Original contribution

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

Alfredo Blanes MD, PhD\(^a\), Salvador J. Diaz-Cano MD, PhD, FRCPath\(^{a,b,*}\)

\(^{a}\)Department of Pathology, University Hospital of Malaga, 29010 Malaga, Spain
\(^{b}\)Department of Pathology, King’s College Hospital and King’s College London School of Medicine, University of London, London SE5 9RS, UK

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

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 23 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 ± 2.52 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.

© 2006 Elsevier Inc. All rights reserved.

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
show a consistent relationship with the histologic diagnosis. Ploidy and clonality patterns are heterogeneous and do not endocrine organs. However, the results reported for DNA combined with proliferation and apoptosis [13-15]: 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-μm 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 HhaI (New England Biolabs, Beverly, Mass) digestion (0.8 U/μL), 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 μg/mL bovine serum albumin, 100 μg/mL mussel glycogen) at 37°C for 4 to 16 hours. A mimicker (0.3 μg of double-stranded and XhoI-linearized φX174-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 HhaI concentration.

HhaI 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 μL 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 μg/mL bovine serum albumin). Both HhaI-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 HhaI, 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-μL 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 mm²) 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 = (nπ/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 incubated with polyclonal horse serum and sections incubated in a moist chamber. Nonspecific binding was developed under microscopic control, using 3,3'-diaminobenzidine tetrahydrochloride with 0.3% H₂O₂ 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 (adenocortical cells from normal areas) [34]. The internal controls were used for setting the G0/G1 cell limits and calculating the DNA index of each G0/G1 peak (>10% of measured cells with evidence of G2 + M cells) [16,35]. DNA histograms were used to calculate the proliferation rate (PR = S + G2 + M/G0 + G1 + S + G2 + M), 5e exceeding rate (5eER 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-μm-thick sections were microdissected, and nuclear preparations were stained with propidium iodide 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× SSC (20 minutes at 80°C) and digested with pronase (500 μg/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 MgCl₂, 1 mmol/L DTT, 250 μg/mL bovine serum albumin, 5 μmol/L of each dATP, dCTP, dGTP, as well as 3.25 μmol/L dTTP, and 1.75 μmol/L 11-digoxigenin-dUTP), at 37°C in a moist chamber. The incorporated digoxigenin-dUMP's 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.
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% ± 7.30%; ACAs, 38.64% ± 8.74%; ACCs, 41.26% ± 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 SDMF/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 SDMF/HPF as independent predictive variable (Table 2). With the use of the classification function coefficients, ACNHs were defined by $SDMF/HPF \leq 0.07$, ACAs by $0.07 < SDMF/HPF \leq 0.30$, and ACCs by $SDMF/HPF > 0.30$.

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 SDMF/HPF and MF/50 HPFs: $SDMF/HPF = 0.0183 \times MF/50 \text{ HPFs} + 0.2367$, $R^2 = 0.9125$ for malignant monoclonal lesions; $SDMF/HPF = 0.0716 \times MF/50 \text{ HPFs} + 0.0314$, $R^2 = 0.8275$ for benign

| Table 1 | Proliferation and DNA ploidy results in adrenocortical proliferative lesions by clonal pattern |
|---------|---------------------------------------------------------------|
|         | Benign polyclonal (mean ± SD) | Benign monoclonal (mean ± SD) | Malignant monoclonal (mean ± SD) |
| MF/50 HPF | 0.69 ± 1.03 | 0.90 ± 1.18 | 14.69 ± 15.16 |
| SDMF/HPF  | 0.07 ± 0.10 | 0.10 ± 0.09 | 0.51 ± 0.29  |
| MF/1000 cells | 0.12 ± 0.20 | 0.24 ± 0.30 | 3.52 ± 3.73  |
| 2c (%) | 79.02 ± 5.19 | 72.88 ± 16.44 | 39.69 ± 23.65 |
| 2c-4c (%) | 17.21 ± 4.27 | 19.10 ± 9.51 | 42.71 ± 25.81 |
| 4c (%) | 3.64 ± 2.20 | 7.25 ± 7.52 | 11.62 ± 17.11 |
| 5cER | 0.14 ± 0.15 | 1.00 ± 1.74 | 5.65 ± 6.15 |

Statistically significant for Benign vs. Malignant
Statistically significant for Polyclonal vs. Monoclonal
(Benign lesions only)
monoclonal lesions; and $SD_{MF/HPF} = 0.0911 \text{ MF/50 HPFs} + 0.0077$, $R^2 = 0.9485$ 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 $G_0/G_1$ 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].

| 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. 2 Proliferation (Ki-67) and apoptosis (ISEL) by clonal patterns in adrenocortical proliferative lesions. The presence of flow cytometric detectable apoptosis ($G_0/G_1$ 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.
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/HPF} 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/HPF} ≥ 0.30 is highly suggestive of malignancy). The isolated evaluation of SD_{MF/HPF} 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 interfase [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.

References

[1] DeLellis RA. Does the evaluation of proliferative activity predict malignancy of prognosis in endocrine tumors? HUM PATHOL 1995; 26:131–4.

[2] DeLellis RA. Proliferation markers in neuroendocrine tumors: useful or useless? A critical reappraisal. Verh Dtsch Ges Pathol 1997;81: 53–61.

[3] Weiss LM. Comparative histologic study of 43 metastasizing and nonmetastasizing adrenocortical tumors. Am J Surg Pathol 1984; 8:163–9.

[4] Weiss LM, Medeiros LJ, Vickery Jr AL. Pathologic features of prognostic significance in adrenocortical carcinoma. Am J Surg Pathol 1989;13:202–6.

[5] Hough AJ, Hollifield JW, Page DL, et al. Prognostic factors in adrenal cortical tumors. A mathematical analysis of clinical and morphologic data. Am J Clin Pathol 1979;72:390–9.

[6] van Slooten H, Schaberg A, Smeenk D, et al. Morphologic characteristics of benign and malignant adrenocortical tumors. Cancer 1985;55:766–73.

[7] Diaz-Cano SJ. Clonality studies in the analysis of adrenal medullary proliferations: application principles and limitations. Endocr Pathol 1998;9:301–16.

[8] Diaz-Cano SJ. Designing a molecular analysis of clonality in tumours. J Pathol 2000;191:343–4.

[9] Diaz-Cano SJ, Blanes A, Wolfe HJ. PCR techniques for clonality assays. Diagn Mol Pathol 2001;10:24–33.

[10] Fialkow PJ. Clonal origin of human tumors. Biochim Biophys Acta 1976;458:283–321.

[11] Fialkow PJ. Primordial cell pool size and lineage relationships of five human cell types. Ann Hum Genet 1973;37:39–48.

[12] Lyon MF. Some milestones in the history of X-chromosome inactivation. Annu Rev Genet 1992;26:16–28.

[13] Diaz-Cano SJ, Blanes A, Rubio J, et al. Molecular evolution and intratumor heterogeneity by topographic compartments in muscle-invasive transitional cell carcinoma of the urinary bladder. Lab Invest 2000;80:279–89.

[14] Diaz-Cano SJ, de Miguel M, Blanes A, et al. Clonality as expression of distinctive cell kinetics patterns in nodular hyperplasias and adenomas of the adrenal cortex. Am J Pathol 2000;156:311–9.

[15] Diaz-Cano SJ, de Miguel M, Blanes A, et al. Clonal patterns in phaeochromocytomas and MEN-2A adrenal medullary hyperplasias: histologic and kinetic correlates. J Pathol 2000;192:221–8.

[16] Diaz-Cano S, Gonzalez-Campora R, Rios-Martin JJ, et al. Nuclear DNA patterns in adrenal cortex proliferative lesions. Virchows Arch A Pathol Anat Histopathol 1993;423:323–8.

[17] Sasano H, Imatani A, Shizawa S, et al. Cell proliferation and apoptosis in normal and pathologic human adrenal. Mod Pathol 1995;8:11–7.

[18] Hosaka Y, Rainwater LM, Grant CS, et al. Adrenocortical carcinoma: nuclear deoxyribonucleic acid ploidy studied by flow cytometry. Surgery 1987;102:1027–34.

[19] Joensuu H, Klemi PJ. DNA aneuploidy in adenomas of endocrine organs. Am J Pathol 1988;132:145–51.

[20] Baithung SI, Naase M, Blanes A, et al. Molecular and kinetic features of bladder transitional cell carcinomas. Biological and clinical implications. Virchows Arch A 2001;438:289–97.

[21] Pozo L, Camacho F, Rios-Martin JJ, et al. Cell proliferation in skin tumors with ductal differentiation: patterns and diagnostic applications. J Cutan Pathol 2000;27:292–7.

[22] Blanes A, Sanchez-Carrillo JJ, Diaz-Cano SJ. Topographic molecular profile of phaeochromocytomas: role of somatic down-regulation of mismatch repair. J Clin Endocrinol Metab 2006;91:1150–8.

[23] Lloyd R, DeLellis R, Heitz P, et al. Pathology and genetics of tumours of endocrine organs. Geneva: WHO Press; 2004.

[24] Lloyd RV, Douglas BR. Endocrine diseases. (First series). Washington (DC): American Registry of Pathology/Armed Forces Institute of Pathology; 2002.

[25] Diaz-Cano SJ, Brady SP. DNA extraction from formalin-fixed, paraffin-embedded tissues: protein digestion as a limiting step for retrieval of high-quality DNA. Diagn Mol Pathol 1997;6: 342–6.

[26] Allen RC, Zoghbi HY, Moseley AB, et al. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Genet 1992;51:1229–39.

[27] Sleddens HF, Oostra BA, Brinkmann AO, et al. Trinucleotide repeat polymorphism in the androgen receptor gene (AR). Nucleic Acids Res 1992;20:1427.

[28] Diaz-Cano SJ, de Miguel M, Blanes A, et al. Contribution of the microvesSEL network to the clonal and kinetic profiles of adrenal cortical proliferative lesions. HUM PATHOL 2001;32:1252–9.

[29] van Diest PJ, Baak JP, Matze-Cok P, et al. Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the
Multicenter Morphometric Mammary Carcinoma Project [see comments]. Hum Pathol 1992;23:603–7.

[30] Harjacek M, Diaz-Cano S, Alman BA, et al. Prominent expression of mRNA for proinflammatory cytokines in synovium in patients with juvenile rheumatoid arthritis or chronic lyme arthritis. J Rheumatol 2000;27:497–503.

[31] Koch M, de Miguel M, Höfler H, et al. Kinetic profiles of intraepithelial and invasive prostatic neoplasias: the key role of down-regulated apoptosis in tumor progression. Virchows Arch 2000;436:413–20.

[32] Blanes A, Rubio J, Martinez A, et al. Kinetic profiles by topographic compartments in muscle-invasive transitional cell carcinomas of the bladder: role of TP53 and NF1 genes. Am J Clin Pathol 2002;118:93–100.

[33] Bibbo M, Bartels PH, Dytch HE, et al. Cell image analysis. In: Bibbo M, editor. Comprehensive cytopathology. Philadelphia: WB Saunders; 1991. p. 965–83.

[34] Sherwood SW, Schimke RT. Cell cycle analysis of apoptosis using flow cytometry. Methods Cell Biol 1995;46:77–97.

[35] Dressler LG. Controls, standards, and histogram interpretation in DNA flow cytometry. Methods Cell Biol 1990;33:157–71.

[36] Dressler LG, Bartow SA. DNA flow cytometry in solid tumors: practical aspects and clinical applications. Semin Diagn Pathol 1989;6:55–82.

[37] Hedley DW, Friedlander ML, Taylor JW, et al. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 1983;31:1333–5.

[38] Beuschlein F, Reincke M, Karl M, et al. Clonal composition of human adrenocortical neoplasms. Cancer Res 1994;54:4927–32.

[39] Jackson CE, Cerny JC, Block MA, et al. Probable clonal origin of aldosteronomas versus multicellular origin of parathyroid “adenomas”. Surgery 1982;92:875–9.

[40] Knudson Jr AG. Mutation and cancer: a personal odyssey. Adv Cancer Res 1995;67:1–23.

[41] Salomon RN, Diaz-Cano S. Introduction to apoptosis. Diagn Mol Pathol 1995;4:235–8.

[42] Cordon-Cardo C. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 1995;147:545–60.

[43] Hicks DG, LiVolsi VA, Neidich JA, et al. Clonal analysis of solitary follicular nodules in the thyroid. Am J Pathol 1990;137:553–62.

[44] Jacoby LB, Hedley-Whyte ET, Pulaski K, et al. Clonal origin of pituitary adenomas. J Neurosurg 1990;73:731–5.

[45] Nowell PC. The clonal evolution of tumor cell populations. Science 1976;194:23–8.

[46] Dvorak HF, Sioussat TM, Brown LF, et al. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. J Exp Med 1991;174:1275–8.

[47] Thomas GA, Williams D, Williams ED. The clonal origin of thyroid nodules and adenomas. Am J Pathol 1989;134:141–7.

[48] Stojadinovic A, Ghossein RA, Hoos A, et al. Adrenocortical carcinoma: clinical, morphologic, and molecular characterization. J Clin Oncol 2002;20:941–50.