel electrophoresis. Incompletely digested samples were phenol-chloroform purified and redigested with higher *Hha*I concentration.

*Hha*I was then inactivated by phenol-chloroform extraction as described [25]. DNA was precipitated with ice-cold absolute ethanol in the presence of 0.3 mol/L sodium acetate (pH 5.2) and resuspended in 10 μ L of polymerase chain reaction (PCR) buffer (10 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, and 100 μ g/mL bovine serum albumin). Both *Hha*I-digested DNA and undigested DNA were then used for PCR amplification of the first exon CAG repeat of the human androgen receptor gene (HUMARA) that included a DNA sequence recognized by *Hha*I, which is consistently methylated in the inactive HUMARA allele only [9,13,14,26,27]. The reactions were run in duplicate and optimized for a 10- μ L reaction in a Perkin-Elmer thermal cycler model 480 (Perkin-Elmer, Norwalk, Conn)

The whole PCR volume was electrophoresed into 0.75-mm-thick 8% nondenaturing polyacrylamide gel at 5 V/cm and processed as described [9]: gels fixed with 7% acetic acid (5 minutes), dried under vacuum (80°C, 40 minutes), and put inside a developing cassette containing

one intensifying screen and preflashed films (Kodak XAR, Kodak, Rochester, NY; 16–48 hours, -70°C). The autoradiograms were developed using an automated processor Kodak-Omat 100 (Kodak).

Interpretation and inclusion criteria in each sample were as reported [9]. Allelic imbalance was densitometrically evaluated (EC model 910 optical densitometer, EC Apparatus, St Petersburg, FL), and evidence of monoclonal proliferation was considered to be allele ratios 4:1 or higher with the normalized *HhaI*-digested samples. Sample normalization was done in relation to the corresponding undigested sample and tissue controls. Only informative cases (2 different alleles in *HhaI*-undigested and *HhaI*-digested samples) were included in the final analysis [8,9,14,15,28].

2.3. Mitotic figure counting

Mitotic figures were screened in the same 50 high-power fields (HPFs) used for DNA cytometry and DNA extraction in each compartment (7.140 mm^2) as reported [29], beginning in the most cellular area. Both the number of positive nuclei per HPF and the number of neoplastic cells intercepted by the microscope field diameter (n) were registered. The last score was used to estimate the number of neoplastic cells/HPF (N) using the formula $N = (n\pi/4)^2$ as reported [30,31] and expressed per 1000 tumor cells. Both the mean and SD were calculated as representative scores per compartment and patient.

2.4. Immunohistochemical expression of Ki-67

The sections were mounted on positively charged slides (Superfrost Plus, Fisher Scientific, Fair Lawn, NJ), baked at 60°C for 2 hours, and processed as described [13,22,28,32]. After routine dewaxing and rehydration, endogenous peroxidase quenching, and antigens heat retrieval, the slides were transferred to a moist chamber. Nonspecific binding was blocked with polyclonal horse serum and sections incubated with monoclonal primary antibodies (overnight, 4°C , $4\text{ }\mu\text{g/mL}$, Dianova, Hamburg, Germany). Then, sections were serially incubated with biotinylated antimouse antibody and peroxidase-labeled avidin-biotin complex. The reaction was developed under microscopic control, using 3,3'-diaminobenzidine tetrahydrochloride with 0.3% H_2O_2 as chromogen (Sigma, St Louis, Mo), and the sections counterstained with hematoxylin. Both positive (reactive lymph node) and negative (omitting the primary antibody) controls were simultaneously run.

2.5. Slide cytometric analysis of DNA content

Feulgen-stained sections were used for DNA quantification [33], according to validated protocols for such material [16,31,33]. Cell Analysis System 200 and Quantitative DNA Analysis software (Becton Dickinson, Franklin Lakes, NJ) were used for the densitometric evaluation. At least 200 complete, nonoverlapping, and focused nuclei (or the whole

lesion if smaller) were measured in every case, beginning in the most cellular area until completion in consecutive HPFs.

External diploid controls were used to determine DNA indices (complete rat hepatocytes and lymphocytes from reactive lymph nodes) and to standardize the nuclear area/DNA content analysis (adrenocortical cells from normal areas) [34]. The internal controls were used for setting the G_0/G_1 cell limits and calculating the DNA index of each G_0/G_1 peak ($>10\%$ of measured cells with evidence of $\text{G}_2 + \text{M}$ cells) [16,35]. DNA histograms were used to calculate the proliferation rate ($\text{PR} = \text{S} + \text{G}_2 + \text{M}/\text{G}_0 + \text{G}_1 + \text{S} + \text{G}_2 + \text{M}$), 5c exceeding rate (5cER or percentage of nonoctaploid cells with DNA content exceeding 5c), and percentage of tetraploid cells (DNA index, 1.85–2.25) [16,31,36].

2.6. Flow cytometric analysis of nuclear DNA

Serial $50\text{-}\mu\text{m}$ -thick sections were microdissected, and nuclear preparations were stained with propidium iodine after RNase A digestion to study DNA ploidy [37]. DNA quantification parameters included DNA indices and proliferation rates as described [14,35,36] and the scatter analysis of nuclear area and DNA content to identify apoptotic cells in each cell-cycle phase (low nuclear area for a given DNA content in each cell-cycle phase), when coupled with in situ end labeling (ISEL, see below). [14,34]. External diploid controls from paraffin-embedded tissues (lymphocyte from reactive lymph nodes and adrenocortical cells from histologically normal adrenal glands) were used to determine DNA indices and to standardize the nuclear area/DNA content analysis (considering only adrenocortical cells for the last purpose) [14]. Proliferation rate was calculated as described for slide cytometry, using the rectangular model for cell-cycle histogram evaluation [35].

2.7. In situ end labeling of fragmented DNA

Extensive DNA fragmentation associated with apoptosis was detected by ISEL as reported [14,15,31,32]. After routine dewaxing and hydration, the sections were incubated in $2\times\text{ SSC}$ (20 minutes at 80°C) and digested with pronase ($500\text{ }\mu\text{g/mL}$, 25 minutes, room temperature) in a moist chamber.

DNA fragments were labeled on 5'-protruding termini by incubating the sections with the Klenow fragment of *Escherichia coli* DNA polymerase I (20 U/mL in 50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L MgCl_2 , 1 mmol/L DTT, $250\text{ }\mu\text{g/mL}$ bovine serum albumin, $5\text{ }\mu\text{mol/L}$ of each dATP, dCTP, dGTP, as well as $3.25\text{ }\mu\text{mol/L}$ dTTP, and $1.75\text{ }\mu\text{mol/L}$ 11-digoxigenin-dUTP), at 37°C in a moist chamber. The incorporated digoxigenin-dUTPs were immunoenzymatically detected by using antidigoxigenin Fab fragments labeled with alkaline phosphatase (7.5 U/mL , in 100 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% bovine serum albumin) for 4 hours at room temperature. The reactions were developed with the mixture nitroblue tetrazolium-X phosphate in 100 mmol/L Tris-HCl (pH 9.5), 100 mmol/L

NaCl, 50 mmol/L MgCl₂ under microscopic control. Appropriate controls were simultaneously run, including positive (reactive lymph node), negative (same conditions omitting DNA polymerase I), and enzymatic (DNase I digestion before the end labeling). The enzymatic controls were used to reliably establish the positivity threshold in each sample.

2.8. Quantification of positive nuclei and statistical analysis

At least 50 HPFs (7.6 mm²) were screened in each pathologic group, beginning in the most cellular area (from the biggest nodule for ACNHs). The number of positive nuclei was expressed per high power fields and per 1000 tumor cells, and the mean and SD calculated in each pathologic condition and patient as described [14,15,30,31]. The positivity threshold was experimentally established at the positive control in each staining batch. Only nuclei with staining features similar to those of their corresponding positive control were considered positive for any marker.

Quantitative variables were compared by diagnostic groups (ACNHs, ACAs, ACCs) and clonality patterns

(polyclonal, monoclonal). Variables showing normal distribution were analyzed using 2-tailed Student *t* test, whereas analyses of variance were used for variables with nonparametric distribution. Normal distribution was tested by the Kolmogorov-Smirnoff test. Results were considered statistically significant if *P* < .05 in 2-tailed distributions. Finally, the MFC thresholds for each diagnostic category (ACNHs, ACAs, ACCs) using discriminant functions and the correlation between different MF scores were calculated.

3. Results

Polyclonal patterns were observed in 48 (77.4%) of 62 informative ACNHs and 7 (12.5%) of 56 informative ACAs, and monoclonal in 14 (22.6%) of 62 ACNHs, 49 (87.55%) of 56 ACAs, and 21 (100.0%) of 21 ACCs (Fig. 1). Noninformative cases (unbalanced methylation of androgen receptor alleles in control tissues) were excluded from further analyses (7 ACNHs, 8 ACAs, and 2 ACCs). Consistent methylation patterns were detected in both peripheral and internal samples from the same cortical nodule or tumor.

Fig. 1 Clonality and DNA ploidy patterns in adrenocortical proliferative lesions. Monoclonal pattern correlated with increased proportion of tetraploid and hypertetraploid cells in the DNA histogram, whereas malignancy was mainly defined by heterogeneous distribution of mitotic figures.

Table 1 Proliferation and DNA ploidy results in adrenocortical proliferative lesions by clonal pattern

	Benign polyclonal (mean $\pm$ SD)	Benign monoclonal (mean $\pm$ SD)	Malignant monoclonal (mean $\pm$ SD)
MF/50 HPF	0.69 $\pm$ 1.03	0.90 $\pm$ 1.18	14.69 $\pm$ 15.16
SD _{MF/HPF}	0.07 $\pm$ 0.10	0.10 $\pm$ 0.09	0.51 $\pm$ 0.29
MF/1000 cells	0.12 $\pm$ 0.20	0.24 $\pm$ 0.30	3.52 $\pm$ 3.73
MF/1000 cells (SD)	0.22 $\pm$ 0.37	0.36 $\pm$ 0.42	1.96 $\pm$ 1.51
2c (%)	79.02 $\pm$ 5.19	72.88 $\pm$ 16.44	39.69 $\pm$ 23.65
2c-4c (%)	17.21 $\pm$ 4.27	19.10 $\pm$ 9.51	42.71 $\pm$ 25.81
4c (%)	3.64 $\pm$ 2.20	7.25 $\pm$ 7.52	11.62 $\pm$ 17.11
5cER	0.14 $\pm$ 0.15	1.00 $\pm$ 1.74	5.65 $\pm$ 6.15

Most ACNHs were diploid (95.7%, 66/69), whereas 51 (79.7%) of 64 ACAs and 9 (39.1%) of 23 ACCs showed diploid DNA content. All hyperdiploid neoplasms (11/13 informative ACAs and 13/14 informative ACCs) revealed monoclonal patterns, whereas 3 hyperdiploid ACNHs were found noninformative for clonality assay. Only tetraploid percentage ($P = .0496$) and 5cER ($P = .0352$) distinguished polyclonal from monoclonal benign lesions (Table 1). Malignancy in monoclonal lesions significantly correlated with lower percentage of diploid cells and higher percentage of tetraploid cells as well.

No significant differences were found for proliferation and ISEL indices after DNA-ploidy stratification, although hyperdiploid neoplasms tended to show greater scores than diploid ones. A heterogeneous immunohistochemical expression of Ki-67 was observed with high variability of expression from field to field (ACNHs, 37.74% $\pm$ 7.30%; ACAs, 38.64% $\pm$ 8.74%; ACCs, 41.26% $\pm$ 8.74%). There were no statistically significant differences in the immunohistochemical expression of Ki-67 according to topography or diagnostic group. Proliferation rates were significantly higher in polyclonal lesions with G₀/G₁ apoptosis than in polyclonal lesions with no G₀/G₁ apoptosis ($P = .005$) and in monoclonal lesions with no G₀/G₁ apoptosis than in

monoclonal lesions with G₀/G₁ apoptosis (Fig. 2; $P = .009$). Apoptotic cells were revealed in G₀/G₁ phase in monoclonal ACNHs (78.6%, 11/14) and polyclonal ACAs (71.4%, 5/7), whereas they were present in any cell-cycle phase in all ACCs. Both MF/50 HPFs and SD_{MF/HPF} progressively increased from ACNHs through ACAs to ACCs, but did not differentiate polyclonal from monoclonal benign lesions.

Kruskal-Wallis 1-way analysis of variance showed statistically significant differences for all mitotic indices ($P < .0001$), distinguishing benign from malignant lesions, but did not differentiate polyclonal from monoclonal benign lesions (Table 1). For diagnostic purposes, a stepwise discriminant analysis selected SD_{MF/HPF} as independent predictive variable (Table 2). With the use of the classification function coefficients, ACNHs were defined by SD_{MF/HPF} $\leq$ 0.07, ACAs by 0.07 < SD_{MF/HPF} $\leq$ 0.30, and ACCs by SD_{MF/HPF} > 0.30.

The regression analyses of equivalent MFC variables showed a linear correlation between MFC/50 HPFs and MFC/1000 cells for both the total scores ($R^2 = 0.8572$) and the SD ($R^2 = 0.8417$), and between SD_{MF/HPF} and MF/50 HPFs: SD_{MF/HPF} = 0.0183 MF/50 HPFs + 0.2367, $R^2 = 0.9125$ for malignant monoclonal lesions; SD_{MF/HPF} = 0.0716 MF/50 HPFs + 0.0314, $R^2 = 0.8275$ for benign

Fig. 2 Proliferation (Ki-67) and apoptosis (ISEL) by clonal patterns in adrenocortical proliferative lesions. The presence of flow cytometric detectable apoptosis (G₀/G₁ cells) correlated with higher proliferation in benign polyclonal lesions (direct correlation) and with lower proliferation in benign monoclonal lesions (inverse correlation). Malignant monoclonal lesions always revealed apoptotic cells in any cell-cycle phase.

monoclonal lesions; and $SD_{MF/HPF} = 0.0911 \text{ MF}/50 \text{ HPFs} + 0.0077$, $R^2 = 0.9485$ for benign polyclonal lesions.

4. Discussion

The most important findings were the key role of mitosis abnormalities during adrenocortical neoplastic transformation and the heterogeneous kinetic pattern of these lesions. Monoclonal lesions are characterized by the accumulation of tetraploid and hypertetraploid cells, suggesting nondisjunctional mitoses. Adrenocortical carcinomas also reveal heterogeneously distributed proliferating cells and decreased proportion of euploid cells.

DNA-ploidy results confirmed the differences between polyclonal and monoclonal proliferative lesions. Nondiploid DNA contents were found in 60.9% (14/23) of ACCs, 21.3% (13/64) of ACAs, and only 4.3% (3/69) of ACNHs. Those cases preferentially revealed hyperdiploid G₀/G₁ cells and monoclonal patterns in 86% of informative cases. The DNA content analysis has been reported as diagnostically useless in differentiating benign from malignant conditions [16,18,19]. However, the close association found between DNA aneuploidy and monoclonal proliferation supports the neoplastic nature of ACAs [14], and the heterogeneous clonal profile of ACNHs (predominantly polyclonal) and ACAs (predominantly monoclonal) suggests a multistep process [14,38–40].

Table 2 Stepwise discriminant analyses for MF counting ($SD_{MF/HPF}$) in adrenocortical proliferative lesions					
Classification function coefficients					
	Benign polyclonal	Benign monoclonal	Malignant monoclonal		
$SD_{MF/HPF}$	2.12	4.00	20.97		
Constant	−1.15	−1.29	−6.35		
Study classification					
Final diagnosis classification	Percent correct	Benign polyclonal	Benign monoclonal	Malignant monoclonal	Total
Benign polyclonal	69.6	48	21	0	69
Benign monoclonal	50.0	30	32	2	64
Malignant monoclonal	78.3	0	5	18	23
Total	62.8	78	58	20	156

Fig. 3 Mitosis cycle and DNA-ploidy patterns in adrenocortical proliferative lesions. The DNA content analysis allows the separation of cell-cycle phases, being G₀/G₁ cells diploid, S cells hyperdiploid, and G₂ + M cells tetraploid. The increased fraction of tetraploid cells in monoclonal adrenocortical lesions suggests the accumulation of either diploid G₂ + M cells before cytokinesis or tetraploid G₀/G₁ cells. The presence of any of those cell types suggests either defective DNA replication with G₂ arrest or defective segregation of sister chromatids (nondisjunctional mitosis), resulting in tetraploid G₀/G₁ cells accumulation. If the DNA defect(s) cannot be repaired, G₂-arrested cells will eventually undergo apoptosis, unless a dysfunctional DNA repair system allows the cells to progress in the cell cycle.

This combined clonality-DNA ploidy study of adrenocortical lesions also revealed accumulation of tetra- and hypertetraploid cells in monoclonal lesions, suggesting the presence of cycling tetraploid G₀/G₁ cells (Fig. 3). Benign monoclonal adrenocortical lesions reveal simultaneous apoptosis downregulation and proliferation upregulation, and promote a stromal vascular reaction to support this demanding cell kinetics [14,28]. The inverted proliferation/apoptosis relationship in monoclonal adrenocortical lesions also provides a functional basis for cellular selection, leading to clonal expansion (if proliferation predominates) or regression (if apoptosis dominates) [7,8,13,17,41,42]. The significantly increased rate of hypertetraploid cells (high 5cER correlated with monoclonal patterns) would support the presence of cycling tetraploid G₀/G₁ cells, which suggests associated abnormalities in the anaphase checkpoint (Fig. 3).

Heterogeneity is the hallmark of cell kinetics during the neoplastic transformation of adrenocortical cells, which could be explained by the following. (a) Embryological

reasons, such as the patch-size concept (relative size of contiguous cellular regions of the same lineage), can determine the clonal pattern. Any kinetic advantage in small cell groups sharing the same origin would result in their preferential growth, thus yielding an overall monoclonal pattern in early developmental stages, early neoplasms, and precancerous conditions [43-45], but can also be responsible for polyclonal patterns if 2 or more patches are mixed together. (b) Pathologically, any tumor growth requires stromal and vascular changes to be effective [28,46]. Adrenocortical adenomas tend to show vascular ectasia and hemorrhage, especially in the internal area, and have revealed bigger vascular areas of sinusoid-like structures than ACNHs [28]. The dense thin-walled blood vessel network in endocrine organs should certainly contribute to that finding, providing also perivascular stromal cells. Those nonepithelial components have been proposed as key elements of epithelial cell growth by either secretion of stimulatory factors or lack of inhibitory factors in thyroid nodules and polyclonal pheochromocytomas [15,16,47].

The multistep tumorigenesis assumes that tumor progression evolves through genetic events, providing some proliferative or invasive advantages to a small number of cells, resulting in tumor heterogeneity. This cell selection process is better revealed by dispersion variables, such as SD. $SD_{MF/HPH}$ thus evaluates one aspect of tumor heterogeneity.

Adrenocortical carcinomas in this series have revealed heterogeneously distributed proliferating cells with significantly higher $SD_{MF/HPF}$ than benign lesions and decreased proportion of euploid cells. Mitotic figure counting is the most important variable for ACC diagnosis [3-6,48], with its variability recorded as a confusing factor. Mitotic count and tumor stage are essential in determining prognosis for patients with ACCs [48], but the molecular complexity and heterogeneity of these neoplasms are such that targeted therapy needs to be patient specific. This issue has diagnostic implications because greater SD increases group overlapping and, therefore, the number of cases with uncertain diagnosis. Result variability has been higher for MFC than for $SD_{MF/HPF}$, which explains the selection of $SD_{MF/HPF}$ as a better diagnostic variable ($SD_{MF/HPH} \geq 0.30$ is highly suggestive of malignancy). The isolated evaluation of $SD_{MF/HPH}$ revealed a sensitivity of 76.9% and a specificity of 98.0%. However, proper evaluation requires screening several HPFs because of both the low proliferation index of adrenocortical lesions and the short period of M phase compared with the interphase [3-6]. Despite requiring some extra time, such arguments should encourage pathologists to include this approach as a diagnostic tool.

In conclusion, mitosis abnormalities play a key role during adrenocortical neoplastic transformation and a heterogeneous kinetic pattern is characteristic. Anaphase checkpoint abnormalities would contribute to the accumulation of both tetraploid and hypertetraploid cells in monoclonal lesions and suggest nondisjunctional mitoses. Adrenocortical carcinomas also reveal heterogeneously distributed proliferating cells (high $SD_{MF/HPF}$) and decreased proportion of euploid cells.

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